EXAMINING DIOXIN-MEDIATED ALTERATIONS IN PULMONARY IMMUNE CELL FUNCTION DURING INFLUENZA VIRUS INFECTION: EFFECTS ON CYTOLYTIC ACTIVITY AND INTERFERON GAMMA

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of HALEY D. NEFF-LAFORD find it satisfactory and recommend that it be accepted.

Chair

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Abstract

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2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent immunotoxicant with the highest binding affinity for a transcriptional regulator known as the aryl hydrocarbon receptor (AhR). Using a murine influenza virus model, we have shown that mice treated with TCDD die from an otherwise non-lethal infection, and have fewer cytotoxic T lymphocytes (CTL) in their lungs. This reduction in the number of CTL is critical because CTL are the principal cells responsible for eliminating virus in a primary infection. In spite of fewer CTL, TCDD-treated mice that survive infection successfully eliminate the virus. We hypothesized that a non-CTL-mediated mechanism was responsible for killing the virus-infected cells in the lung. However, we found that exposure to TCDD did not increase anti-viral cytokine levels or NK cell activity in the lung during infection. Instead, we found that the CTL, although fewer in number, are sufficient for viral clearance from the lungs of immunocompromised mice.

In addition to decreasing the CTL response, exposure to TCDD elevates pulmonary interferon gamma (IFNγ) levels. This is of interest because while some level of IFNγ is important for anti-viral immunity, excessive IFNγ production is associated with enhanced inflammation and tissue damage. We found that influenza virus infection stimulates IFNγ production by multiple immune cell types. Interestingly, the majority of the IFNγ in the lungs of both vehicle- and TCDD-treated mice is produced by phagocytic cells, a non-typical source of IFNγ. The elevated IFNγ production correlates with an increase in inducible nitric oxide synthase (iNOS) levels in the lungs of TCDD-treated mice. Using CD45.2AhR$^{-/-}$→ CD45.1AhR$^{+/+}$ bone marrow chimeric mice we found that AhR-driven events external to the immune system mediate the exacerbated levels of IFNγ and iNOS during infection. Furthermore, we found that a novel iNOS-mediated mechanism may be responsible for the elevated IFNγ levels in the lung. Given that chronic inflammatory diseases of the lower respiratory tract are on the rise worldwide, and that high levels of both IFNγ and iNOS have been associated with the pathology of these diseases, our data suggest that environmental exposure to AhR ligands may contribute to the development of these disorders.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS……………………………………………………………….iii

ABSTRACT………………………………………………………………………………vii

LIST OF FIGURES……………………………………………………………………….xi

CHAPTER

1. INTRODUCTION

Introduction………………………………………………………………………1

Sources and exposure……………………………………………………….....2

Mechanism of toxicity…………………………………………………………...5

Toxicity in humans……………………………………………………………....9

Toxicity in laboratory animals………………………………………………….11

Objectives………………………………………………………………………20

2. FEWER CTL, NOT ENHANCED NK CELLS, ARE SUFFICIENT FOR VIRAL CLEARANCE FROM THE LUNGS OF IMMUNOCOMPROMISED MICE

Abstract…………………………………………………………………………23

Introduction……………………………………………………………………..23

Materials and Methods…………………………………………………………28

Results…………………………………………………………………………..33

Discussion……………………………………………………………………...42

3. POLLUTANTS AND THE ETIOLOGY OF RESPIRATORY DISEASE: A NOVEL MECHANISM OF AHR-MEDIATED DEREGULATION OF IFNγ PRODUCTION BY PHAGOCYTIC CELLS

Abstract…………………………………………………………………………46

Introduction……………………………………………………………………..47

Materials and Methods…………………………………………………………49

Results……………………………………………………………………………55

Discussion………………………………………………………………………...67

4. SUMMARY AND FUTURE DIRECTIONS

Summary…………………………………………………………………………71

Future Directions………………………………………………………………74

APPENDICES

Appendix A – The role of IFNγ in the decreased host resistance of TCDD-treated mice: studies using IFNγ-deficient mice……………………………………..80

Appendix B - Examining whether depletion of a cellular source of IFNγ increases survival of TCDD-treated, influenza virus-infected mice……………..82

Appendix C – TCDD-mediated effects on NFκB levels during influenza virus infection……………...……………………………………………………..86

Appendix D – The role of cytokines in the stimulation of IFNγ production………....90

Appendix E – Characterizing the role of the AhR in deregulated immune responses to influenza virus infection: studies using multiple AhR ligands………93

LIST OF REFERENCES……………………………………………………………100

LIST OF FIGURES

1.1 Structures and WHO TEFs for TCDD and other common pollutants……………………….3

1.2 The AhR activation pathway..……………………………………………………………6

2.1 Exposure to TCDD does not impair viral clearance……..…………………………….34

2.2 Exposure to TCDD does not alter levels of proinflammatory cytokines in lung lavage fluid from influenza virus-infected mice…………………….………………………..36

2.3 AhR activation increases the percent and number of NK1.1$^+$ cells in the lungs of virus-infected mice…………………………………………………….……………………….37

2.4 CTL, although fewer in number, are responsible for eliminated virus-infected cells from lungs of mice treated with TCDD……………………………………….……………….39

2.5 Activation of the AhR does not enhance the cytolytic activity of NK cells.....………….40

2.6 Exposure to TCDD does not enhance IFNγ production by NK cells or CD8$^+$ T cells in the lung………………………………………………………………………………………41

3.1 Activation of the AhR increases the percentage of IFNγ-producing cells in the lung, but the majority are neither CD3$^+$ nor NK1.1$^+$…………………………….………….…...56

3.2 The majority of the IFNγ-producing cells in the lungs of TCDD-treated mice are Gr1$^+$ and CD11b$^+$…………………………………………………………….…………..58

3.3 An AhR-mediated signal from the lung drives aberrant IFNγ production by macrophages and neutrophils……………………………………………………….…………62

3.4 Activation of the AhR increases iNOS levels in the lung during influenza virus infection…………………………………………………………...…………..64

3.5 Activation of the AhR in the lung, not in the immune system, induces iNOS expression by epithelial cells and alveolar macrophages……………………………………….………65

3.6 Pulmonary IFNγ levels are not elevated in TCDD-treated, iNOS-deficient mice.………66

5.1 Interferon gamma is essential for survival from the HKx31 strain of influenza A virus..81

5.2 Intranasal administration of Clodronate-containing liposomes successfully depletes F4/80+ cells from the lung airways ……………………………………………………...83

5.3 Alveolar macrophages are essential for survival from influenza virus infection…...…..83

5.4 Intranasal administration of PBS- or Clodronate-containing liposomes five days after pulmonary viral infection causes significant mortality ..……………………………….84

5.5 The NFκB signaling pathway……………………………………………………………86

5.6 p65 levels are not affected by exposure to TCDD during influenza virus infection…….87

5.7 Exposure to TCDD during influenza virus infection does not alter *p*-IκB protein levels 88

5.8 The AhR and p65 subunit of NFκB do not coimmunoprecipitate…………………….……89

5.9 Activation of the AhR decreases IL-12 levels in the lung during viral infection….……91

5.10 Exposure to TCDD does not alter IL-15 levels in the lung……………….…………...92

5.11 Exposure to TCDD does not alter IFN mRNA expression in the lung………………....92

5.12 Structures and WHO TEFs for TCDD and other selected AhR ligands.……………..……..94

5.13 Activation of the AhR decreases host resistance to influenza virus infection……..…….95

5.14 Exposure to TCDD decreases CD8+ T cell numbers in the lung…………………..…….95

5.15 Exposure to TCDD or PCB 126 increases pulmonary neutrophil numbers during infection……………………………………………………………………………..…..96

5.16 Activation of the AhR alters IL-12 and IFNγ levels in the lung during infection…..…..97

CHAPTER ONE

Introduction

Polyhalogenated aromatic hydrocarbons (PHAH) belong to a family of structurally-related compounds, which includes dibenzodioxins, dibenzofurans and biphenyls. PHAH are very lipophilic and halogenation renders them relatively resistant to biochemical degradation (1). Therefore, these chemicals generally have long half-lives and tend to bioaccumulate in mammalian tissues (2). In fact, the most toxic member of this family, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has an estimated half-life of 7-11 years in humans (3) and at least 10 years in the soil (4). Given the stability and persistence of these chemicals, many studies have been conducted to determine the toxicity of PHAH. As a result, TCDD and other PHAH have been shown to cause numerous toxic effects in humans and laboratory animals. In particular, these compounds cause significant toxicity in many organ systems including the liver and the lung, as well as in the reproductive and immune systems. In fact, the immune system is especially sensitive to the effects of these chemicals because exposure to very low doses causes significant immunosuppression, elevated inflammation, and decreased host resistance.

While the immunotoxic effects have been well documented, the specific mechanisms that drive TCDD-mediated immunotoxicity are not fully elucidated. The overall goal of this thesis project was to characterize the effects of exposure to TCDD on the immune response to influenza virus. Specifically, these studies focus on understanding; 1) how TCDD affects viral clearance in the lung, and 2) the mechanisms that drive overproduction of IFNγ in the lungs of TCDD-treated mice.

I. Sources and exposure

PHAH are widely disbursed and persistent environmental contaminants that are produced by a multitude of anthropogenic and natural processes. Some PHAH, such as polychlorinated biphenyls (PCBs), have high chemical and thermal stability, and thus, were produced for industrial purposes as early as the 1920's. Due to these properties, PCBs were widely used as dielectric fluids in transformers and capacitors, as plasticizers, and as components of carbonless copy paper. PCB production and use continued until health and environmental concerns led to their regulation and decreased production in the late 20th Century (5). However, in spite of the usefulness of PCBs, most PHAH, including dioxins and furans, are produced as unwanted byproducts of many manufacturing processes. These include industrial chlorine bleaching (such as bleaching of paper products) and the production of chlorinated compounds including precursors to polyvinyl chloride or PVC (5). Currently, the main source of PHAH in industrialized countries is the incineration of chlorine-containing organic compounds in medicinal and municipal wastes (5-7).

Many PHAH, including dioxins and coplanar PCBs, elicit their toxic effects via activation of the aryl hydrocarbon receptor (AhR). The relative toxicities of these compounds are determined by examining the relationship between the potency of the ligand and its ability to cause AhR-mediated induction of cytochrome P4501A1 (8). Using this relationship, each ligand is given a toxic equivalency factor (TEF). TCDD has the highest binding affinity for the AhR, and thus is the most toxic member of the PHAH family. As a result, TCDD has been given a TEF of 1 (9). The TEF of all other AhR ligands is determined by comparing their toxic potency to that of TCDD. Examples of the chemical structures and TEFs of some AhR ligands are shown

in Figure 1.1. However, not all PHAH bind to the AhR (e.g. non-coplanar PCBs), and thus do not have a TEF.

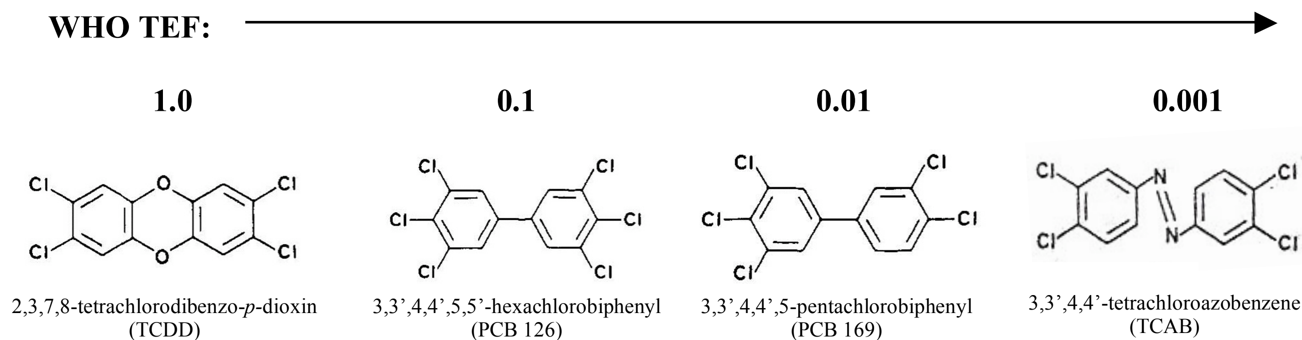

Figure 1.1 Structures and WHO TEFs for TCDD and other common pollutants. While the ligands have similar structures, their binding affinities for the AhR and subsequent toxicities are quite different. Structures adapted from Goldstein and Safe, 1989 (8), TEFs taken from Gilpin *et al.* 2003 (10).

PHAH have distinctive chemical and physical properties including high lipophilicity, low volatility, and resistance to degradation, which taken together allow for bioaccumulation of these chemicals in the environment. Therefore, the majority of human exposure is through the consumption of contaminated foodstuffs, especially food containing high levels of animal fats. For example, freshwater and marine fish, pork, beef, poultry and dairy products are the most significant food-related sources of dioxins (11,12). In fact, in contrast to acute exposures, humans consume relatively low amounts during their everyday lives. In 2000, a report from the United States Environmental Pollution Agency estimated that the daily dietary intake of dioxins, furans and coplanar PCBs in the United States is approximately 1 pg TEF/kg body weight. This exposure is close to the tolerable daily intake (1-4 pg TEF/kg body weight) set by health authorities in several countries including the United States, United Kingdom, Germany, Japan, Belgium and Australia (13).

While daily exposure of humans is generally low, with few reported overt adverse health effects, several incidents have occurred in which people have been exposed to high levels of dioxins and PCBs via inadvertent contamination of the food supply, industrial accidents, or deliberate poisoning. For example, TCDD in particular became a public concern when it was discovered as a contaminant of the herbicides 2,4-D and 2,4,5-T. These compounds were the primary components of the defoliant Agent Orange as well as many other herbicides that were utilized in Southeast Asia during the Vietnam War. This led to the exposure of many people to TCDD. Because of this, multiple studies have been performed on Vietnam War Veterans in order to characterize adverse effects resulting from this accidental exposure (14,15). In addition, an explosion of a chemical manufacturing plant in Seveso, Italy in 1976 resulted in the acute exposure of more than 17,000 people to high levels of dioxins. Subsequently, almost 30 years of epidemiological research has been performed on this population (16). Furthermore, in 1968 and 1979, people in Japan (Yusho) and Taiwan (Yu-chen) respectively, were poisoned following the consumption of PCB-contaminated rice oil, leading to multiple toxic effects.

More recently, in 1999, a mixture of PCBs contaminated with dioxins was accidentally added to recycled fat stock that was used in the production of animal feed in Belgium. This contamination resulted in a major food crisis throughout Belgium, which led to the development of a large PCB/dioxin food-monitoring program (17). Fortunately, this monitoring program aided in the fast detection and containment of another contamination in January of 2006, which called for the quarantine of over 100 Belgian pork and poultry farms (18). In 2005, Ukrainian presidential candidate, Viktor Yushchenko was intentionally poisoned with a large dose of dioxin. At the time of his overt illness, experts estimated that his dioxin body burden was at least 1000 times that of the average person (19). While most of these well-documented exposures

have been the result of accidents, the threat of using these chemicals for bioterrorism has now come to the forefront of public attention, and it remains to be seen whether dioxin will become a prevalent agent of terrorism.

II. Mechanism of toxicity

Most, if not all of the toxic effects of PHAHs are mediated by activation of the aryl hydrocarbon receptor (AhR). The AhR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family of DNA binding proteins. The AhR is a novel member of this family because it is an orphan receptor and the only PAS protein known to bind to exogenous ligands. Since the AhR is a ligand-activated transcription factor, its activation is thought to lead to toxicity by the inappropriate activation of Ah-responsive genes. However, for many of the known consequences of exposure to TCDD (or other AhR agonists) the target genes affected by AhR activation have yet to be identified.

Prior to ligand binding, the AhR is found in the cytoplasm associated with several chaperone proteins including a dimer of heat shock protein 90 (hsp90, 20), a c-Src protein kinase (21), and an immunophilin-related protein (AIP1, 22). AhR ligands tend to be highly lipophilic, and thus are able to diffuse across the plasma membrane and bind to the AhR in the cytoplasm. Once ligand is bound, the chaperone proteins dissociate and the AhR:ligand complex enters the nucleus where it binds to another bHLH-PAS protein called the AhR nuclear translocator (Arnt). Binding to Arnt allows the AhR:ligand complex to convert into its high affinity DNA binding form (23,24), which then binds to core sequences (5'-GCGTG-3'), referred to as AhR response elements (AhRE) on the upstream regulatory regions of many AhR-responsive genes (Figure 1.2). This binding either upregulates or suppresses gene expression depending upon 1) the other

proteins that bind to the AhR and 2) the conformational change induced by AhR activation on the promoter region of the gene.

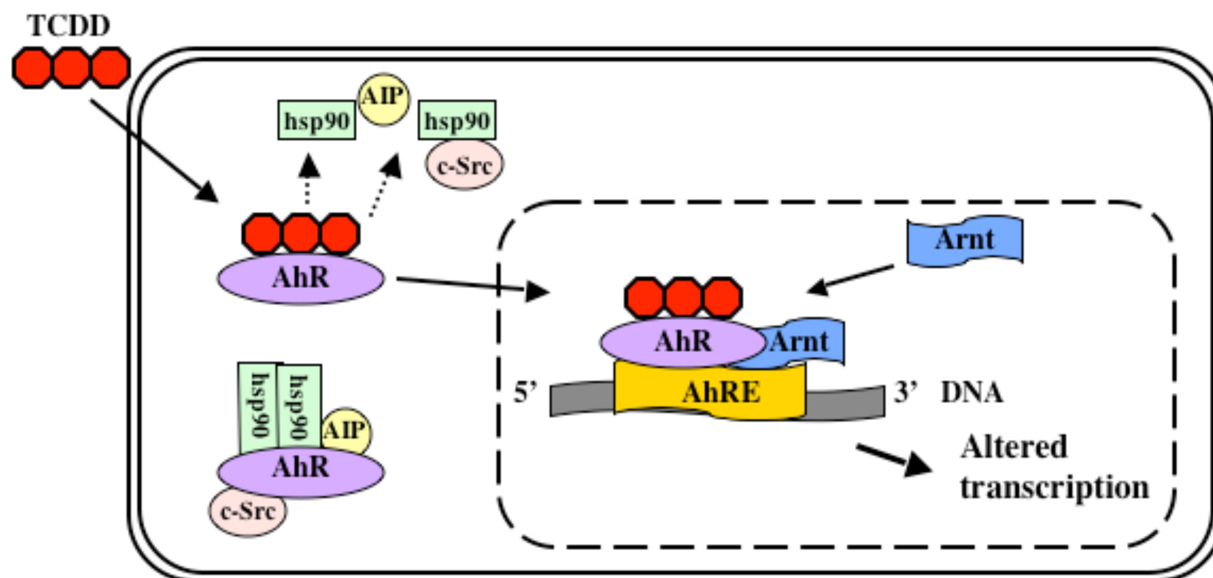

Figure 1.2 The AhR activation pathway. See text for details. Abbreviations include: AhR nuclear translocator (Arnt); heat shock protein 90 (hsp90); AhR-interacting protein (AIP); src protein kinase (c-Src); and AhR response element (AhRE). Adapted from Whitlock (25).

While many of the toxic effects of TCDD and other PHAHs are known to be driven by direct AhR-DNA binding, studies have shown that there are likely alternative mechanisms by which AhR activation leads to altered cellular function. These include protein phosphorylation, transcription factor crosstalk, competition for Arnt binding and interference with the physiological function of an endogenous ligand. Summaries of evidence for each of these mechanisms are listed below.

A. Protein phosphorylation and transcription factor crosstalk

Exposure to TCDD alters intracellular signaling pathways that involve protein phosphorylation, calcium, or transcription factors such as estrogen, NFκB and AP-1 (26-

28). One possible mechanism for these changes is that TCDD binding to the AhR causes c-Src dissociation in the cytoplasm, which then goes on to phosphorylate growth factor receptors and recruit other signaling proteins (21,29). In addition, TCDD is a potent antagonist of estrogen-mediated signaling and can both deregulate estrogen receptor expression as well as increase estrogen metabolism (30-32). Interaction between the AhR and estrogen may also occur by the AhR binding to AhRE that overlap with estrogen response elements (ERE) on the promoter regions of estrogen-regulated genes (33-35). It is also possible that the AhR competes with the estrogen receptor for SRC-1 and RIP 140 binding (30). Thus, exposure to TCDD or other PHAHs may lead to indirect AhR-mediated changes in cell signaling via disruption of estrogen-mediated pathways. Furthermore, the AhR has been shown to associate with other transcription factors including the p65 subunit of NFκB (28). The AhR:p65 complex is thought to bind to either AhRE or κB sites on the promoter regions of AhR- and NFκB-inducible genes, thus providing another mechanism for altered cellular function.

B. Competition for Arnt binding

In addition, by binding to Arnt, the active AhR may prevent Arnt from associating with other proteins. Since Arnt is important for regulating several diverse cellular responses including hypoxia, glucose deprivation and angiogenesis (36), sequesterization of Arnt by the AhR may lead to deregulation of many signaling pathways. For example, Arnt dimerizes with another member of the bHLH-PAS family, hypoxia-inducing factor 1α (HIF1α). This dimerization allows for HIF1α-mediated signaling and studies have shown that the AhR and HIF1α compete for the use of Arnt as a partner (37-39). Arnt

also dimerizes with other proteins including the transcription factors Single Minded (SIM) 1 and 2 (40). While the SIM1/Arnt complex causes transactivation, SIM2 competes with SIM1 for Arnt binding and quenches the SIM1/Arnt signaling, suggesting that there is antagonism between these two pathways. Therefore, it is possible that the AhR competes with many other proteins for Arnt, thus potentially causing alterations in a wide range of cellular processes.

C. Interference with physiological function and endogenous ligand binding

Another alternative to direct AhR-DNA binding is that by interacting with exogenous chemicals, the AhR is not able to perform its physiological function. Although the endogenous ligand and function of the AhR are not currently known, many researchers have discovered that the AhR binds to many naturally-occurring compounds such as tryptophan metabolites, indole 3 carbinol, carotenoids, flavonoids, indirubin, indigo and polyphenolics (41-45). Furthermore, the AhR may play a role in a diverse range of biological functions including liver and cardiac development, cell proliferation, cell cycle regulation and cytokine production (26,28,46-55). These studies suggest that the AhR likely has many diverse physiological functions that have yet to be characterized. However, by understanding the mechanism(s) that drive(s) AhR-mediated toxicity, we may be able to determine not only the endogenous function of this receptor, but also how to prevent the toxic effects of its activation by environmental contaminants.

III. Toxicity in humans

While human exposure studies are limited, the most characteristic and commonly observed PHAH-induced lesion in humans is chloracne (56,57). Chloracne is characterized as a persistent acne-like disease, which presents with the formation of cystic acne, hyperplasia and hyperpigmentation of the skin (58). Interestingly, this condition was described as early as 1897-1899 in workers that were exposed to tarry wastes remaining from the production of chlorine (59). Since then, incidences of chloracne have been reported in almost every population that has been highly exposed. This includes the recently poisoned Russian President Viktor Yushchenko, those exposed during the Seveso, Yusho and Yucheng incidents, as well as laboratory workers exposed to pure TCDD (60,61). Although the cases of chloracne in Seveso residents resolved within 10 years of the accident, it can persist for over 30 years after the original exposure in other populations (62). Thus, while this condition may not be life threatening, it is a clear biomarker of PHAH exposure.

Although chloracne is the most obvious symptom of human PHAH exposure, other toxic effects have been reported in humans including immune system and reproductive system defects as well as developmental abnormalities. In the context of immune system deficiencies, elevated PHAH levels have been associated with an increase in the development of otitis media and overall infection rates in Canadian Inuits (63,64). This population is an interesting epidemiological group because they consume large quantities of high-fat fish and whale products, which causes the accumulation of high PHAH levels in these individuals when compared to the general public (65). Furthermore, exposure to PHAHs has been associated with an increase in the development of skin and respiratory infections, chronic obstructive pulmonary

disease (COPD), alterations in antibody production and changes in the number and function of peripheral lymphocytes (66,67).

PHAH exposure in humans has also been associated with reproductive and developmental toxicity. For example, the incidence of abnormally low testosterone levels was 2-4 times higher in trichlorophenol workers with elevated serum TCDD concentrations (20-244 pg/g) compared to those in unexposed controls (68). Likewise, developmental effects have been reported in children born to highly exposed mothers in Taiwan and Japan. These children had low birth weights, decreased height and muscle development, and abnormal lung auscultation compared to control populations (69-72). This decrease in birth weight was also observed in infants born to PCB- and TCDD-exposed mothers in northern Europe and the Netherlands (32). Many of these findings are supported by data from studies using PHAH-exposed laboratory animals, and will be discussed more thoroughly in upcoming paragraphs.

In 1997, TCDD was classified as a class I human carcinogen by the International Agency for Research on Cancer (IARC) and again by the United States Environmental Protection Agency (USEPA) in 2000 (11,73). Studies have characterized TCDD as a multi-site carcinogen in humans, due to its potent tumor promoting effects. While TCDD has been shown to cause cancer in experimental animals, correlations between cancer rates and TCDD exposure in humans have been harder to demonstrate. The classification of TCDD as a known human carcinogen was based primarily on animal studies and the increased overall cancer mortality from highly exposed industrial workers in the United States, the Netherlands, Germany and other industrialized-countries (74). Various other studies on PHAH-exposed populations show an increased risk for soft tissue carcinomas, respiratory cancers, rectal and liver cancers as well as non-Hodgkin's lymphoma (75-77). While these correlations have been documented, many of

these studies, were performed on small populations, thus making statistical power a limiting factor. Although more evidence about the role of TCDD in carcinogenesis is likely to come, these limited studies suggest that exposure to TCDD increases the risk of developing multiple types of cancer.

IV. Toxicity in laboratory animals

In contrast to the limited amount of data on TCDD-mediated toxicity in humans, numerous studies examining the effects of TCDD and other PHAHs have been performed in animal models. While we have learned much about the toxic effects of PHAH from animal studies, it has been difficult to extrapolate the findings of these studies to potential human health risks due to the variation of sensitivity across species. One endpoint of TCDD-mediated toxicity that demonstrates this interspecies variation is lethality. For example, the LD_{50} of TCDD in guinea pigs is only 0.6-2 μg/kg, whereas in mice it ranges from 114-284 μg/kg. Interestingly, hamsters seem to be the least sensitive rodents to TCDD-mediated lethality with an LD_{50} of > 3000 μg/kg (reviewed by 78). Moreover, not only are there significant differences between species, there are also intraspecies variations. For instance, in Long Evans rats the LD_{50} is as low as 10-20 μg/kg, but it is greater than 9600 μg/kg in the Han/Wistar rat strain (79).

Interestingly, by observing these differences, researchers were able to determine that the ability of TCDD to cause toxicity is not only AhR-mediated, but it depends on the specific allele of the AhR that is expressed. For example, in mice, four different AhR alleles have been identified, three with high binding affinities ($Ah^{b\text{-}1}$, $Ah^{b\text{-}2}$, $Ah^{b\text{-}3}$) and one with low binding affinity (Ah^{d}) (80). These findings are important because they not only help explain why different species have varying sensitivities to the toxic effects of TCDD, but also present a

reason why some individuals in the same population may respond differently than others due to their genetic makeup. By using these multiple animal models and dosing paradigms, we have gained insight to the mechanism that drives TCDD-mediated toxicity (i.e. AhR activation). Furthermore, we have been able to characterize the toxic effects caused by exposure to this pollutant, many of which are summarized in the following paragraphs.

A. Wasting syndrome and thymic atrophy

The development of cachexia is one of the most common symptoms of exposure to lethal doses of TCDD and related compounds. During this syndrome, animals begin to lose weight shortly after exposure and this weight loss continues slowly until death. This syndrome has been attributed to a decrease in food intake, not a change in metabolism and nutrient absorption (81). However, the wasting syndrome is not solely responsible for death as (82) showed that the weight loss, but not lethality can be prevented by parenteral feeding.

Another hallmark of TCDD exposure is thymic atrophy, which is characterized by reduced T cell numbers in the thymus. While extensive studies have been performed in many laboratories over the years, the mechanisms that drive TCDD-mediated thymic atrophy are not fully understood. However, it is unlikely that thymic atrophy in adult animals contributes to the immunosuppressive effects of TCDD, since the thymus does not play an active role in adult immunity (83) and the doses required to cause thymic atrophy are significantly higher than those that cause immunosuppression (84-86). Furthermore, the T cell response to SRBC in thymectomized adult mice was suppressed by TCDD treatment (87,88), again suggesting that the immunotoxic effects of TCDD are unlikely related to thymic atrophy.

B. Hepatotoxicity

The liver is a well-characterized target organ of TCDD-mediated toxicity. In fact, one of the hallmark effects of exposure to TCDD is the induction of three cytochrome P450 enzymes: cyp1A1, cyp1A2 and cyp1B1. Generally, these enzymes oxidize xenobiotics, including some polyaromatic hydrocarbons, plant products such as flavones and some pharmaceuticals. While many PHAH strongly induce these enzymes, some (including TCDD) are not readily oxidized due to the presence and placement of halogen atoms (5). This lack of degradation causes accumulation of these chemicals in the liver, which in combination with elevated metabolic enzymes leads to hepatomegaly, inflammatory cell infiltration, steatosis, multinucleated hepatocytes and focal necrosis, which is usually located in centrilobular regions (reviewed in 89). Recently, using cyp knockout mice, TCDD-induced hepatoxicity has been attributed to elevated cyp1A1 and 1A2 expression (90,91). Therefore, it is clear that treatment with TCDD causes significant liver damage and these endpoints, specifically the induction of metabolizing enzymes, are often used as biomarkers of TCDD exposure.

C. Tumor promotion and tumor suppression

In addition to the hepatotoxic effects of TCDD described above, the liver has been recognized as a cancer endpoint for dioxin in chronic exposure studies in rodents. The carcinogenic effects of TCDD are not due to direct genotoxicity. Instead, TCDD is a potent tumor promoter and in addition to the liver it increases tumor development in the lung, adrenal cortex, nasal turbinates, thyroid gland and tongue (92,93). On the other hand, TCDD

has tumor suppressive properties, characterized by a significant decrease in the incidence of uterine, mammary gland, and pituitary tumors in female rats and reduced development of adrenal and pancreatic tumors in males (92). This decrease in hormone-dependent cancers is likely related to the ability of TCDD to alter estrogen signaling and metabolism. These findings demonstrate that activation of the AhR has both tumor promoting and tumor suppressing action, depending on which tissues are involved.

D. Reproductive toxicity and teratogenicity

Many studies have been performed to examine the reproductive and teratogenic effects of TCDD and PHAHs in various species including fish, birds, rodents and humans. When administered to adult male rodents, TCDD decreases testis and accessory organ weights, alters testicular morphology and reduces fertility and spermatogenesis. Many of these effects are due to decreased testosterone levels in these animals (94,95). Likewise, exposure to TCDD in adult female rodents reduces uterine weight and fertility, decreases litter size and causes structural deformities in the female gonads (32). Furthermore, defects in mammary gland formation and differentiation have been observed in both rats and mice following exposure to TCDD (96-98). This decrease in mammary development may impair the ability of these animals to support their offspring.

Moreover, recent studies show that the developing fetus is extremely sensitive to TCDD-mediated toxicity. For example, gross abnormalities such as cleft palate and hydronephrosis have been documented in prenatally-exposed animals (32). Interestingly, male offspring are especially sensitive to the toxic effects of *in utero* and lactational exposure to TCDD, which is demonstrated by reduced sperm counts, decreased reproductive organ weight and altered

sexual behavior in these animals (99). Developmental exposure to TCDD also affects the female reproductive system by decreasing fertility and altering estrous cycles in rats (99). Furthermore, exposure to TCDD *in utero* and during lactation causes immune system impairment during adulthood (100,101), suggesting that prenatal exposure to TCDD causes lasting changes in many organ systems.

E. Immunotoxicity

TCDD is a well-characterized immunosuppressant, with immunotoxic effects observed at doses that are not overtly toxic to the animal and do not cause thymic atrophy (84). While the molecular mechanisms and direct cellular targets of TCDD remain unknown, numerous studies have shown that exposure to TCDD suppresses both the antibody and cell-mediated arms of the immune system (reviewed in 102, 103). This significant immunosuppression is thought to contribute to decreased host resistance of TCDD-treated mice to many different pathogens including bacteria (Salmonella and Listeria) and viruses (Herpesvirus type II and influenza A) (104). In fact, the increased mortality observed in TCDD-treated, influenza virus-infected mice represents one of the most sensitive adverse effects of PHAH reported to date (105). In addition to its overt effects on host resistance, the effects of TCDD on specific cells of the immune system have been arduously studied. The findings of many of these studies are described in further detail below.

1. TCDD and lymphocytes

a. TCDD and T cells

In vivo exposure to TCDD causes a dose-dependent suppression of T cell number and functions, including proliferation, differentiation and cytokine production, and T cell-dependent B cell responses (102). Specifically, exposure to TCDD impairs T cell proliferation (106-110), suppresses DTH responses (111), decreases cytolytic activity (85,112) and alters cytokine production in response to multiple antigens (106,107,109,113-115).

While TCDD-mediated effects on T cells have been laboriously studied, the mechanisms that drive the decreased T cell function are not fully understood. However, it is known that exposure to TCDD impairs T cell functions via an AhR-mediated mechanism (116, and our unpublished data) and that T cells can be direct targets of TCDD-mediated toxicity (117). Unfortunately, the mechanisms that cause TCDD-mediated T cell suppression have been difficult to identify because TCDD does not affect T cells in *vitro* (118). However, several different mechanisms have been shown to responsible for AhR-mediated decreased T cell responses, including increased apoptosis (119), induction of anergy (107) and inhibition by suppressor T cells (120). The findings of these numerous studies demonstrate that TCDD causes significant suppression of T cell expansion and function via multiple different mechanisms.

b. TCDD and B cells

Similar to T cells, B cell functions are suppressed following exposure to TCDD. For example, *in vitro* and *in vivo* exposure to TCDD decreases B cell proliferation and antibody (IgM) production (88,112,113,115,121-124). Also like T cells, B cells express the AhR, thus making them possible direct targets of AhR-mediated toxicity (125,126). In fact, inhibition of IgM protein secretion from B cells following exposure to various dioxins was shown to be dependent upon AhR binding affinity (126). In addition, two AhREs have been located in the heavy chain of Ig μ, (127), again suggesting that these cells are direct targets of AhR activation. In contrast, however, Nagai *et al.* (128) recently showed using a model of OVA immunization, that the suppression of antibody production by B cells was due to a decrease in G-protein-linked signaling in $CD4^+$ T cells, suggesting that B cells are not direct targets of TCDD-mediated toxicity. Therefore, although exposure to TCDD clearly alters B cell functions, the mechanisms by which TCDD causes these changes are not fully elucidated.

c. TCDD and NK cells

Although many groups have characterized the immunosuppressive effects of AhR agonists on B and T lymphocytes, very few studies have examined the effects of TCDD on NK cell function, and their findings are not consistent. Specifically, one study showed that NK cell activity was *increased* in the blood and spleens of mice exposed to TCDD (129). In contrast, Yang *et al*. (130), observed that NK cell cytolytic activity was *decreased* in the lungs of TCDD-treated, influenza virus-infected rats. Two additional studies have examined the effects TCDD exposure on the cytolytic activity of NK cells.

Using Yac-1 cells as targets, both of these studies demonstrated that NK cell-specific cytolytic activity was *not altered* in spleen cells isolated from naïve, TCDD-treated mice (131,132). Therefore, unlike lymphocytes, studies examining the immunomodulatory effects of TCDD on NK cells are limited and their findings are inconsistent.

2. TCDD and non-lymphocytes

a. TCDD and phagocytic cells

While many studies have examined the effects of TCDD on lymphocytes, much less is known about how TCDD alters phagocytic cell function. Similar to NK cells, the limited studies examining the effects of TCDD on macrophages and neutrophils have varying results. On the one hand, several laboratories have reported that TCDD decreases or does not alter macrophage and neutrophil numbers and cellular functions at the site of antigen challenge. For example, exposure to TCDD decreased LPS-induced production of IL-1 by splenic macrophages (133), and neutrophils isolated from the spleen, peritoneum or blood of TCDD-treated mice had a decreased ability to lyse neutrophil sensitive tumor cell targets (134). In contrast, multiple studies have shown that TCDD does not affect macrophage number or function. For example, our laboratory and others have shown that exposure to TCDD does not increase the number of alveolar macrophages in the lungs of TCDD-treated, influenza virus-infected mice (115,135). Furthermore, TCDD does not affect peritoneal macrophage cytolytic activity, phagocytosis, H_2O_2 or cytokine (IL-1, IL-6, IL-8) production (104,131,132,136,137). Likewise, TCDD-exposure does not alter the production of superoxide anion and hydrogen peroxide by peritoneal or pulmonary neutrophils (138,139).

On the other hand, several studies have shown that exposure to TCDD not only increases the number of macrophages and neutrophils at the site of antigen challenge, but alters the function of these cells. Specifically, TCDD elevates the number of peritoneal macrophages and their activity toward the antigen sheep red blood cells (140). In addition, the number of neutrophils in the lungs of TCDD-treated mice is increased following influenza virus infection (114,115,135,139). Furthermore, TCDD induces the production of tumor necrosis factor (TNF) and reactive oxygen species by peritoneal macrophages both *in vivo* and *in vitro* (141,142). Choi *et al.* also showed that superoxide anion production by neutrophils isolated from the blood and spleens of TCDD-treated, P815 tumor cell challenged mice is elevated compared to levels in control animals (134). These findings are of interest because macrophages and neutrophils play an extensive role in the development of inflammation. The fact that TCDD can increase the number of these cells at the site of antigen challenge, as well as elevate the production of pro-inflammatory mediators by these cells suggests that exposure to TCDD may lead to the development and/or progression of inflammatory disorders. Therefore, it is of interest to further investigate the mechanisms that underlie TCDD-mediated activation of phagocytic cells.

b. TCDD and inflammatory cytokines

Studies have shown that exposure to TCDD disrupts the immune response by altering the production of pro-inflammatory cytokines produced by many different cell types including T cells, macrophages and NK cells. For example, treatment with TCDD alters the production of interferon αβ (IFNαβ), IFNγ, interleukin-1 (IL-1), IL-12, and tumor

necrosis factor-α (TNFα) in several different exposure paradigms (107,115,142-146). Interestingly, TCDD can cause site-specific effects on cytokine production, leading to both increased and decreased levels of the same cytokine, in different organs. For example, in the context of infection with influenza virus, exposure to TCDD prior to infection greatly suppresses IFNγ production by T cells in the draining lymph node (107,115). In contrast, in the same animals, exposure to TCDD during infection causes a 2 to 4-fold increase in the amount of IFNγ present in the lungs of TCDD-treated mice (115). Thus, it is likely that deregulated cytokine production following exposure to TCDD may lead to an exacerbated inflammatory response which may play a role in TCDD-mediated decreased survival to infection.

V. Objectives

In order to delineate the mechanisms of TCDD-mediated immunotoxicity, our laboratory uses a model of infection with a murine-adapted strain of human influenza A virus (H3N2). Perturbation of the pulmonary immune response to influenza virus is of interest because the lung is the site of exposure to many AhR ligands, including dioxins and components of diesel exhaust and cigarette smoke. Furthermore, in mice and humans, successful recovery from influenza virus infection requires activation of the innate and adaptive arms of the immune system, both of which are disrupted by exposure to TCDD. Thus, using a model of infection with influenza A virus provides an elegant mechanism for examining the effects of TCDD on both innate and adaptive immunity that is relevant to human health.

Studies in our laboratory have shown that mice treated with TCDD have a dose-dependent decrease in survival following a sublethal infection with influenza virus (114,115).

Exposure to TCDD also decreases the number of virus-clearing, cytotoxic T lymphocytes (CTL) in the lung by 50-80% (115,147). This finding is important because CTL are the primary cell type responsible for clearing influenza virus from the lung (reviewed in 148, 149). Interestingly, in spite of fewer CTL numbers in the lungs of TCDD-treated mice, the cytolytic activity of isolated immune cells from the lung is equivalent between TCDD and vehicle-treated mice, and TCDD-treated mice clear the virus at the same rate as vehicle-treated controls (115, 147). While it is clear that TCDD-treated mice do not die from enhanced pulmonary viral loads, it is not known what mechanisms are responsible for viral clearance, or what ultimately leads to the decreased survival of these animals.

Dysregulation of the innate immune response could account for the decreased host resistance of TCDD-treated mice. While many groups have studied the immunosuppressive effects of TCDD on B and T lymphocytes, much less is known about the effects of AhR activation on cellular components of the innate immune response. We do know that exposure to TCDD disrupts the early immune response by altering the production of cytokines by cells of the innate immune system. For example, studies have shown that exposure to TCDD modulates the production of cytokines produced by macrophages and NK cells, including IFNαβ, IFNγ, IL-1, and TNFα in several different exposure paradigms (107,115,142-146). In the context of infection with influenza virus, exposure to TCDD prior to infection greatly alters pulmonary IFNγ levels, demonstrated by a 4-fold increase in the amount of IFNγ present in lung lavage fluid isolated from TCDD-treated mice. This finding is significant because while some IFNγ is important in the immune response to viral infection, excess IFNγ can lead to severe inflammation and tissue damage (150-153). Thus, the enhanced pulmonary IFNγ levels might account for the decreased survival of TCDD-treated mice.

While studies in our laboratory have shown that TCDD-treated, influenza virus-infected mice do not die from enhanced pulmonary viral burdens, the mechanism that underlies their decreased survival is unknown. In addition, since there are fewer CTL in the lungs of TCDD-treated mice, the finding that pulmonary cytolytic activity is not decreased suggests that another mechanism besides CTL is responsible for viral clearance. Therefore, the goals of the studies presented in this dissertation were 1) to determine the mechanism responsible for viral clearance from the lungs of TCDD-treated mice; 2) to characterize the mechanism that drives the excessive IFNγ production in the lung; and 3) to examine the role of the excess IFNγ in the decreased host resistance of these animals.

CHAPTER TWO

Fewer CTL, not enhanced NK cells, are sufficient for viral clearance from the lungs of immunocompromised mice[1]

ABSTRACT

Activation of the aryl hydrocarbon receptor (AhR) causes numerous defects in anti-viral immunity, including suppressed CTL generation and impaired host resistance. However, despite a reduced CTL response, mice that survive infection clear the virus. Therefore, we examined the contribution of NK cells and pro-inflammatory cytokines to viral clearance in influenza virus-infected mice exposed to TCDD, the most potent AhR agonist. Infection caused transient increases in pulmonary TNFα, IL-1 and IFNα/β levels, but neither the kinetics nor magnitude of this response was affected by AhR activation. No IL-18 was detected at any time point examined. Exposure to TCDD enhanced NK cell numbers in the lung but did not affect their IFNγ production. Furthermore, depletion of NK cells did not alter anti-viral cytolytic activity. In contrast, removal of $CD8^+$ T cells ablated virus-specific cytolytic activity. These results demonstrate that the pulmonary CTL response to influenza virus is robust and few CTL are necessary for viral clearance.

INTRODUCTION

Infection with influenza virus causes epidemic disease and leads to substantial morbidity and mortality (154,155). Since the virus is transmitted via large-particle aerosols, the initial site

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[1] Data from this chapter were reprinted from: *Cellular Immunology*, 226, Neff-LaFord, H.D., Vorderstrasse, B.A., and Lawrence, B.P., Fewer CTL, not enhanced NK cells, are sufficient for viral clearance from the lungs of immunocompromised mice, p. 54-64 (2003), with permission from Elsevier.

of infection is usually the nasopharynx. The infection generally remains localized, with viral replication restricted to respiratory epithelial cells (156). The mechanisms mediating viral clearance have been extensively studied, and it is apparent that redundant host protective pathways are employed by the immune system in response to this pathogen. This redundancy is demonstrated by the fact that, while killing of infected epithelial cells by $CD8^+$ T cells constitutes the main mechanism of viral clearance in a primary infection, clearance of influenza virus also occurs in the absence of $CD8^+$ T cells (reviewed by 148, 149). For example, Eichelberger *et al.* (157) and Allan *et al.* (158) demonstrated that $CD4^+$ T cells eliminate virus-infected cells when $CD8^+$ T cells are depleted. However, depletion of both $CD4^+$ and $CD8^+$ T cells results in a fatal infection (159), demonstrating that host resistance and viral clearance are T cell-dependent.

In addition to T cells, NK cells form an important component of the immune response to viral infection. NK cells recognize and kill virus-infected cells, and early production of IFNγ by NK cells plays a critical role in the activation of antigen-specific T cells and anti-viral immunity (160). The importance of NK cells in the immune response to infection with influenza virus has been established. For example, local ablation of pulmonary NK cells increases morbidity and mortality in mice and hamsters infected with influenza virus (161). *In vivo* depletion of NK cells also results in defects in the capacity of $CD8^+$ T cells to mature into CTL, suggesting that, in addition to having potent anti-viral activity, NK cells are important immunoregulatory cells (162,163).

Cytokines are important non-cellular mechanisms in the immune response to influenza virus, and IFNα/β, TNFα and IL-1 in particular play important roles in anti-viral immunity (164). The anti-viral effects of IFNα/β, including inhibition of viral replication, are mediated by

several IFN-inducible gene products and by the interaction of secreted IFNα/β with components of the immune system (165,166). For instance, secreted IFNα/β are potent activators of NK cells, altering their expression of cell surface molecules, cytolytic activity, trafficking and cytokine production (167,168). Likewise, IL-1 and TNFα activate NK cells, inducing IFNγ production and enhancing cytolytic activity (169,170). IL-1 and TNFα also have potent anti-viral activity, upregulate the expression of co-stimulatory molecules on APC, and alter adhesion molecule expression on epithelial and endothelial cells. In addition to IFNα/β, IL-1 and TNFα, IL-18 has been implicated as a critical factor in the early response to viral infection. While the *in vivo* role of IL-18 is less clearly defined, IL-18 is a potent inducer of IFNγ, and viral infection of cultured cells leads to rapid induction of IL-18 (171). Thus, collectively, these cytokines act to decrease viral replication, further activate CTL and NK cells, and regulate the production of downstream cytokines, including IFNγ (170-172).

Our previous work (107,114,115,147) and that of others (105,131,135,173) demonstrate that activation of the aryl hydrocarbon receptor (AhR) disrupts the immune response to infection with influenza virus. The AhR is a member of the bHLH-PAS protein family of transcription factors. While the endogenous biological function and ligands of the AhR are unknown, this receptor binds to a large group of structurally-related xenobiotics including polychlorinated dioxins and polychlorinated biphenyls (PCBs) (42,174). The pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) binds the AhR with the highest affinity and is used as a prototypic ligand to study the toxic and biological consequences of AhR activation (reviewed by 42).

Many immune cells, including T cells, express the AhR, and activation of the receptor is thought to drive functional changes in these cells, altering the regulatory balance of the immune

system. In fact, TCDD is one of the most immunosuppressive chemicals known (102). Activation of the AhR by TCDD and related compounds suppresses T cell-dependent responses to many antigens, resulting in defects in both cell-mediated and humoral immune responses. For example, our laboratory has shown that mice exposed to TCDD have an impaired CTL response and altered virus-specific antibody production following infection with influenza virus (115). With regard to the CTL response, proliferation of virus-specific $CD8^+$ T cells is decreased by 90% in mediastinal lymph nodes (MLN) from TCDD-treated mice, and the infection-associated production of IFNγ and IL-2 is also suppressed (107,115). This reduction in the $CD8^+$ T cell response in the lymph node correlates with 50-80% fewer virus-specific effector CTL in lungs of TCDD-exposed mice as compared to vehicle-treated control mice. In addition to these changes in immune function, activation of the AhR impairs host resistance to influenza virus to the extent that about 50% of TCDD-treated mice challenged with a sublethal infection die within seven to ten days.

Due to the suppressed response of $CD8^+$ T cells in the MLN, the reduced number of CTL in the lungs, and the decreased ability of TCDD-treated mice to survive a sublethal infection, we expected that decreased host resistance was simply due to the fact that mice could not eliminate the virus. However, mice exposed to TCDD that survive the infection successfully clear the virus (105,147). Furthermore, in spite of the decreased number of CTL in the lung, the cytolytic activity of pulmonary immune cells isolated from vehicle- and TCDD-treated mice is equivalent in an *ex vivo* chromium release assay using virus-infected target cells (115). Since there are considerably fewer CTL in the lungs of mice exposed to TCDD, the equivalent cytolytic activity and viral clearance are surprising and raise questions about AhR-mediated induction of cytolytic and anti-viral mechanisms that compensate for a suppressed CTL response.

The purpose of this study was to characterize the compensatory mechanisms responsible for the killing of virus-infected cells in the lungs of TCDD-treated mice. Based on previous studies, a compensatory CD4-driven mechanism of viral clearance is unlikely, as AhR activation decreased the number and percentage of $CD4^+$ lymphocytes in lung airways and interstitial spaces (147, and our unpublished observations). In contrast to lack of evidence for a CD4-mediated compensatory mechanism, activation of the AhR results in pulmonary IFNγ levels that are at least 4-fold higher in the lungs of infected mice (115). Given that NK cells produce IFNγ during viral infection, and that these cells are essential for the development of virus-specific CTL (162,163), the elevated levels of IFNγ suggest that activation of NK cells may underlie the compensatory anti-viral mechanisms invoked in mice exposed to the potent AhR agonist, TCDD. To test this hypothesis, we examined the effects of AhR activation on the magnitude and kinetics of NK cell recruitment to the lung and tested whether exposure to TCDD altered their cytolytic activity and IFNγ production. Additionally, to determine whether differential production of anti-viral cytokines contributes to viral clearance and accounts for enhanced IFNγ levels and NK cell activation, we examined the effects of AhR activation on the levels of IL-1, TNFα, IFNα/β and IL-18 in lung lavage fluid.

MATERIALS AND METHODS

Mice

Female C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used at 7-9 weeks of age. Animals were housed 3-5 per cage in microisolation units in a pathogen-free facility at Washington State University. Animals were given food and water *ad libitum*, and all procedures were conducted in accordance with protocols approved by the University Animal Care and Use Committee.

TCDD exposure and influenza virus infection

Mice were dosed orally with 10 μg/kg TCDD (≥99% purity, Cambridge Isotope Laboratories, Inc. Woburn, MA) dissolved in anisole and diluted in peanut oil. This dose of TCDD is well below the LD_{50} in C57Bl/6 mice (≥ 120 μg/kg; 175). Control mice received an equivalent volume of peanut oil-anisole vehicle. TCDD or vehicle was administered one day prior to intranasal (i.n.) infection. Anesthetized mice (Avertin, 2,2,2-tribromoethanol, Sigma-Aldrich, St. Louis, MO) were infected with 120 hemagglutinating units (HAU) of influenza virus A/HKx31 (H3N2) diluted into 25 μl of sterile PBS. In vehicle-treated mice this dose of virus does not typically cause mortality (115, 147, 176). Mock-infected, vehicle- or TCDD-treated mice received an equivalent volume of PBS (i.n). Animals were sacrificed by asphyxiation with CO_2 at various times relative to infection.

Collection of bronchoalveolar lavage (BAL) cells

Cells infiltrating the lung airways were collected by lavage as described by (158). Briefly, following intubation of the trachea, BAL cells were collected by rinsing the lungs with

three sequential 1 ml washes of RPMI 1640 media containing 1% BSA and 10 mM HEPES. Media from the first wash was retained as BAL fluid. Cells recovered from all three washes were pooled and enumerated using a Coulter Counter (Beckman Coulter, Miami, FL).

Collection of total lung cells

Total lung-derived immune cells were obtained by digesting whole lung tissue with collagenase. Lungs were digested in RPMI 1640 media containing 0.7mg/ml collagenase A (Roche Molecular Biochemicals, Indianapolis, IN), 30μg/ml deoxyribonuclease I (Sigma-Aldrich) and 1% BSA. Following a 90 min incubation at 37°C with 5% CO_2, cell suspensions were layered over Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and centrifuged at room temperature for 20 minutes at 1000 x g. The leukocyte-containing fraction was collected and washed, and cells were enumerated using a Coulter Counter. Greater than 99% of the cells in this fraction were $CD45^+$.

Virus titer

Virus titer was determined using Madin-Darby canine kidney (MDCK) cells followed by a standard hemagglutination assay (177). Briefly, frozen lungs from individual animals were resuspended in 1.2 ml ice-cold RPMI, containing 0.01% BSA, and homogenized using a Tissue Tearor (Biospec Products, Bartlesville, OK). Homogenates were clarified by centrifugation (1000 x g, 4°C, 10 min) and 100 μl of the supernatant was added to 5 x 10^4 viable MDCK cells in eight serial four-fold dilutions. After 4 days incubation at 37°C with 5% CO_2, the culture supernatants were tested for the presence of viral hemagglutinin activity using chicken

erythrocytes (Colorado Serum Co., Denver, CO). Lung virus titers are expressed as the dilution of lung homogenate at which 50% of the MDCK cells revealed virus growth (178).

Bioassays

TNF levels were determined using an L929 bioassay as described by Flick and Gifford (179). Briefly, confluent cultures of L929 cells in 96-well flat-bottomed tissue culture plates were incubated with actinomycin D for 30 min at 37°C, followed by the addition of serially diluted BAL fluid or TNF standards. After overnight incubation, cells were stained with crystal violet, dried and resolublized with 1% SDS. Absorbance values were read at 595 nm on a BioRad Benchmark plate reader. The standard curve was plotted on a log scale and sample concentrations were calculated using linear regression of the standard curve. The limit of detection was 1 pg/ml.

IL-1 levels in BAL fluid were determined using a NOB-1 bioassay (180). Briefly, IL-1 standards (Endogen, Woburn, MA) or BAL fluid samples were added to $2x10^5$ NOB-1 cells in a flat-bottomed 96-well tissue culture plate. Samples and standards were incubated overnight at 37°C with 5% CO_2. On the following day, the NOB-1 cells were centrifuged at 200 x g for 10 min and 200 μl of supernatant was removed. IL-2 was subsequently measured by ELISA according to the manufacturer's protocol. IL-2 standards and matched antibody pairs were purchased from BD Pharmingen (San Diego, CA).

IFNα/β levels in BAL fluid were determined using a cytoprotective bioassay with L929 cells infected with vesicular stomatitis virus (VSV) (181). Recombinant murine IFNα and IFNβ (NIAID Reference Reagent Repository, Gaithersburg, MD) were used to generate the standard

curve. VSV was a gift from Dr. Thomas Jerrells (Omaha VA Medical Center, Omaha, NE). The limit of detection was 2 IU/ml.

ELISA

TNFα and IL-18 levels in BAL fluid were measured using cytokine-specific sandwich ELISAs according to the manufacturers' recommended protocols. TNFα standards and matched antibody pairs were purchased from Endogen (Woburn, MA). IL-18 levels were examined using both an OptEIA™ IL-18 ELISA kit from BD Pharmingen and matched IL-18 antibody pairs from R&D Systems (Minneapolis, MN). The limits of detection for TNFα and IL-18 were 50 pg/ml and 31.3 pg/ml respectively.

***Ex vivo* depletion of NK1.1$^+$ and CD8$^+$ cells**

Cell suspensions were selectively depleted of CD8$^+$ T cells or NK1.1 cells using immunomagnetic beads (Dynal Biotech, Oslo, Norway). BAL cells collected from10 mice were pooled to make a single group. For each treatment group (vehicle or TCDD), three separate pools of BAL cells were made. CD8$^+$ T cells were depleted using anti-CD8α-coated magnetic beads at a 10:1 bead-to-target cell ratio. NK1.1$^+$ cells were removed by first incubating with a purified mouse anti-mouse NK1.1 antibody (Caltag Laboratories, Burlingame, CA) followed by incubation with anti-mouse IgG-coated magnetic beads at a 10:1 bead-to-target cell ratio. Efficiency of depletion was 85-95%, as determined by flow cytometry.

Cytotoxicity assays

Influenza virus-specific cytolytic activity was assessed using a standard 5-h ^{51}Cr-release assay, as described by Nonacs *et al.* (182). Briefly, BAL cells were incubated with ^{51}Cr-labeled, influenza virus-infected EL-4 tumor cells at E:T ratios from 100:1 to 3:1. Cells were incubated for 5 hr at 37°C, 5% CO_2, and released radioactivity was measured using a gamma counter (LKB-Wallac, San Francisco, CA). BAL cells obtained from mock-infected (PBS-treated) mice exposed to either vehicle or TCDD were used as controls. NK cell-specific cytolytic activity against Yac-1 tumor cells was also measured using a standard 5-h ^{51}Cr-release assay (105). BAL cells or total lung cells were incubated with ^{51}Cr-labeled Yac-1 tumor cells at E:T ratios from 50:1 to 3:1. Released radioactivity was counted using a gamma counter.

Immunophenotypic analyses

Lung lavage cells or total lung cells were incubated with combinations of monoclonal antibodies (mAbs) including PE-labeled anti-NK1.1 and TC- or APC-labeled anti-CD8α (Caltag Laboratories, Burlingame, CA). Appropriately labeled isotype-matched mAbs were used to determine nonspecific fluorescence. For all experiments, listmode data were collected on 25,000-50,000 stained cells using a FACScan or FACSort flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using WinList software (Verity Software, Topsham, ME).

Intracellular staining for IFNγ following peptide re-stimulation

Cells were cultured in 24-well plates at 37°C with 5% CO_2 for 5 h in the presence of 12.5 U/ml rmIL-2 (R&D Systems), 1 μM viral peptide ($NP_{366-374}$ ASNENMETM), and 10 μg/ml Brefeldin A (Sigma-Aldrich). Cells were stained with PE-labeled anti-NK1.1 and FITC-labeled

anti-CD8α antibodies, then fixed, permeabilized with 1% saponin, and incubated with an APC-labeled anti-IFNγ mAb (BD Pharmingen) or isotypic control for 30 min at room temperature. Cells were analyzed for phenotype and IFNγ production by flow cytometry as described in the immunophenotypic analysis section.

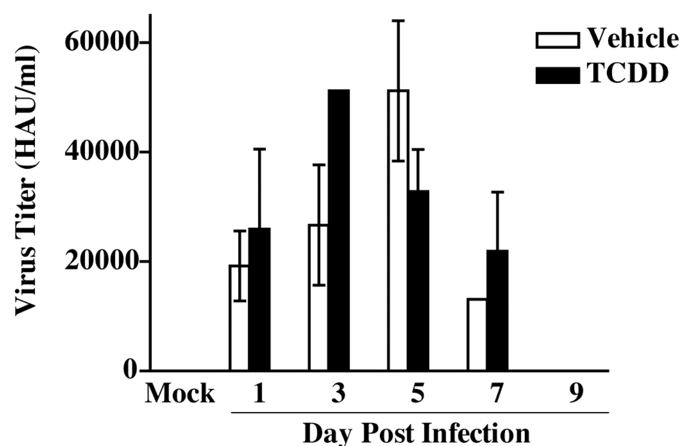
Statistics

Statistical analyses were performed using StatView statistical software (SAS, Cary, NC). Using one-way ANOVA, followed by *post hoc* Fisher's PLSD tests, differences between independent variables were compared over time and between each treatment group. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

Mice treated with TCDD do not have enhanced pulmonary virus burdens and clear influenza A virus from their lungs.

The virus titer of lung homogenates from vehicle- and TCDD-treated mice was measured over the course of infection. Similar pulmonary viral burdens were detected in vehicle- and TCDD-treated mice throughout infection, and mice from both treatment groups had completely cleared the virus from their lungs by day 9 (Figure 2.1). This is consistent with the previously reported time course of influenza virus replication in and clearance from lungs of immunocompetent and TCDD-treated mice (105,147,158,183,184). Therefore, although exposure to TCDD suppresses the CTL response to influenza virus (115,147) TCDD-treated mice do not have higher or more persistent pulmonary viral burdens.



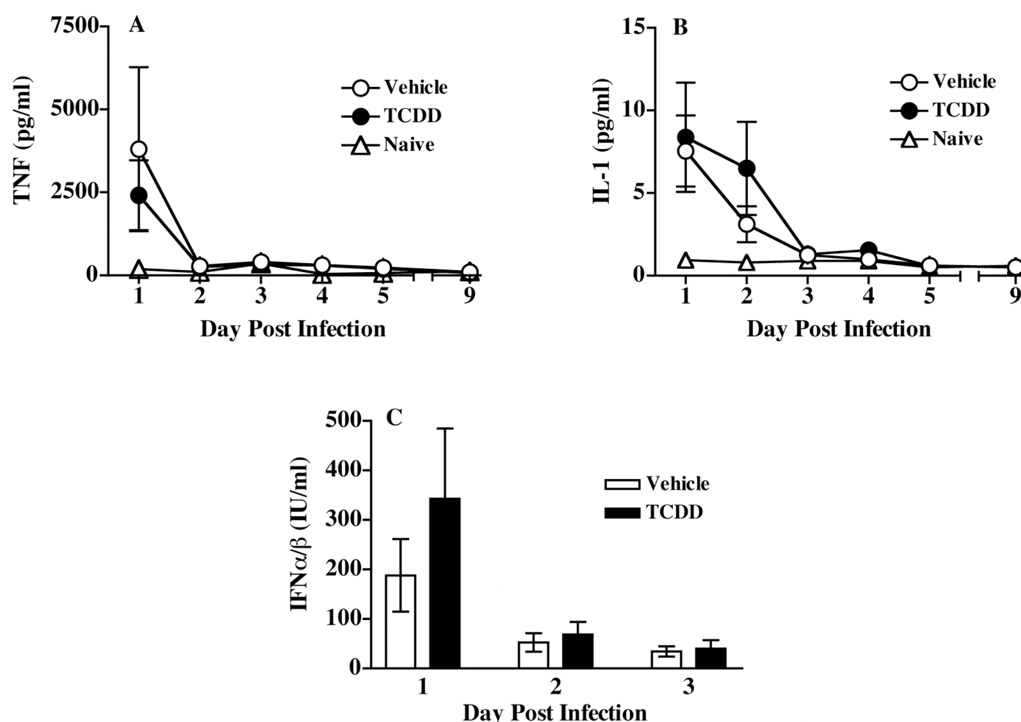
<u>Figure 2.1</u> Exposure to TCDD does not impair viral clearance. C57Bl/6 mice (*n* = 4-5 per group per day) were given vehicle or TCDD (10 μg/kg) orally one day prior to intranasal infection with 120 HAU of influenza virus strain A/HKx31. Animals were sacrificed on the indicated day relative to infection and pulmonary virus titers of individual infected mice were determined by endpoint titration in MDCK cells. The results are expressed as the mean (± SEM) virus titer in hemagglutinating units (HAU)/ml for vehicle- (open bars) and TCDD-treated (closed bars) mice. Lung homogenates from mock-infected mice had no hemagglutination activity. These data are representative of at least three separate experiments.

Activation of the AhR does not affect levels of anti-viral cytokines in lung lavage fluid from mice infected with influenza A virus.

Given that previous studies have shown that exposure to AhR agonists increases levels of IFNα/β, IL-1 and TNF in other model systems (142-146) and that these cytokines have anti-viral properties, one possible mechanism by which AhR activation induces compensatory anti-viral mechanisms is via enhanced production of these cytokines. To test this theory, we compared the infection-associated production of IFNα/β, IL-1 and TNF in lungs of vehicle- and TCDD-treated mice.

As expected, infection with influenza virus increased IL-1, TNF and IFNα/β levels in lung lavage fluid (Figure 2.2). This increase was very rapid and transient, with levels of all cytokines tested returning to baseline by three days after infection. Exposure to TCDD did not

alter the magnitude or kinetics of the infection-associated production of these cytokines in the lung. Therefore, it is unlikely that altered production of TNFα, IL-1, or IFNα/β is responsible for eliminating the virus-infected cells from the lungs of TCDD-treated mice or driving other compensatory mechanisms. Due to the fact that exposure to TCDD greatly elevates IFNγ levels in the lungs of infected mice (115), we examined whether AhR activation also increased the level of IL-18 (formerly designated as IFNγ-inducing factor, 170,185) in the lung. Infection with influenza virus *in vitro* stimulates IL-18 production by macrophages (171), suggesting that the enhanced IFNγ levels observed in TCDD-treated mice may be due to elevated IL-18 production. Therefore, IL-18 levels in lung lavage fluid from vehicle- and TCDD-treated mice were measured over the course of infection starting on day 1. No IL-18 was detected in lung lavage fluid from mice in either treatment group at any time point examined (data not shown; note: IL-18-specific ELISAs were performed using kits from two separate vendors). These results suggest that IL-18 is utilized *in vivo* too rapidly to be measured, is not produced in this immune response, or is below the limit of detection of the assay.



<u>Figure 2.2</u> **Exposure to TCDD does not alter levels of pro-inflammatory cytokines in lung lavage fluid from influenza virus-infected mice.** Infected mice (*n* = 6-10 per group per day) were treated as described in Figure 2.1. Lung lavage fluid was collected from two naïve mice at each time point. Animals were sacrificed on the indicated day relative to infection, and TNF (A), IL-1 (B), and IFNα/β (C) levels in bronchoalveolar lavage (BAL) fluid were determined by bioassay. Similar results were obtained when TNFα levels were measured by TNFα-specific ELISA (data not shown). Error bars represent the SEM. These experiments were repeated three separate times with comparable results.

Exposure to TCDD increases the percent and number of NK cells in the lungs of mice infected with influenza A virus.

Given that NK cells can kill cells infected with influenza virus, enhanced NK cell number or activity could underlie the clearance of virus-infected cells from the lungs of immunocompromised mice. Therefore, we examined whether exposure to TCDD increased the quantity of NK cells in the lungs of influenza virus-infected mice. Specifically, we determined the percent and number of NK1.1^{+} cells present in the airways (BAL cells) and in the total lung using flow cytometry (Figure 2.3). Interestingly, in contrast to the suppressed recruitment of

CD8$^+$ T cells, there was a two-fold increase in the percentage of NK1.1$^+$ cells in the lungs of TCDD-treated mice (Figures 2.3A and B). Consistent with this increased percentage, the number of NK1.1$^+$ cells recovered from the intact lungs of TCDD-treated mice was also enhanced (Figure 2.3D). Thus, activation of the AhR enhances both the percent and number of NK1.1$^+$ cells in the lung, suggesting that NK cells could compensate for the decreased pulmonary CTL numbers.

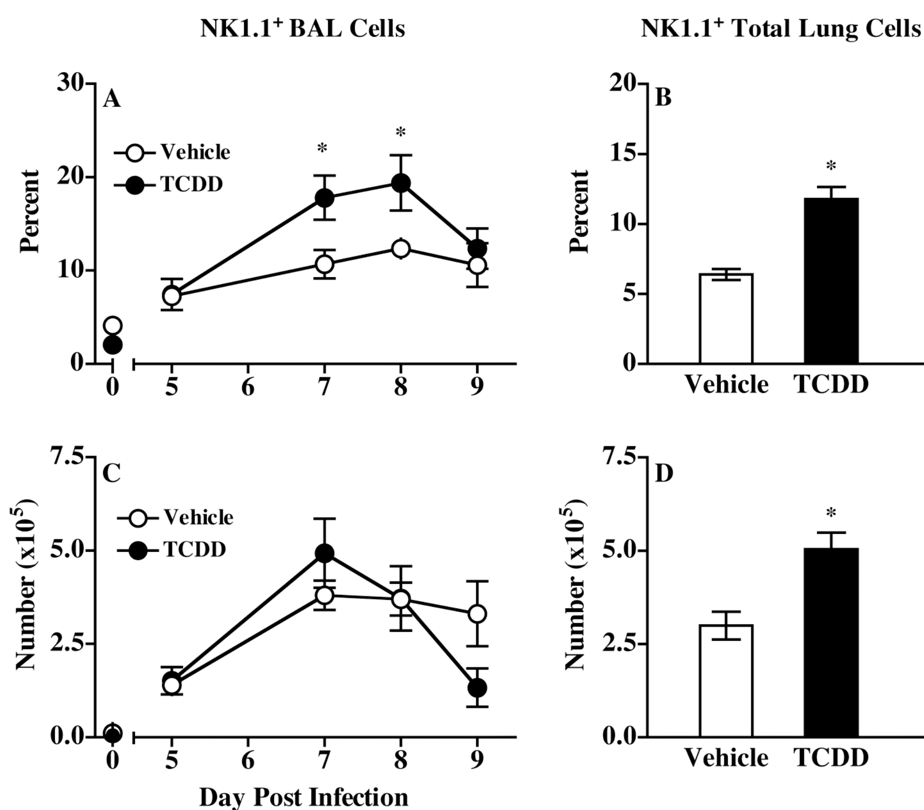
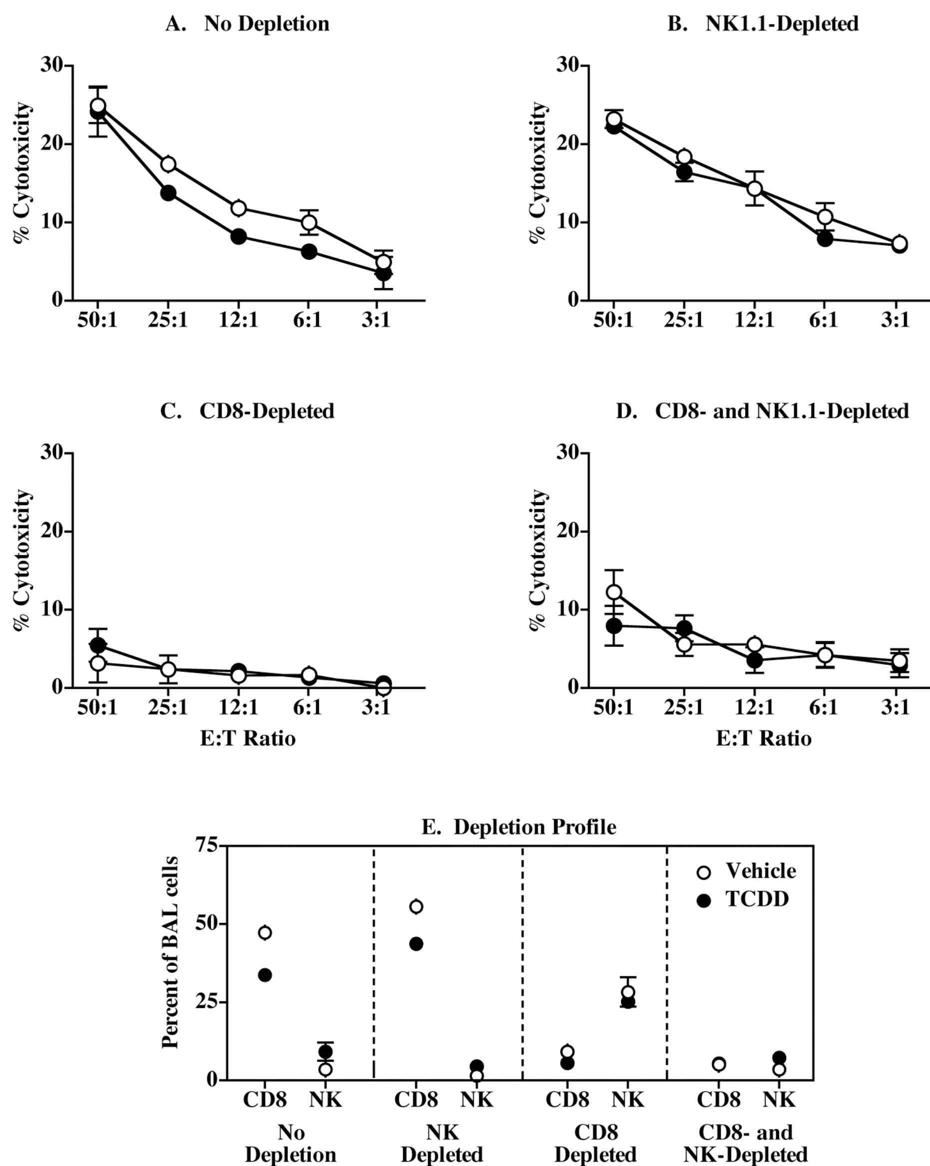


Figure 2.3 AhR activation increases the percent and number of NK1.1$^+$ cells in the lungs of virus-infected mice. Mice were treated as described in Figure 2.1. Data represent the average percentage and number of NK1.1$^+$ cells in the lungs of vehicle-treated and TCDD-treated mice. NK cells recovered from the lung airways (A, C) were enumerated from BAL cell populations collected on days 5-9 following infection. BAL cells collected from mock-infected mice treated with either vehicle or TCDD served as controls. To examine the quantity of NK cells in the lung as a whole, mice were sacrificed seven days after infection and the average percentage (B) and number (D) of NK1.1$^+$ cells in the entire lung was determined. Error bars indicate the SEM, and an asterisk indicates a significant difference compared to vehicle-treated mice ($p \leq 0.05$). Each treatment group consisted of 5-8 mice on all days tested. Results are representative of at least three separate experiments.

AhR activation does not enhance pulmonary NK cell function.

To further explore the potential for AhR-mediated mechanisms to influence NK cell-mediated viral clearance, we tested the cytolytic activity of NK cells in the lungs of vehicle- and TCDD-treated mice. First, we examined the effects of AhR activation on the virus-specific cytolytic activity of both NK cells and $CD8^+$ T cells. To accomplish this, BAL cells were isolated from vehicle- and TCDD-treated mice nine days after infection. This day was selected because it is the peak day of virus-specific pulmonary cytolytic activity (147). In fact, at earlier time points isolated BAL cells exhibit little virus-specific cytolytic activity. BAL cells were subsequently depleted of $NK1.1^+$ cells, $CD8^+$ cells or both populations. Following depletion, virus-specific cytolytic activity was measured using influenza virus-infected target cells in a standard ^{51}Cr-release assay (Figure 2.4).

Consistent with previous studies, BAL cells from vehicle- and TCDD-treated mice had equivalent cytolytic activity against virus-infected targets. This is in spite of the reduced percentage of $CD8^+$ T cells in the lungs of mice treated with TCDD (Figure 2.4A). Removal of $NK1.1^+$ cells had no effect on cytolytic activity in either treatment group (Figure 2.4B). In contrast, when $CD8^+$ T cells alone or both $NK1.1^+$ cells and $CD8^+$ T cells were depleted, cytolytic activity was ablated in lung cells isolated from either vehicle- or TCDD-treated mice (Figure 2.4C & D). Collectively, these results suggest that the CTL, although fewer in number, are able to eliminate the virus-infected cells from the lungs of TCDD-treated mice.



<u>Figure 2.4</u> CTL, although fewer in number, are responsible for eliminating virus-infected cells from lungs of mice treated with TCDD. Mice were treated as described in Figure 2.1, and lung lavage cells were collected nine days after infection. BAL cells collected from 10 mice were pooled to make a single group. For each treatment group (vehicle or TCDD), three separate pools of BAL cells were made. Cells were left untreated (A) or depleted of NK1.1$^+$ cells (B), CD8$^+$ T cells (C), or both populations (D) using immunomagnetic beads. Following depletion, cells were incubated for 5h at 37°C, 5% CO_2 with ^{51}Cr-labeled, virus-infected EL-4 cells. Data represent the average percent cytotoxicity (±SEM) of lung cells isolated from vehicle-treated (open circles) and TCDD-treated (filled circles) mice. In addition, prior to and following depletion, an aliquot of cells was removed and the percentage of CD8$^+$ T cells and NK1.1$^+$ cells was determined using flow cytometry (E).

To corroborate these data suggesting that enhanced NK cell cytolytic activity does not underlie viral clearance in mice exposed to TCDD, we tested NK cell-specific cytolytic activity against Yac-1 tumor cells. Lung cells were isolated from vehicle- and TCDD-treated mice on days 5, 7, 8 and 9 after infection. While cells isolated from the lungs of both vehicle- and TCDD-treated mice were able to lyse Yac-1 tumor cells, treatment with TCDD did not augment this NK cell-specific cytolytic activity (Figure 2.5). These results provide further evidence that activation of the AhR during infection with influenza virus does not enhance the cytolytic activity of NK cells.

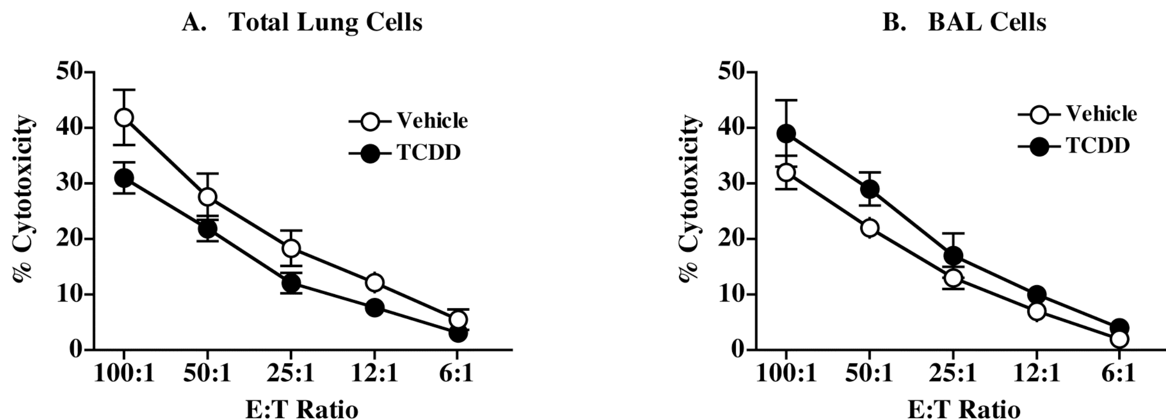
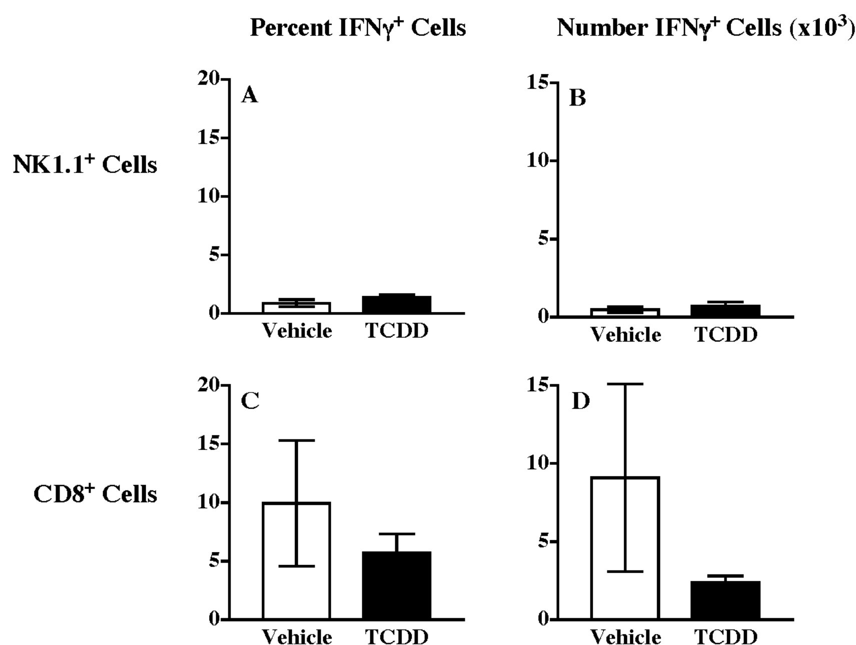


Figure 2.5 Activation of the AhR does not enhance the cytolytic activity of NK cells. In vitro cytolytic activity of NK cells was compared using Yac-1 tumor cells as targets. Mice (5-6 per group) were treated as described in Figure 2.1 and sacrificed on day 7 (A, total lung cells) or 8 (B, BAL cells) post infection with influenza virus. The percent cytotoxicity was calculated and data points represent the mean (±SEM). Similar results were obtained when BAL cells or total lung cells were isolated from vehicle- and TCDD-treated mice on days 5 and 9 post-infection.

In addition to killing virus-infected cells, NK cells produce IFNγ in response to infection. Furthermore, IFNγ produced by NK cells can activate $CD8^+$ T cells, thus helping in the development of the CTL response (162,186-188). Although activation of the AhR did not enhance the cytolytic function of NK cells, it is possible that exposure to TCDD increases IFNγ production by NK cells, thereby indirectly enhancing $CD8^+$ T cell function. To test the effects of

AhR activation on this aspect of NK cell function, IFNγ producing cells were enumerated 7 days after infection, which is two days prior to peak cytolytic activity in the lung. An additional reason for selecting day 7 of infection is that this is the day of maximal NK cell numbers (Figure 2.3) and IFNγ levels (115) in the lungs of TCDD-treated mice.



<u>Figure 2.6</u> Exposure to TCDD does not enhance IFNγ production by NK cells or CD8$^+$ T cells in the lung. Mice (4-5) per group were treated as described in Figure 2.1. Animals were sacrificed 7 days after infection, the peak day of NK cell number and IFNγ production in the lung. The percent and number of lung airway NK1.1$^+$ cells (A, B) and CD8$^+$ T cells (C, D) producing IFNγ was determined by flow cytometry. The results are expressed as the mean (±SEM) percent and number for vehicle- (open bars) and TCDD-treated (closed bars) mice. Results are representative of three separate experiments.

As shown in Figure 2.6A and B, very few NK1.1$^+$ cells produced IFNγ and treatment with TCDD did not significantly alter the percentage or number of IFNγ-producing NK cells in the lung airways. In addition, exposure to TCDD did not enhance the production of IFNγ by CD8$^+$ cells as depicted in panels C and D. Therefore, taken together, these data demonstrate that

activation of the AhR does not enhance the cytolytic function or the production of IFNγ by NK cells in the lungs of virus-infected mice.

DISCUSSION

In mice, activation of the AhR during infection with influenza virus results in reduced recruitment of CTL to the lung and an impaired ability to resist a sublethal infection. However, we and others (105,147) have shown that these animals do not demonstrate defects in viral clearance. Thus, the goal of this study was to determine what mechanism accounts for the killing of the virus-infected cells in the lungs of these immunocompromised mice. Based on our previous observation that exposure to TCDD, the most potent ligand for the AhR, leads to substantial increases in IFNγ in the lungs of infected mice, we posited that NK cells were the most likely compensatory mechanism. Furthermore, since activation of the AhR upregulates TNF and other pro-inflammatory cytokines in other models, and because these cytokines are important anti-viral mediators that activate NK cells, we examined the possible contribution of enhanced production of these cytokines to AhR-mediated compensatory mechanisms for anti-viral immunity.

Exposure to TCDD has been shown to alter the expression of genes encoding anti-viral cytokines (144-146,189,190). In fact, the idea that activation of the AhR dysregulates cytokine production and that these defects underlie the immunomodulatory activity of AhR ligands is supported by a large number of studies (107,109,112,191,192). Therefore, a cytokine-mediated compensatory mechanism was logical. However, the results from this study suggest that activation of the AhR does not alter the early, infection-associated production of IL-1, TNFα and IFNα/β in the lung, making it unlikely that aberrant production of these cytokines provides

compensatory or host protective anti-viral activity. Additionally, we were unable to detect IL-18 following *in vivo* infection with influenza virus. Therefore, although IL-18 production by macrophages following *in vitro* infection has been reported (171), our data indicate that IL-18 is either utilized very rapidly *in vivo*, is not produced in the lung during infection with influenza virus, or is below the level of detection of the assay.

In the absence of observed effects on these cytokines in the lungs of TCDD-treated mice, an increase in the NK cell-mediated anti-viral response was an attractive alternate mechanism for viral clearance. In addition, this NK cell-mediated compensatory hypothesis correlated well with the increased number of NK cells in the lungs of TCDD-treated mice. Unfortunately, attempts to test this hypothesis by depleting NK cells *in vivo* were unsuccessful due to significant mortality in both the anti-asialoGM1 and control antibody groups of TCDD-treated mice (our unpublished observations). However, *ex vivo* depletion of NK cells demonstrated that exposure to TCDD does not enhance the cytolytic function of NK cells.

Although many groups have studied the immunosuppressive effects of AhR agonists on B and T lymphocytes, very few studies have examined the effects of AhR activation on NK cell function. Two previous studies examined the effects of AhR activation on the cytolytic activity of murine NK cells using Yac-1 cells as targets (131,132). Both of these studies demonstrated that NK cell-specific cytolytic activity was not altered in spleen cells isolated from naïve, TCDD-treated mice. Our results are consistent with these studies and extend these observations by demonstrating that NK cells in the lungs of infected TCDD-treated mice do not have enhanced virus-specific cytolytic activity. In addition, our data demonstrate that activation of the AhR does not enhance another NK cell effector function, the ability to produce IFNγ. Therefore,

the findings of this study contribute to the evidence that, unlike the potent immunomodulatory effects on T and B lymphocytes, activation of the AhR does not alter the activity of NK cells.

In fact, contrary to our hypothesis, the results from this study demonstrate that fewer CTL, not enhanced NK cells, are sufficient for viral clearance from the lungs of TCDD-treated mice. At first glance, this finding is somewhat surprising. However, when one compares the kinetics of viral growth and clearance with the influx of $CD8^+$ T cells to the lung, it becomes evident that viral clearance actually commences during a time when very few CTL are present in the lung. For example, the number of $CD8^+$ T cells and virus-specific cytolytic activity in the lung peak 8-10 days after infection, a time at which there is no detectable virus in the lung (115,147,183). In other words, CTL are detected in the lung at points in time after influenza virus has been cleared and are likely produced in numbers that exceed what is necessary to clear the infection. This idea is supported by the observation that CTL precursors are present in the lung for at least 4 days after the time that infectious virus is cleared from the lung (193). Collectively, these studies provide evidence that although current methods for measuring $CD8^+$ T cells in the lung indicate that they arrive relatively late in the response, it is likely that a small number of CTL are present during the early stages of infection and that these cells, although few in number, clear the virus. The reason for their continued arrival and expansion subsequent to viral clearance remains unclear.

Another enigma that remains is the mechanistic relationship between activation of the AhR and impaired host resistance. If the reduced CTL response is ample, why then do the mice die? Several separate mechanisms may underlie the observed defects in host resistance. One possibility is that the excess IFNγ found in the lungs of TCDD-treated mice is detrimental; however, it has been difficult to experimentally determine whether this is the case. We

examined whether IFNγ-deficient mice were less susceptible to mortality induced by AhR activation, but found that these animals succumbed to viral infection similar to wild type, TCDD-treated mice. Furthermore, vehicle-treated, IFNγ-deficient mice also had decreased host resistance compared to wild-type controls (our unpublished observations). Therefore, while it seems likely that some level of IFNγ is protective in response to viral infection, the consequences of excess IFNγ in the lungs remain unclear. Another immunomodulatory effect of exposure to TCDD is the increased recruitment of neutrophils to the lungs of infected mice (114,115,135). Although the mechanisms that drive neutrophilia are not yet known, activation of the AhR has been reported to enhance inflammation and neutrophil recruitment in response to other antigens (134,142). Therefore, taken together, the neutrophilia and excessive IFNγ production make immune-mediated pathology a plausible mechanism for AhR-driven defects in host resistance.

In summary, the findings of this study demonstrate that the CTL response to influenza virus is extremely robust and that relatively few CTL are essential for viral clearance from the lung. Likewise, our data indicate that AhR-mediated decreases in host resistance are not likely due to suppression of the CTL response. Instead, other mechanisms, such as alterations in the recruitment of neutrophils to the lung or inappropriate production of IFNγ, may result in impaired host resistance to respiratory viral infection. The findings presented here also demonstrate that, in contrast to the suppressive effects on T lymphocytes, NK cell function appears less sensitive to perturbation by TCDD. Given that numerous chemicals bind to and activate the AhR, and that the response to infection with influenza virus is similar to events elicited during infection with other pathogens, our findings expand the current understanding of how AhR activation affects immunity in general.

CHAPTER THREE

Pollutants and the etiology of respiratory disease: a novel mechanism of AhR-mediated deregulation of IFNγ production by phagocytic cells[1]

ABSTRACT

We have previously shown that exposure of mice to the Ah receptor (AhR) ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to infection with influenza A virus results in 4-fold higher levels of IFNγ in the lung and decreases survival to an otherwise non-lethal infection. Using Ah receptor (AhR)-deficient mice, we show here that the AhR mediates the elevated IFNγ levels in the lungs of infected mice. These findings are significant because excessive IFNγ production is associated with enhanced inflammation and severe tissue damage in many model systems. Influenza virus infection elicited IFNγ production by many different immune cell types including $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, γδ T cells, dendritic cells, macrophages and neutrophils. Interestingly, we show here that the majority of the excess IFNγ in the lungs of both vehicle- and TCDD-treated mice comes from alveolar macrophages and neutrophils. Furthermore, elevated IFNγ levels correlate with an increase in iNOS in the lungs of TCDD-treated mice. The elevated IFNγ production correlates with an increase in inducible nitric oxide synthase (iNOS) levels in the lungs of TCDD-treated mice. Using CD45.2AhR$^{-/-}$→ CD45.1AhR$^{+/+}$ bone marrow chimeric mice we found that AhR-driven events external to the immune system mediate the exacerbated levels of IFNγ and iNOS during infection. Furthermore, we found that a feedback mechanism likely occurs between IFNγ and iNOS and in

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[1] Data will be submitted for publication in the following manuscript: Neff-LaFord, H.D., Teske, S., and Lawrence, B.P. (2006). Pollutants and the etiology of respiratory disease: a novel mechanism of AhR-mediated deregulation of IFNγ production by phagocytic cells.

doing so determined that a novel iNOS-mediated mechanism may be responsible for the elevated IFNγ levels in the lung. Given that chronic inflammatory diseases of the lower respiratory tract are on the rise worldwide, and that high levels of both IFNγ and iNOS have been associated with the pathology of these diseases, our data suggest that environmental exposure to AhR ligands may contribute to the development of these disorders.

INTRODUCTION

The cytokine interferon-gamma (IFNγ) has been long considered an important first line of defense during viral infections, and influences both innate and adaptive immunity. For example, IFNγ plays a role in the innate immune response by upregulating the expression of MHC class I and II molecules on antigen presenting cells (APC) and stimulating the function of macrophages and natural killer (NK) cells (194-196). Furthermore, IFNγ contributes to adaptive responses by increasing monocyte and T cell-recruiting chemokines, stimulating cytotoxic T lymphocytes (CTL), and directing antibody isotype switching (196-198). Thus, via effects on many different cell types, IFNγ plays an important role in the both the early and late phases of immune responses to viral infections.

While some level of IFNγ is important for anti-viral immunity, excessive IFNγ production leads to downstream pathology characterized by enhanced inflammation and severe tissue damage in many organ systems including the lung, liver, CNS and eye (150-153,199). Although much is known about the downstream consequences of IFNγ, the factors that regulate the production of this cytokine are less understood. Until very recently, the production of IFNγ was thought to be restricted to natural killer (NK) cells and activated $CD4^+$ and $CD8^+$ T cells.

Many cytokines (e.g., IL-2, IL-12, IL-18 and IFNα/β) can increase IFNγ production by T cells and NK cells (170,172,177,200-205), and IFNγ expression in T cells can be controlled by multiple transcriptional regulators including NFκB, NFAT and ATF2 (206-208). However, in addition to NK cells and T cells, several other cellular sources of IFNγ have been reported recently including γ∂T cells, NKT cells, macrophages, dendritic cells and B cells (171,209-213). In contrast to T cell-mediated production of IFNγ, very little is known about the regulation of IFNγ production by these cell types.

We have discovered that during influenza virus infection, activation of the aryl hydrocarbon receptor (AhR) by the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or 'dioxin') results in 4-fold higher IFNγ levels and an increase in the duration of elevated IFNγ in the lung (115). This observation is of interest because the AhR is a ligand-activated transcription factor expressed in cells of the immune system and the lung (214-217). It is generally considered an orphan receptor because it has been difficult to identify an endogenous ligand. However, in addition to TCDD, numerous other pollutants activate the AhR, including 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 (42,218). In addition to environmental contaminants, many plant-derived natural compounds and tryptophan metabolites bind the AhR (41-44). Therefore, exposure to AhR ligands occurs daily through ingestion and inhalation.

It is not clear why some individuals infected with influenza virus have significant pathology whereas others have relatively mild symptoms. Our data suggest that via activation of the AhR, environmental factors influence disease severity by altering host resistance mechanisms. Epidemiological evidence supports this idea, as exposure to pollutants containing

AhR agonists correlates with diminished host resistance, altered immune function, and an increased incidence of influenza and other respiratory pathogens (11,16,60,67,104,189,219-221). Moreover, in addition to elevating pulmonary IFNγ levels, exposure of mice to TCDD increases neutrophil recruitment to the lung and impairs survival following infection with influenza virus (115,139,147). Interestingly, decreased survival does not appear to be caused by impaired viral clearance, but by elevated pulmonary inflammation (115,139,222). In the present study, we sought to identify the cellular sources of IFNγ in the lung, and to determine the mechanism that drives AhR-mediated enhancement of IFNγ production. While conducting these studies, we have identified a novel cellular source of IFNγ during influenza virus infection, and novel mechanism for the regulation of IFNγ production in the lung.

MATERIALS AND METHODS

Animals and treatment

Four-to-six week old female C57Bl/6 ($CD45.2^+$ phenotype), B6-LY5.2/Cr congenic mice ($CD45.1^+$ phenotype), IFNγR-deficient (B6.129S7-$Ifngr1^{tm1Agt/J}$), and iNOS-deficient (B6.129P2-$Nos2^{tm1Lau/J}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or NCI-Frederick (Frederick, MD). A colony of AhR-deficient (B6.129-$Ahr^{tm1Bra/J}$) mice was maintained at Washington State University, and bred as previously described (139). Animals were housed 3-5 per cage under pathogen-free conditions on a 12/12 h light cycle with food and water available *ad libitum*. All treatment was in accordance with protocols approved by the Institutional Animal Care and Use Committee.

TCDD (≥99% purity, Cambridge Isotope Laboratories, Inc. Woburn, MA) was dissolved in anisole and diluted in peanut oil. Mice were gavaged with TCDD (10μg/kg body weight) or

peanut oil vehicle one day prior to infection. This dose of TCDD is not overtly toxic and is well below the LD_{50} in C57Bl/6 mice (175). Animals were anesthetized with Avertin (2,2,2-tribromoethanol, Sigma-Aldrich, St. Louis, MO) prior to intranasal inoculation with 120 hemagglutinating units of influenza virus, strain A/HKx31 (H3N2), diluted in 25μL of sterile PBS. In vehicle-treated mice this dose of virus does not typically cause mortality (114,115,176). Mock-infected mice received 25μl sterile endotoxin-tested PBS and were included as non-immune controls. Animals were sacrificed at various times relative to infection by asphyxiation with CO_2.

Generation of bone marrow chimeric mice

Four-week old, female B6-CD45.2$^+$ mice and B6-CD45.1$^+$ congenic mice were purchased from NCI-Frederick. Mice received sterile-filtered, acidified water (pH 3.0) supplemented with 1 mg/ml oxytetracycline HCL (terramycin), and were fed irradiated food (Pico-Vac mouse diet 20, Purina Mills) beginning one week prior to irradiation. Mice were maintained on this regimen throughout the study. Anesthetized B6-CD45.1$^+$ mice were irradiated with two separate doses of 600 rad with 3.5 hours between doses (Philips SL-15 linear accelerator, Radiology Dept., Washington State University College of Veterinary Medicine). Administering the radiation in two intervals reduces irradiation-associated toxicity (223,224) and allows for a cumulative dose of 1200 rad. One hour after the second radiation treatment, 1.5 x 10^6 bone marrow cells from either B6-CD45.2$^+$ AhR$^{+/+}$ or B6-CD45.2 AhR$^{-/-}$ donor mice were intravenously (i.v.) injected into irradiated B6-CD45.1$^+$ AhR$^{+/+}$ recipient mice. Irradiated B6-CD45.1$^+$ AhR$^{+/+}$ mice that did not receive donor bone marrow cells served as controls for the irradiation, and died within two weeks of radiation exposure. Chimerism was confirmed via

flow cytometry, which showed that >95% of the bone marrow cells were CD45.2^{+}. These chimeric mice were determined to be immunocompetent regardless of the AhR status of the immune system as both AhR$^{-/-}$ → AhR$^{+/+}$ and AhR$^{+/+}$ → AhR$^{+/+}$ mice could survive challenge with influenza virus (data not shown). Four weeks after irradiation and bone marrow reconstitution, the chimeric mice were treated with vehicle or TCDD (10 μg/kg) one day prior to infection with 120 HAU influenza virus, as described above.

Collection of immune cells

Cells in the lung airways were collected by bronchoalveolar lavage as previously described (115,158). Media from the first 1 ml wash of the airway was retained as BAL fluid. Cells recovered from all three 1 ml washes were pooled and enumerated. Total lung-derived immune cells were obtained by digesting intact lungs (i.e., unlavaged) with collagenase (RPMI 1640 media containing 0.7 mg/ml collagenase A (Worthington Biochemical Corp., Lakewood, NJ), 30 μg/ml deoxyribonuclease I (Sigma-Aldrich), 2.5% FBS and 10 mM HEPES). Immune cells from interstitial spaces of the lung were obtained by first removing airway cells by lavage, and then digesting the lung with collagenase-containing media. Following a 25 min incubation at 37°C, 5% CO_2, lung cell suspensions were layered over Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and centrifuged at room temperature for 20 minutes at 1000 x g. The leukocyte-containing fraction was collected and washed. Greater than 99% of the cells in this fraction are CD45^{+} (our unpublished observations).

Immunophenotypic analyses

Cells were incubated with the following previously-titrated monoclonal antibodies (mAbs): FITC- or PE-labeled anti-NK1.1, PE-labeled anti-CD3, FITC- or TC-labeled anti-CD8α, FITC- or TC-labeled anti-F4/80 (Caltag Laboratories, Burlingame, CA); and FITC- or TC-labeled anti-CD11b, PE-labeled anti-GR1 and FITC-labeled anti-CD4 (BD Biosciences). Biotinylated mAbs were used in combination with SpectralRed™-streptavidin or FITC neutralite avidin (Southern Biotechnology Associates, Birmingham, AL). Appropriately-labeled, isotype-matched mAbs were used to determine nonspecific fluorescence. For all experiments listmode data was collected on 10,000-50,000 stained cells using a FACScan or FACSort flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). To identify IFNγ-producing cells, cells were incubated in 24-well plates at 37°C, 5% CO_2 for 5 hours in the presence of 12.5 U/ml rmIL-2 (R&D Systems), 1 μM influenza virus nucleoprotein peptide ($NP_{366-374}$ ASNENMETM), and 10 μg/ml Brefeldin A (Sigma-Aldrich). Cells were stained with antibodies specific for cell surface molecules (as described above), fixed with 2% formalin, permeabilized with 1% saponin, and incubated with an APC-labeled anti-IFNγ mAb (BD Biosciences or eBiosciences, San Diego, CA) or isotypic control mAb for 30 min at room temperature.

ELISA

IFNγ levels in BAL fluid were measured using an IFNγ-specific sandwich ELISA according to the manufacturer's recommended protocols. IFNγ standards and matched antibody pairs were purchased from BD Biosciences (San Diego, CA). The limit of detection for this assay is 250 pg/ml.

Immunoblotting

Whole lungs or previously-lavaged lungs were snap frozen in liquid nitrogen and stored at -80°C prior to homogenization. Frozen lungs from individual animals were thawed in 0.5 or 1 ml of cold homogenization buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.6% NP-40, 10 μg/ml aprotinin and leupeptin, and 20 μg/ml PMSF). Lungs were homogenized using a Tissue Tearor (Biospec Products, Bartlesville, OK) at high speed for 40 sec and debris was removed by centrifugation (2400 rpm, 4°C, 30 sec). Supernatants were incubated on ice for 5 min then spun at 6000 x g for 5 min at 4°C. Supernatants were collected and protein concentrations were determined using a BCA protein assay (Pierce Endogen, Rockford, IL). All samples were normalized to a concentration of 3 mg/ml with Laemmli sample buffer and boiled for 10 min. Samples were subsequently run on 8% SDS gels at 50μg per sample, transferred to nitrocellulose membranes and blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline. Membranes were probed with a rabbit polyclonal anti-iNOS antibody at a 1:500 dilution (Cayman Chemical, Ann Arbor, MI) followed by horseradish-peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich, 1:20,000 dilution). Antibody complexes were visualized using chemiluminescent reagents (SuperSignal West Dura Extended Duration Substrate, Pierce Endogen).

Immunohistochemistry

Whole lungs were perfused with 10% formalin, embedded in paraffin, sliced and mounted onto glass slides (WADDL, Washington State University). Tissue slices were deparaffinized using xylenes, then rehydrated with decreasing percentages of ethanol followed by a final rinse with dH_2O. Tissue sections were incubated with 1% saponin in endotoxin-tested

PBS for 30 min at 25°C, rinsed with buffer (0.25M NaCl, 20mM Tris, 0.125% Tween-20 in endotoxin-tested water, pH=7.5) and incubated with 3% H_2O_2 for 5 min. Non-specific binding was blocked using 3% normal goat serum for 25 min. Sections were exposed to a polyclonal rabbit anti-murine iNOS antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) at a 50-fold dilution overnight at 4°C. Slides were rinsed and incubated for 45 min at 37°C with an HRP-conjugated goat anti-rabbit IgG, $F(ab')_2$ antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:100 dilution. Cells expressing iNOS were identified using an AEC or DAB substrate for 40 min (Vector Laboratories, Burlingame, CA). Samples were counterstained with hematoxylin (Vector Laboratories) and coverslips were mounted with aqueous mounting medium for AEC-stained samples (Serotec, Raleigh, NC), or permanent mounting medium for DAB-stained samples (Vector Laboratories).

Statistics

Statistical analyses were performed with StatView statistical software (SAS, Cary, NC) or Prism (GraphPad Software, Inc., San Diego, CA). Differences between independent variables were compared over time and between each treatment group using one-way ANOVA, followed by *post hoc* Fisher's protected least significant difference (PLSD) tests. Data collected at a single point in time were analyzed using a one-tailed Students' *t*-test. A value of $p \leq 0.05$ was considered significant.

RESULTS

Activation of the AhR increases the percentage of IFNγ-producing cells in the lung, the majority of which are $Gr1^+CD11b^+$.

Exposure to TCDD greatly elevates infection-associated production of IFNγ in the lung (114,115), with the peak levels occurring seven days after infection (Figure 3.1, panel A). However, the mechanism responsible for these exacerbated levels in the lung is unclear. It is unlikely that an increase in systemic IFNγ levels is responsible for elevated pulmonary levels since no IFNγ is detected in the blood (data not shown), and IFNγ levels in the peribronchiolar lymph node are decreased following exposure to TCDD (107,115).

We hypothesized that inappropriate activation of the AhR during infection increases the frequency of IFNγ-producing cells in the lung, leading to elevated IFNγ levels in lung lavage fluid. We show here that the elevated IFNγ levels in the lung are dependent upon AhR activation (Figure 3.1B) and are due to an increase in the overall amount of IFNγ-producing cells in the lungs of TCDD-treated, virus-infected mice (Figure 3.1C).

We next set out to determine the cellular sources of this excess IFNγ. We first examined whether AhR activation increases IFNγ production by T cells ($CD3^+$) and NK cells ($NK1.1^+$). In Figure 1D, we show that although both of these cell types produce IFNγ in response to infection, neither T cells nor NK cells are responsible for the majority of IFNγ produced in the lungs of either vehicle- or TCDD-treated, influenza virus-infected mice. These findings are consistent with separate studies where immune cells isolated from the lungs of vehicle- and TCDD-treated, virus-infected mice produced IFNγ in the absence of both $CD8^+$ T cells and NK cells (data not shown). These findings suggest that another cell type is responsible for the majority of the IFNγ produced in the lungs of both vehicle- and TCDD-treated mice.

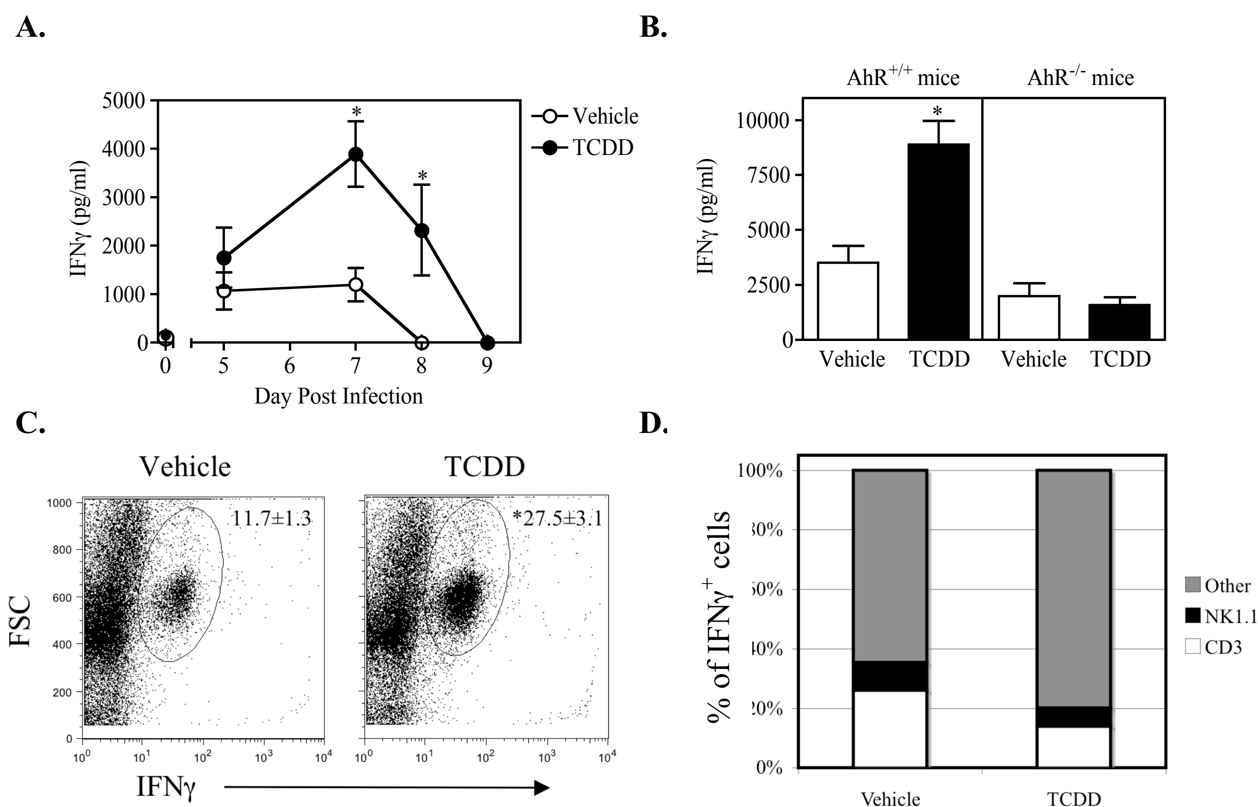


Figure 3.1 Activation of the AhR increases the percentage of IFNγ-producing cells in the lung, but the majority are neither CD3$^+$ nor NK1.1$^+$. C57Bl/6 (AhR$^{+/+}$) or AhR$^{-/-}$ mice were given peanut oil vehicle or TCDD (10μg/kg) orally one day prior to intranasal infection with 120 HAU of influenza virus strain A/HKx31. (A.) Mice were sacrificed on the indicated days relative to infection, and IFNγ levels in the lung lavage fluid were determined by ELISA. (B.) Seven days post infection, IFNγ levels in the lung lavage fluid of wild type and AhR$^{-/-}$ mice were compared. (C.) The percentage of IFNγ-producing cells in the lungs of vehicle- and TCDD-treated AhR$^{+/+}$ mice was determined seven days after infection. (D.) The relative levels of CD3$^+$ and NK1.1$^+$ IFNγ$^+$ cells are depicted. An * indicates a significant difference from vehicle-treated mice ($p \leq 0.05$). These data are representative of at least 3 separate experiments, n=4-6 per treatment group per day.

We next identified the immune cell types in the lung that produce IFNγ and found that, in addition to T cells and NK cells, macrophages (CD11b$^+$), dendritic cells (CD11c$^+$), B cells (CD19$^+$), and neutrophils (Gr1$^+$) also produce IFNγ in response to influenza virus infection, regardless of TCDD-exposure (Figure 3.2A). Since neutrophils have not been previously reported to produce IFNγ during influenza virus infection, we further characterized these cells

using flow cytometry. We found that the majority of the IFNγ-producing cells in the lungs of both vehicle- and TCDD-treated mice are $Gr1^+$ and $CD11b^+$, but do not express CD3, NK1.1, CD11c, or γδTCR (Figure 3.2B). Furthermore, exposure to TCDD increased IFNγ production by the $Gr1^+CD11b^+$ cells, suggesting that activation of the AhR deregulates phagocytic cell function. We also determined that the IFNγ-producing $Gr1^+$ and $CD11b^+$ cells are found in the airways and interstitium of both vehicle- and TCDD-treated mice (Figure 3.2B), and that exposure to TCDD greatly elevates IFNγ production by $Gr1^+$ cells, regardless of CD11b status. Furthermore, these IFNγ-producing cells are adherent to plastic, which supports the idea that these cells are not lymphocytes. The data shown here are novel for two reasons. First, we determined that the majority of the IFNγ in the lung seven days after influenza virus infection is produced by non-lymphocyte sources. Second, AhR activation increases the frequency of IFNγ-producing phagocytic cells in the lung.

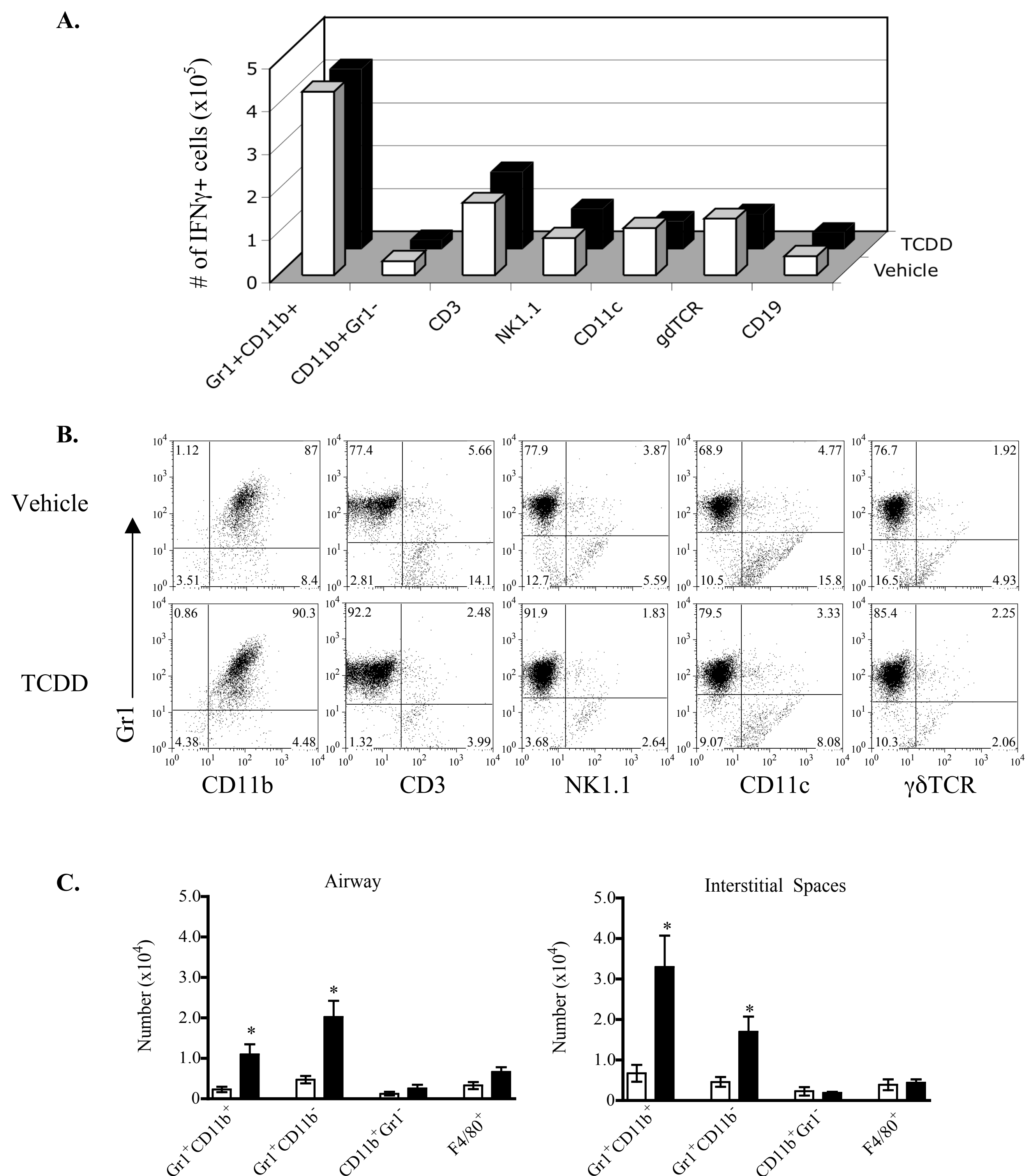


Figure 3.2 The majority of the IFNγ-producing cells in the lungs of TCDD-treated mice are $Gr1^+$ and $CD11b^+$. C57Bl/6 mice were treated as described in Figure 3.1. Seven days post infection, immune cells were isolated from whole lungs and re-stimulated with IL-2 and viral peptide for 5 hr in the presence of Brefeldin A. (A.) The phenotype of $IFN\gamma^+$ cells was determined using flow cytometry, and the number of $IFN\gamma^+$ cells that express the listed cell-surface markers is shown. (B.) Histograms depict the percentage of $IFN\gamma^+Gr1^+$ cells with various

phenotypes in the lungs of vehicle- and TCDD-treated mice. (C.) Graphs show the average number of IFNγ^{+} cells isolated from the lung airways or interstitial spaces of vehicle- (clear bars) and TCDD-treated (filled bars) mice. Note: these cells were adherent to plastic, and thus, lymphocyte populations are not included in these graphs. An * indicates a statistically significant difference compared to the vehicle-treated controls ($p \leq 0.05$). Error bars represent the SEM and experiments were repeated three times with comparable results, n=4 to 6 per group.

Neither IFNγ-stimulating cytokines nor direct AhR:AhRE binding in the ifng gene increases IFNγ production by phagocytic cells.

We next set out to determine the mechanism by which activation of the AhR deregulates the production of IFNγ by phagocytic cells. Unfortunately, little is known about the signals that control IFNγ production by phagocytic cells. However, there is much data demonstrating that IFNγ production by lymphocytes can be induced by IL-12, IL-18, IFNα/β and IL-2 (170,172,177,200-205). However, we have previously shown that exposure to TCDD suppresses pulmonary IL-12 levels (114,115) and does not alter IFNα/β or IL-18 levels in the lung during infection (222). It is also unlikely that IL-2 plays a role in the over production of IFNγ in the lungs of TCDD-treated, influenza virus-infected mice, since we observe a significant decrease in the levels of IL-2 in the lymph nodes of these animals and do not detect its production in the lung (107, 115, and our unpublished observations). Therefore, cytokines known to stimulate IFNγ production by T cells and NK cells do not appear to drive the IFNγ production by phagocytic cells in the lungs of either vehicle- or TCDD-treated, influenza virus-infected mice.

With regard to the elevated IFNγ levels in the lungs of TCDD-treated mice, direct AhR:AhRE binding may be responsible for this deregulated response. When activated, the ligand-bound AhR binds to AhR response elements (AhRE) on the upstream regulatory regions of Ah-responsive genes. Since the elevated IFNγ levels in the lung are AhR-dependent, we

would expect that the AhR directly upregulates its expression by binding to AhRE on the IFNγ gene. To examine whether this occurs, we searched for AhRE binding sites 3000 bp up- and downstream of the IFNγ gene transcription start site using 1) a manual search of the annotated gene sequence, 2) Genomatix software (www.genomatix.de), and 3) Vector NTI (Invitrogen, Carlsbad, CA). The core sequence GCGTC was used to identify AhRE. None of the search strategies identified a consensus AhRE in the upstream regulatory region of the IFNγ gene. Additionally, we searched the IFNγ gene sequence for the newly described AhRE II binding site (CATG{N_6}CTATG) (225,226) and as with the AhRE, did not find AhRE II binding sites in the IFNγ gene. Thus, it is unlikely that direct AhR:AhRE interaction in the promoter region of the IFNγ gene is responsible for its elevated production.

An AhR-mediated signal extrinsic to the immune system drives inappropriate IFNγ production by phagocytic cells.

We have been unable to link the elevated pulmonary IFNγ levels in the lungs of TCDD-treated mice to an increase in IFNγ-inducing cytokines or direct AhR:AhRE binding. Likewise, stimulation of IFNγ production by freshly isolated (alveolar) or cultured (alveolar and peritoneal) macrophages following influenza virus infection *in vitro,* has been unsuccessful regardless of TCDD exposure (data not shown). Collectively, these data suggest that the production of IFNγ by phagocytic cells may not be a direct target of AhR-mediated toxicity. To test this, we developed bone marrow chimeric mice to determine whether AhR activation within immune cells is directly responsible for elevated IFNγ production by phagocytic cells. For these studies, lethally irradiated wild type congenic mice (B6-CD45.1^{+}AhR$^{+/+}$) received donor immune cells

isolated from either B6-CD45.2^{+}AhR$^{+/+}$ control wild-type mice or B6-CD45.2^{+}AhR$^{-/-}$ AhR-deficient mice. Similar to previous findings, TCDD-treated wild-type chimeric (AhR$^{+/+}$ → AhR$^{+/+}$) mice had at least 6-fold higher levels of IFNγ in their lungs compared to vehicle-treated wild-type chimeric controls (Figure 3.3A). Interestingly, TCDD-treated mice that received AhR-deficient immune cells (AhR$^{-/-}$ → AhR$^{+/+}$) had IFNγ levels similar to those in the lung lavage fluid of TCDD-treated wild-type chimeric mice. The number of IFNγ-producing cells was also elevated in the lungs of the TCDD-treated AhR$^{-/-}$→ AhR$^{+/+}$ mice at levels similar to those in the TCDD-exposed AhR$^{+/+}$→ AhR$^{+/+}$ controls (Figure 3.3B). Furthermore, we show that >95% of the IFNγ-producing cells lack the AhR (CD45.2^{+}) and express Gr1 and F4/80 cell surface antigens (Figure 3.3C). Taken together, these data suggest that activation of the AhR in the lung, not in immune cells drives the excess IFNγ production by phagocytic cells in the lungs of virus-infected mice.

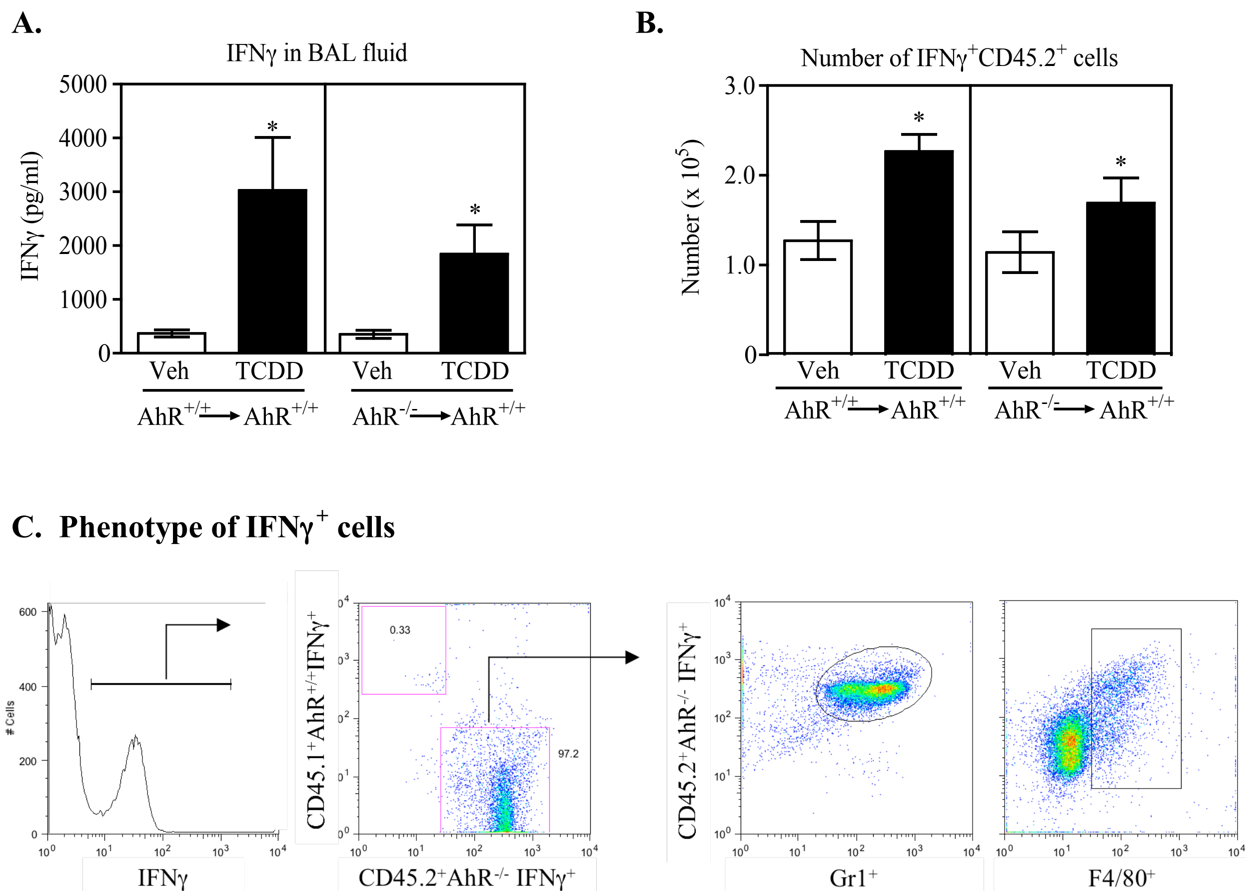


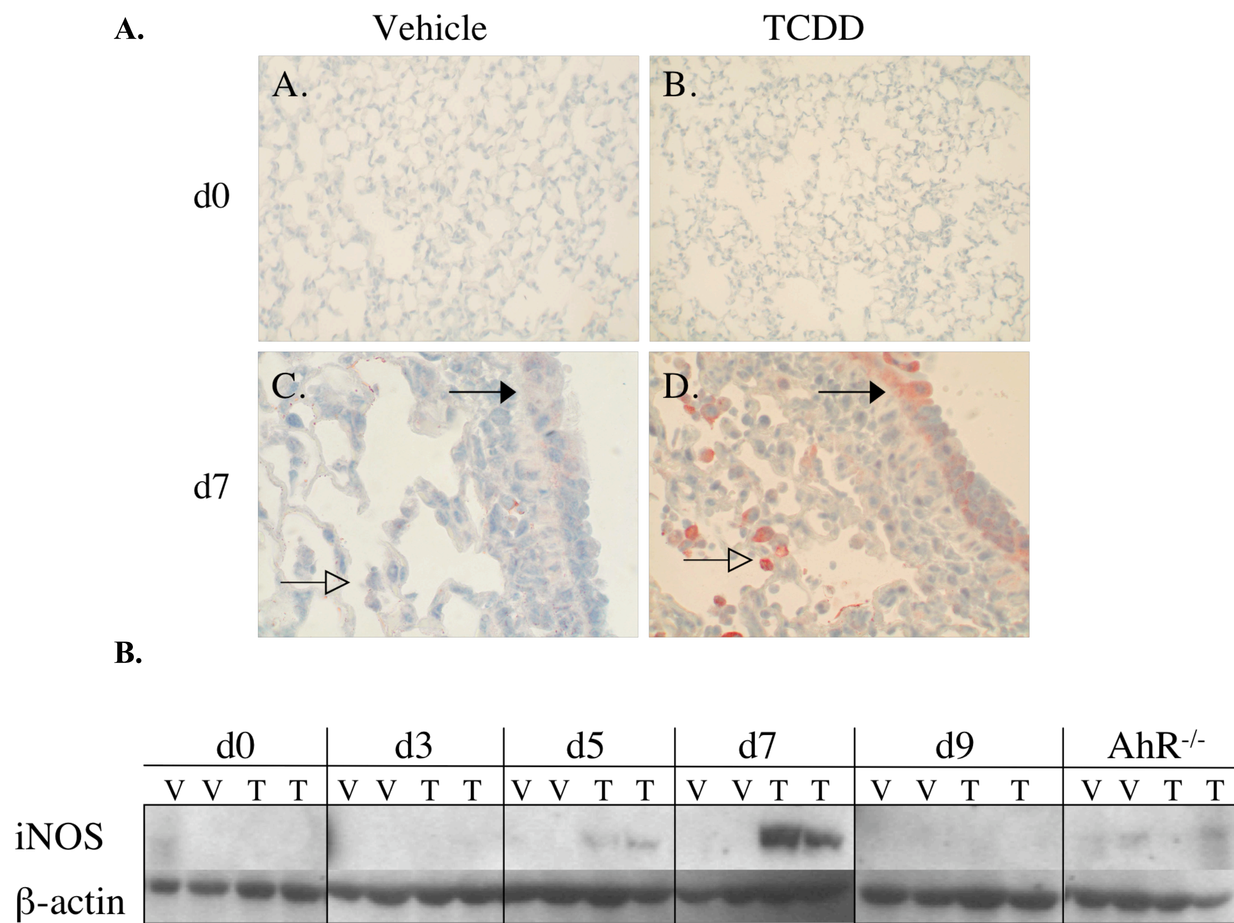
Figure 3.3 An AhR-mediated signal from the lung drives aberrant IFNγ production by macrophages and neutrophils. Bone marrow chimeric mice were produced by reconstituting lethally-irradiated CD45.1$^+$ congenic mice with immune cells derived from either CD45.2$^+$AhR$^{+/+}$ or CD45.2$^+$AhR$^{-/-}$ mice. Four weeks after bone marrow reconstitution, the chimeric mice were treated with either vehicle or TCDD (10μg/kg) one day prior to infection with 120 HAU HKx31. Mice were sacrificed seven days after infection. (A.) IFNγ levels in lung lavage fluid were measured by ELISA. (B.) The number of IFNγ$^+$CD45.2$^+$ (i.e., donor-derived) cells in the lung was determined by flow cytometry. (C.) A histogram depicting IFNγ$^+$ cells in the lungs of AhR$^{-/-}$→AhR$^{+/+}$ is shown. Over 95% of the IFNγ$^+$ cells were from donor mice (i.e., CD45.2$^+$) and express Gr1$^+$ and F4/80. These results are representative of at least two separate experiments. Error bars represent the SEM and an * depicts a statistically significant difference ($p \leq 0.05$) compared to vehicle-treated mice n=5-6 per treatment group per day.

Activation of the AhR elevates pulmonary iNOS levels in macrophages and epithelial cells.

A downstream effect of IFNγ-mediated signaling is increased expression of inducible nitric oxide synthase (iNOS). Aberrant iNOS induction can have pathological consequences in many systems, including during influenza virus-induced pneumonia (227,228). This is primarily

due to overproduction of highly reactive nitric oxide (NO). Since IFNγ can induce iNOS and because TCDD-treated, virus-infected mice have elevated pulmonary levels of IFNγ, we hypothesized that iNOS levels would also be increased in the lungs of these mice. Using immunohistochemistry, we determined that activation of the AhR in the absence of infection, does not induce iNOS expression in the lung (Figure 3.4A, panel B). However, alveolar macrophages and lung epithelial cells express iNOS seven days after infection (Figure 3.4A, panels C and D), with activation of the AhR increasing iNOS expression by both cell types (visualized by more intense staining in these cells, Figure 3.4A, panel D). Likewise, when iNOS protein levels were measured by Western blot, we found that exposure to TCDD greatly elevated iNOS levels in the lungs seven days post infection, and that this increase was dependent upon AhR activation (Figure 3.4B).

While IFNγ is a well-characterized stimulator of iNOS, some studies suggest that iNOS can also induce IFNγ production (229-231). Since the elevated pulmonary IFNγ levels are driven by a signal external to the immune system and because iNOS is highly expressed by lung epithelial cells, we hypothesized that iNOS-mediated signaling may be responsible for increasing IFNγ production. To examine this relationship, we measured iNOS levels in the lungs of bone marrow chimeric mice (Figure 3.5). Similar to our previous findings, exposure to TCDD increased iNOS expression by lung epithelial cells in the lungs of both $AhR^{-/-} \rightarrow AhR^{+/+}$ and $AhR^{+/+} \rightarrow AhR^{+/+}$ mice. Interestingly, we found that exposure to TCDD elevated iNOS levels in macrophages lacking the AhR at similar levels as wild-type cells (Figure 3.5, compare panels F and H). This finding suggests that activation of the AhR in the lung may drive deregulated phagocytic cell immune responses.



<u>Figure 3.4</u> Activation of the AhR increases iNOS levels in the lung during influenza virus infection. C57Bl/6 and $AhR^{-/-}$ mice were treated as described in Figure 3.1, and were sacrificed on the indicated days after infection (n=6 mice per treatment group per day). (A.) iNOS expression in the lungs of vehicle- and TCDD-treated mice was determined by immunohistochemistry 0 and 7 days after infection (AEC substrate was used in these studies). Photographs are representative of 6 samples per treatment group. Lung epithelial cells are denoted with filled arrows, alveolar macrophages with open arrows. Pictures shown in panel A and B were taken at 40X, panels C and D at 100X. (B.) iNOS and β-actin levels in lung homogenates (50 μg) were examined by Western blotting. The top row of each group depicts relative iNOS (130 kD) levels in the lungs of vehicle (V)- and TCDD (T)-treated mice. Vehicle- and TCDD-treated, mock-infected (PBS) samples were included as controls (d0).

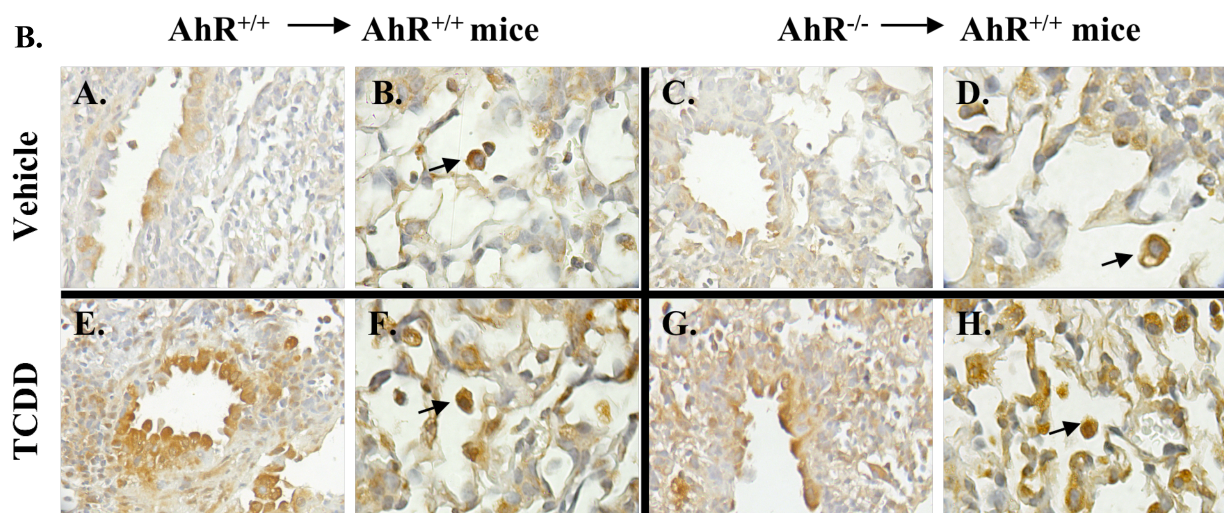
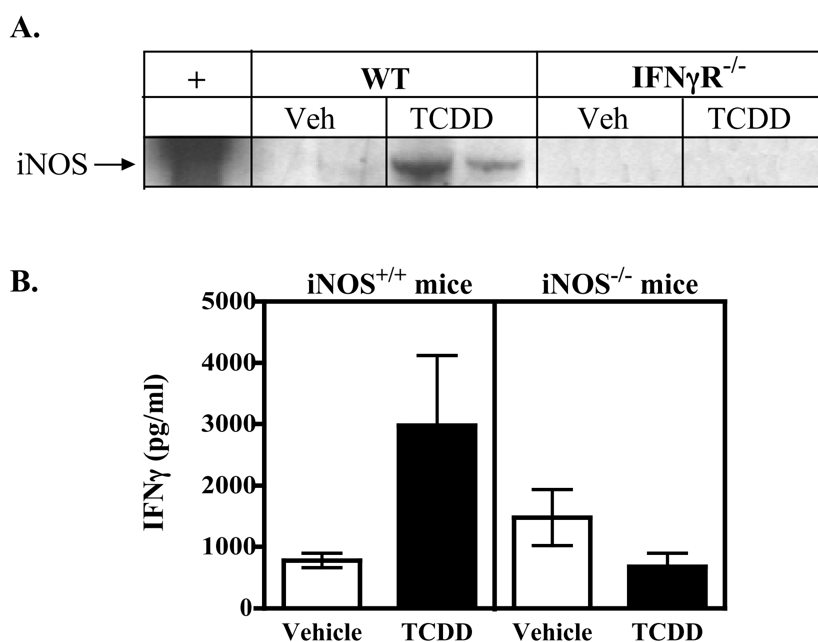


Figure 3.5 Activation of the AhR in the lung, but not in the immune system, induces iNOS expression by epithelial cells and alveolar macrophages. Bone marrow chimeric mice were produced and treated as described in Figure 3.3. Seven days after infection, mice were sacrificed and iNOS expression in the lungs of vehicle- and TCDD-treated mice was determined using immunohistochemistry (visualized by DAB staining). Arrows depict alveolar macrophages. Photographs are representative of at least 5 fields of view from 3-5 samples per treatment group. Pictures shown in panels A, C, E and G were taken at 40X, panels B,D,F, and H at 100X.

Elevated iNOS levels drive the excessive IFNγ production in the lungs of TCDD-treated mice.

We have shown that exposure to TCDD elevates pulmonary levels of IFNγ and iNOS during influenza virus infection. Interestingly, both IFNγ and iNOS reach peak levels in the lungs of TCDD-treated mice seven days post infection (Figures 3.1A and 3.4A). This information, combined with the fact that IFNγ is a well-known inducer of iNOS, caused us to characterize the effects of TCDD on the relationship between these two proteins in the lung during infection. Specifically, we measured iNOS levels in the lungs of IFNγR-deficient mice, and IFNγ levels in the lung lavage fluid of iNOS-deficient mice during infection, with or without TCDD exposure. Similar to what we have previously shown, exposure to TCDD increased iNOS levels in the lungs of wild-type mice (Figure 3.6, panel A). In contrast, TCDD-treated,

IFNγR-deficient mice did not have elevated pulmonary iNOS levels, suggesting that the increased iNOS levels in the lungs of TCDD-treated mice are likely driven by IFNγ. Interestingly, we show that while exposure to TCDD increases IFNγ levels in the lungs of wild-type mice, TCDD-treated iNOS-deficient mice do not have elevated pulmonary IFNγ levels. This finding is of interest because it suggests that an iNOS-dependent pathway may drive the production of excess IFNγ in the lungs of TCDD-treated mice. Furthermore, it appears that IFNγ drives iNOS *and* that iNOS may induce IFNγ production in this model, implying that a possible feedback loop exists between IFNγ and iNOS. Overall, the results shown in Figures 3.5 and 3.6 are interesting because they suggest that activation of the AhR during respiratory viral infection directly affects lung epithelial cells and likely increases IFNγ production by phagocytic cells via a novel iNOS-mediated mechanism.



<u>Figure 3.6</u> Pulmonary IFNγ levels are not elevated in TCDD-treated iNOS-deficient mice. C57Bl/6 (wild-type), IFNγR$^{-/-}$ and iNOS$^{-/-}$ mice were treated as described in Figure 3.1. Mice were sacrificed seven days post infection and lavaged lungs (IFNγR$^{-/-}$) or lung lavage fluid (iNOS$^{-/-}$) was collected. (A.) Lungs were homogenized and iNOS levels were measured by Western blot using 60 μg total protein. LPS-treated macrophages were used as a positive control

(+). Each band is protein taken from an individual animal and these results are representative of 3-9 animals per treatment group. (B). IFNγ levels in the lungs of wild type and $iNOS^{-/-}$ mice were determined by ELISA. Data are representative of two separate experiments (n=3-6 per treatment group).

DISCUSSION

We have previously shown that exposure to TCDD greatly increases IFNγ levels in the lung during influenza virus infection. While IFNγ is clearly important for the immune response to influenza virus, it is well known that excessive production of this cytokine can lead to significant pathology and tissue damage. However, the relationship between AhR activation and deregulated IFNγ production during infection has not been well defined. Thus, the studies shown here were performed to characterize the relationship between AhR activation and aberrant IFNγ production. Overall, the results of these experiments provide several novel pieces of information. First, we found that influenza virus infection alone stimulates IFNγ production by macrophages and neutrophils in the lung. Second, AhR activation increased the production of IFNγ by these cells during infection. Finally, an AhR-mediated signal external to hematopoetic cells drives the excess production of IFNγ, and this signal is likely driven by iNOS activation.

In the process of characterizing the cellular source of the excess IFNγ, we found that in addition to $CD3^+$ T cells and NK cells, B cells, γδ T cells, dendritic cells, macrophages and neutrophils produce IFNγ in response to influenza virus infection. Although the production of IFNγ by B cells and neutrophils has been reported previously in other models (209-212,232,233), to our knowledge, this is the first study documenting IFNγ production by these cell types *in vivo* in the context of influenza virus infection. The findings of these studies are significant because they not only give insight into the effects of AhR activation on different cell types, but they also increase our understanding of the immune response to influenza virus in general. Moreover,

these data are novel because they suggest that the majority of the IFNγ-producing cells in the lung at this time point during infection are not lymphocytes. Furthermore, we show that activation of the AhR triggers phagocytic cells (i.e., macrophages and neutrophils) to inappropriately increase their production of IFNγ.

Unlike T cells, the mechanisms that drive IFNγ production by phagocytic cells have not been well characterized. Our data show that in contrast to IFNγ production by T cells, production of IFNγ by macrophages and neutrophils in the lungs does not appear to be driven by elevated levels of IL-2, IL-12, IL-18 or IFNα/β. Nor do elevated levels of these cytokines drive AhR-mediated increases in IFNγ levels. Moreover, while the excess IFNγ in the lungs of TCDD-treated mice is AhR-dependent, it is not caused by direct AhR:AhRE regulation of IFNγ gene expression. Thus, it is likely that IFNγ production by macrophages and neutrophils is driven by an indirect mechanism that is different from what has been previously described in T cells.

Using bone marrow chimeric mice we determined that an AhR-mediated signal external to the immune system, is responsible for inducing pulmonary IFNγ production and iNOS expression. These findings are significant because they suggest that activation of the AhR has direct effects on lung epithelial cells, not phagocytic cells. This is consistent with the results of several other studies demonstrating that TCDD affects various types of epithelial cells including medial palatal, ureteric and lung airway epithelial cells (137,234-240).

Due to the fact that AhR activation increased iNOS expression by lung epithelial cells, and the excessive IFNγ production by immune cells was driven by an indirect mechanism, we considered that the elevated iNOS levels could be responsible for increased IFNγ production in

the lung. In fact, we show here that activation of the AhR does not increase IFNγ levels in the lungs of influenza virus-infected iNOS-deficient mice. This finding is of interest because it suggests that via an unknown mechanism, iNOS regulates IFNγ production, and that this IFNγ is likely produced by phagocytic cells. To out knowledge, no other studies have examined the role of iNOS in IFNγ production by phagocytic cells, but two laboratories have shown that iNOS plays a role in the production of IFNγ by NK cells (229,230). Thus, although the mechanism is unclear, it is likely that iNOS can regulate IFNγ expression, and that exposure to TCDD activates that pathway in this model.

Although IFNγ-mediated induction of iNOS has been well characterized, there is little known about the mechanisms by which iNOS can drive IFNγ production. However, the transcriptional regulators NFκB, NFAT and ATF2 have been shown to control the expression of IFNγ in T cells as well as regulate iNOS expression (206-208,241-243). Therefore, it is possible that AhR activation alters one or more of these signaling pathways, which subsequently causes deregulated iNOS and IFNγ levels. In fact, the NFκB pathway in particular is a likely target for AhR-mediated deregulation because there is evidence that the AhR and NFκB can associate in liver and mammary cells (28,37). However, our data suggest that the AhR and NFκB p65 subunits do not interact in the lung (see Appendix C), but the effect of TCDD on NFκB activity in the lung during influenza virus infection has not been examined. Thus, it is unclear what role these signaling pathways play in the induction of IFNγ in the lungs of TCDD-treated, virus-infected mice.

The reasons why some individuals have relatively mild symptoms following influenza virus infection, but others have significant pathology is unclear. However, several studies have shown that overproduction of IFNγ leads to hyperinflammation in the lungs of both humans and

experimental animals with chronic obstructive pulmonary disease (COPD) (244-246) and that elevated levels of IFNγ and iNOS correlate with an increased severity of disease-associated pathology in mice with adult respiratory distress syndrome (ARDS). Furthermore, respiratory viral infections (including influenza virus) and AhR activation have also been implicated in the development and/or severity of these chronic respiratory diseases (77,247-250). Our results suggest that activation of the AhR by pollutants deregulates IFNγ production during viral infection via a novel iNOS-mediated pathway, which may provide a possible mechanism for the severity of influenza virus infection and the development of chronic inflammatory disorders of the lung. Thus, by further characterizing the mechanism that drives these AhR-mediated effects, we may increase our knowledge of the causes of these chronic inflammatory diseases of the lower respiratory tract, and provide an experimental link between the environment and the etiology of these diseases.

CHAPTER FOUR
Summary and Future Directions

Summary

The studies presented in this dissertation focused on answering two questions; 1) how does exposure to TCDD affect viral clearance in the lung, and 2) what mechanisms drive the over production of IFNγ in the lungs of TCDD-treated, influenza virus-infected mice? In the process of answering these questions, we have made several novel discoveries, which are described in more detail below. Taken together, these results are not only important for understanding how exposure to TCDD alters immunity, but for characterizing the immune response to influenza virus in general.

We have previously shown that exposure to TCDD significantly suppresses the number of virus-clearing $CD8^+$T cells in the lung without affecting the rate of viral clearance or cytolytic activity of lung immune cells (114,115). Therefore, in Chapter 1 we tested other mechanisms that may compensate for the decreased $CD8^+$ T cell numbers in the lung. We found that exposure to TCDD did not increase IFNα/β, TNFα or IL-1 levels in the lung at any time during infection. Furthermore, while exposure to TCDD increased the numbers of NK cells in the lung seven days post infection it did not alter their cytolytic activity or ability to produce IFNγ. Thus, neither anti-viral cytokines nor NK cells were responsible for the viral clearance. Interestingly, however, we learned that while TCDD clearly suppresses the number of $CD8^+$ T cells in the lung, they are still functional and are responsible for killing the virus-infected cells in the lung.

We have also shown that exposure to TCDD prior to infection causes a 2-4-fold increase in pulmonary IFNγ levels (114,115). Therefore, in Chapter 2, we set out to examine the

mechanisms responsible for the elevated IFNγ production in the lungs of TCDD-treated mice. Using AhR-deficient mice, we show for the first time that the increased IFNγ levels in the lungs of TCDD-treated mice are dependent upon AhR activation. Furthermore, we determined that the AhR-mediated increased IFNγ levels are due to an elevated percentage of IFNγ-producing cells in the lung, not an increase in the amount of IFNγ produced by each cell.

Our studies characterizing the effects of TCDD on IFNγ production led to three important and novel findings. First, we learned that infection alone (i.e., no TCDD) causes many different cell types to produce IFNγ, including $CD8^+$ and $CD4^+$ T cells, NK cells, B cells, macrophages, neutrophils, and dendritic cells. To our knowledge, this is the first study showing IFNγ production by B cells and neutrophils *in vivo* during influenza virus infection. Second, we have found that during infection, the majority of the IFNγ in the lung seems to be produced by $Gr1^+$ and $CD11b^+$ cells, regardless of TCDD exposure. Finally, exposure to TCDD greatly elevates IFNγ production by macrophages and neutrophils during influenza virus infection, which are two non-characteristic sources of IFNγ.

During our investigation into the effects of TCDD on IFNγ production, we determined that activation of the AhR deregulates a downstream effect of IFNγ signaling. This was demonstrated by a significant increase in iNOS levels in the lungs of TCDD-treated mice seven days post infection. Using AhR-deficient mice, we determined that like the increased IFNγ production, the elevated iNOS levels in the lungs of TCDD-treated mice are dependent upon AhR activation. We also found that alveolar macrophages and lung epithelial cells express iNOS in the lungs of both vehicle- and TCDD-treated mice. Interestingly, exposure to TCDD did not alter the cellular sources of iNOS, but appeared to cause increased expression of the enzyme by the same cells.

With regard to the elevated IFNγ levels, we have shown that none of the well-known IFNγ-stimulating cytokines (IFNα/β, IL-2, IL-12, IL-15 and IL-18) are increased following exposure to TCDD. Thus, these cytokines are not likely responsible for increasing IFNγ production in the lung. Second, although the IFNγ levels are AhR-dependent, the IFNγ gene lacks identifiable AhRE or AhREII binding sites. This suggests that direct AhR:AhRE interactions are not responsible for the elevated IFNγ levels in the lungs of TCDD-treated mice. Using bone marrow chimeric mice in which the immune cells are AhR-deficient, but the rest of the tissues possess the AhR, we determined that the excessive production of IFNγ in the lung is driven by an AhR-mediated signal extrinsic to the immune system. This suggests that while macrophages and neutrophils produce the excess IFNγ in the lung, it is not due to direct AhR-mediated changes in these cells.

We have also shown that IFNγ mRNA expression is not increased following TCDD-treatment, suggesting that a post-translational mechanism may play a role in the increased protein levels of this cytokine. Using iNOS-deficient mice, we determined that exposure to TCDD did not increase IFNγ levels in the lungs of these mice. Thus, it is possible that the over-production of IFNγ by phagocytic cells may be driven by increased expression of iNOS in the lung. Fitting with this idea, our data suggest that AhR activation causes a feedback mechanism to occur between IFNγ and iNOS in the lung during infection.

In summary, the findings of these studies demonstrate that although AhR activation by TCDD suppresses the number of $CD8^+$ T cells in the lung, this response is extremely robust and relatively few CTL are needed for pulmonary viral clearance. We also show here that activation of the AhR greatly increases IFNγ levels in the lung, the majority of which is produced by non-typical sources, i.e., phagocytic cells. Furthermore, our results demonstrate that AhR-driven

events external to the immune system underlie the excessive production of IFNγ in the lungs of TCDD-treated mice, and that a novel iNOS-mediated pathway may drive this signal.

Future Directions

While we have investigated the role of iNOS in the regulation of IFNγ using iNOS-deficient mice, another approach should be utilized to confirm this finding. One method we could use would be to block iNOS-mediated signaling at specific time points during infection using a NOS inhibitor, such as L-NMMA (NG-monomethyl-L-arginine). This approach has merit because others have shown that mice treated with L-NMMA have remarkable improvement in the pathological effects of influenza virus infection (227). For these studies, female C57Bl/6 mice would be treated with vehicle or TCDD (10 μg/kg) one day prior to infection with influenza A virus (120 HAU, HKx31). During the time of excessive IFNγ production in the lungs of TCDD-treated mice (i.e. days 5-7 post infection), mice would be treated once daily with 2 mg L-NMMA, or PBS control (i.p.). Seven days after infection, mice would be sacrificed and IFNγ levels will be measured using ELISA. iNOS levels will be determined by Western blot as a measure of L-NMMA efficacy. If iNOS drives the excess IFNγ in the lungs of TCDD-treated mice, we would expect that L-NMMA-treated, TCDD-exposed mice would have similar IFNγ levels as vehicle-treated mice. If iNOS-mediated signaling does not play a role in the elevated pulmonary IFNγ levels, L-NMMA treated mice should have similar IFNγ levels as PBS-treated controls.

Alternatively, because L-NMMA is a non-specific NOS inhibitor, we could use the iNOS-specific inhibitor L-NIL (L-N6-L-iminoethhyl) lysine) for these studies. However, to our knowledge, this inhibitor has not been tested in influenza virus infected mice. Therefore,

preliminary studies would need to be performed to determine an effective dosing strategy and to confirm that administration of this chemical does not alter survival from influenza virus infection.

Our data demonstrated that an AhR-mediated signal from the lung drives the elevated pulmonary IFNγ levels. Some possible targets of AhR-mediated toxicity that can be driven by the lung include mediators of oxidative stress. Specifically, reactive oxygen species, such as superoxide anions can be formed by epithelial cells, influenza virus-activated leukocytes and via xanthine oxidase. Interestingly, the activity of xanthine oxidase is increased during influenza virus infection (251). Moreover, superoxide has been shown to play a role in TCDD-induced lipid peroxidation (252), suggesting that this pathway is sensitive to AhR-mediated alterations. Superoxide anions readily interact with nitric oxide (generated via iNOS activation) to produce a very potent oxidant, peroxynitrite, which connects the generation of superoxide with iNOS. Thus, alterations in mediators of oxidative stress in the lung may provide a pathway by which AhR activation in the lung can drive IFNγ production via an iNOS-mediated mechanism.

Studies in our laboratory have shown that exposure to TCDD prior to influenza virus infection does not upregulate H_2O_2 or superoxide anion production by neutrophils in the lung. However we have not examined the production of these reactive intermediates by the lung parenchyma, (i.e. the source of the signal that drives the elevated pulmonary IFNγ levels). Therefore, we will measure total levels of superoxide anion in the lung lavage fluid taken from vehicle- and TCDD-treated mice. Specifically, female C57Bl/6 mice will be treated with vehicle or TCDD (10 μg/kg) one day prior to i.n. infection with influenza A virus (HKx31). Vehicle and TCDD-treated, mock-infected animals will serve as controls for the infection (day 0). On days 5, 7 and 9 after infection, mice will be sacrificed and lavage will be performed with ice-cold PBS

containing 2 mM EDTA. Cells and cellular debris will be removed by centrifugation (400 x *g* for 5 min) and then filtered with a 0.45 μm syringe filter. In order to be detected, superoxide levels in BAL fluid must be examined immediately using a spectroscopic assay based on SOD-inhibitable reduction of ferricytochrome *c* (253,254). Alternatively, superoxide levels could be measured via a LumiMax superoxide anion detection kit (Stratagene) or via flow cytometry. We expect that superoxide levels will be increased in the lungs of TCDD-treated mice. If so, then xanthine oxidase levels in lung lavage fluid would also be measured as a possible mechanism of superoxide production (using a spectrophotometric assay, described by Massey *et al.* 255). Furthermore, we expect that H_2O_2 levels in the lung will be elevated following AhR activation. Hydrogen peroxide levels in the lung will be measured via flow cytometry as described previously (256). Briefly, collagenase-digested lung cells would be incubated with dihydroethidine (HE, Molecular Probes) for 15 minutes at 37°C in the dark prior to flow cytometric analysis.

The link between superoxide production and IFNγ levels can be examined using mice that overexpress the human extracellular (EC) superoxide dismutase (SOD) gene. Detoxification of superoxide occurs via dismutation that is catalyzed by three major dismutases: Cu/Zn SOD, Mn SOD and EC SOD (251). EC SOD is a major extracellular antioxidant expressed in the lung primarily by alveolar type II pneumocytes (257,258). Suliman *et al.* demonstrated that the detrimental effects of superoxide in the lung can be rescued in influenza virus-infected SOD-transgenic (SOD-TG) mice (251). Specifically, SOD-TG mice have decreased IFNγ levels, iNOS expression and neutrophil numbers in their lungs during influenza virus infection (251), as compared to wild type controls. These data are of interest because they provide a possible link between elevated superoxide levels and increased neutrophil numbers and IFNγ over production,

which we observe in the lungs of TCDD-treated, virus-infected mice. Therefore, SOD-TG (from Dr. Suliman, Duke University) and C57Bl/6 wild-type mice would be treated with vehicle or TCDD one day prior to infection with influenza virus (A/HKx31). Seven days after infection, the peak day of elevated IFNγ and iNOS, mice would be sacrificed and pulmonary IFNγ and iNOS levels will be determined by ELISA and Western blot, respectively. Neutrophil numbers in the lung would be measured by flow cytometry. If the excess IFNγ and iNOS levels are ablated in TCDD-treated, infected SOD-TG mice compared to TCDD-treated, infected wild-type mice, it would suggest that superoxide plays a role in the elevation of these two proteins. However, it is possible that superoxide may be involved in the induction of either IFNγ or iNOS, but not both. So using these mice may help more specifically target the superoxide-driven pathway that is involved in the elevated IFNγ levels in the lungs of TCDD-treated mice.

In addition to altered redox status, increased transcription factor levels and activity may play a role in the elevated IFNγ levels in the lung. For example, exposure to TCDD, elevated iNOS activation and oxidative stress intermediates can induce the AP-1 and NFκB signaling pathways (26,259-263). Although others have shown that the AhR and NFκB p65 associate in mammary and liver cells (28,37), our data do not corroborate their findings (Appendix C). However, while our data suggest that exposure to TCDD does not affect protein levels of NFκB p65 or *p*-IκB during infection, they do not rule out the effects of AhR activation by TCDD on NFκB activity. Therefore, AP-1 and NFκB activity levels will be measured using Gelshift™ assay kits (Active Motif). Activity of these transcription factors will be measured in the nuclear fractions of lung homogenates from vehicle- and TCDD-treated, virus-infected mice. Initially, activity will be determined on days 5 and 7 post infection (prior to and the peak day of IFNγ in the lungs of TCDD-treated mice). If these transcription factors play a role in the elevated IFNγ

levels in the lung, we expect that their activity will be increased in the lungs of TCDD-treated mice. If activity is elevated, further studies could be performed to determine whether these transcription factors bind to the upstream regulatory regions of the IFNγ and iNOS genes using chromatin immunoprecipitation assays (ChIPs).

In conclusion, findings from the studies presented above will help determine the mechanisms that lead to overproduction of IFNγ in the lungs of TCDD-treated, virus-infected mice. Furthermore, they will characterize the effects of TCDD on oxidative stress in the lungs of influenza virus-infected mice, a pathway that has not been well studied. Understanding the mechanisms responsible for AhR-mediated deregulated IFNγ production is of interest because IFNγ has been associated with the development and severity of chronic inflammatory diseases. Furthermore, the number of people suffering chronic respiratory diseases such as ARDS and COPD is on the rise worldwide. Our results suggest that activation of the AhR by pollutants deregulates IFNγ production during viral infection via a novel iNOS-mediated pathway, which may provide a possible mechanism for the development of chronic inflammatory disorders of the lung. Thus, by further characterizing the mechanism that drives these AhR-mediated effects, we may increase our knowledge of the causes of these chronic inflammatory diseases of the lower respiratory tract, and provide an experimental link between the environment and the etiology of these diseases.

APPENDICES

Some of the findings presented in this dissertation show that activation of the AhR increases IFNγ production by macrophages and neutrophils, and elevates iNOS expression in the lung during viral infection. The studies described in the following appendices investigate the relationship between excessive IFNγ production and decreased host resistance to viral infection, the pathways involved in the induction of IFNγ and iNOS, and whether exposure to other AhR ligands deregulates the immune response to influenza virus similarly to TCDD.

Appendix A: The role of IFNγ in the decreased host resistance of TCDD-treated mice: studies using IFNγ-deficient mice.

Appendix B: Examining whether depletion of a cellular source of IFNγ increases survival of TCDD-treated, influenza virus-infected mice.

Appendix C: TCDD-mediated effects on NFκB levels during influenza virus infection.

Appendix D: The role of cytokines in the stimulation of IFNγ production.

Appendix E: Characterizing the role of the AhR in deregulated immune responses to influenza virus infection: studies using multiple AhR ligands.

Appendix A

The role of IFNγ in the decreased host resistance of TCDD-treated mice: studies using IFNγ-deficient mice.

We have previously shown that approximately 50% of mice treated with TCDD are unable to survive an otherwise non-lethal infection with influenza A virus (115). Furthermore, we know that exposure to TCDD causes a four- to ten-fold increase in IFNγ levels in the lungs of influenza virus-infected mice (114,115). Given that aberrant production of IFNγ can lead to excess inflammation and tissue damage (264), we hypothesized that the excess IFNγ in the lungs of infected, TCDD-treated mice contributes to the decreased survival of these animals. To test this, we examined whether IFNγ-deficient (gamma knockout, or GKO) mice were less susceptible to mortality in the context of a sublethal infection.

In Figure 5.1, we show that while 90% of vehicle-treated wild-type mice (open circles) survived the infection, only 40% of vehicle-treated GKO mice lived (open diamonds). TCDD-treated wild-type mice also had elevated levels of mortality, with only 15% of these animals surviving the infection (closed circles). Moreover, only 5% of the GKO mice treated with TCDD survived the infection (closed diamonds). Thus, while other studies have shown that IFNγ-deficient mice can mount a sufficient immune response to influenza virus infection (265), these data suggest that some level of IFNγ is critical for survival from the HKx31 strain of influenza virus.

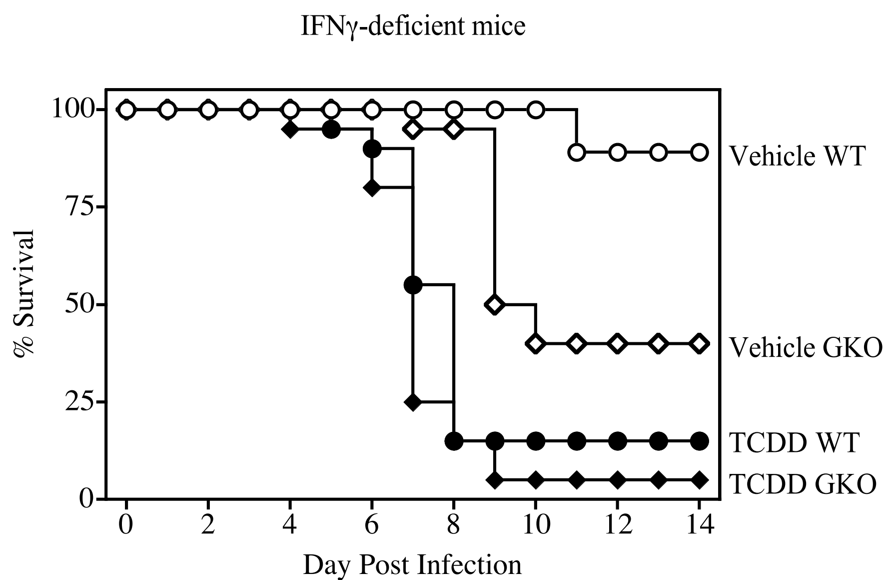


Figure 5.1 Interferon gamma is essential for survival from the HKx31 strain of influenza A virus. Female C57Bl/6 (wild type, WT) or interferon gamma-deficient mice (B6.129S7-*Ifng*^{tm1Ts}, GKO) mice were treated with peanut oil vehicle or TCDD (10 μg/kg body weight) one day prior to i.n. infection with 120 HAU influenza virus (A/HKx31). Survival was monitored for 14 days after infection.

Appendix B

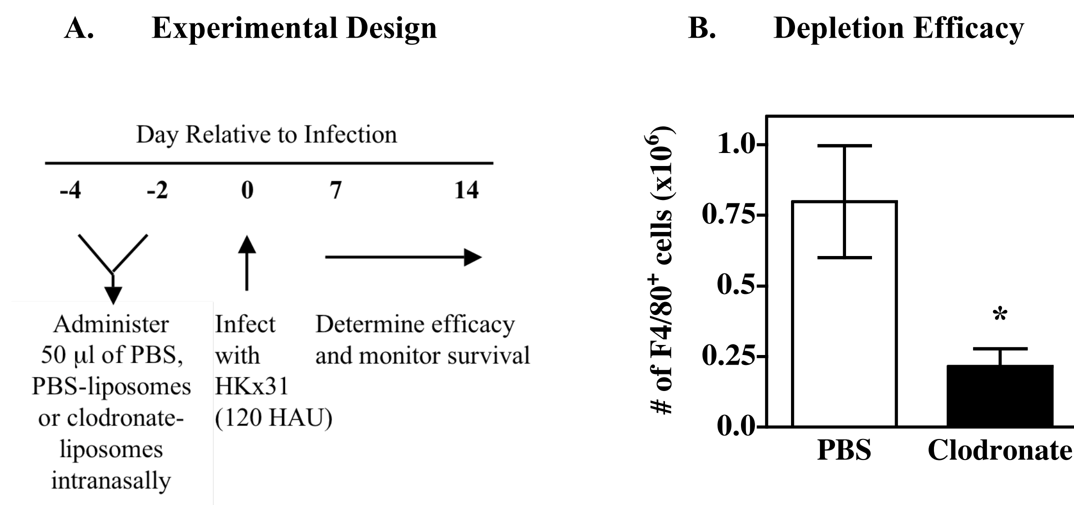
Examining whether depletion of a cellular source of IFNγ increases survival of TCDD-treated, influenza virus-infected mice.

Another approach we used to characterize the relationship between excessive pulmonary IFNγ levels and decreased survival following influenza virus infection was to deplete one of the primary cellular sources of the excess IFNγ in the lung. Our preliminary studies suggested that alveolar macrophages produce a substantial portion of the excess IFNγ in the lungs of TCDD-treated mice (data not shown). Thus, we utilized a method of macrophage-specific depletion for these experiments, which is described in detail below.

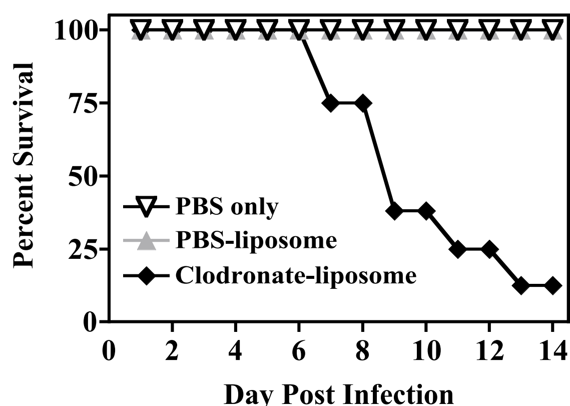
In 1996, van Rooijen *et. al.* discovered that macrophages could be specifically targeted and depleted *in vivo* using liposomal delivery of a drug called Clodronate (266). Clodronate by itself is non-toxic, but when encapsulated by a liposome it is phagocytosed by macrophages, and cannot escape from the cell. This allows the drug to accumulate to toxic levels within the cell and subsequently causes the macrophage to undergo apoptosis. Using intranasal administration of Clodronate-containing liposomes, we sought to experimentally test whether elimination of this cellular source of the excess IFNγ would rescue influenza virus-infected mice from TCDD-mediated mortality.

In our initial approach, 50 μl of PBS- or Clodronate-containing liposomes were administered (i.n.) to untreated mice four and two days prior to infection (i.e., the mice had not received vehicle or TCDD; Figure 5.2, panel A). PBS treatment alone (i.e., no liposomes) was included as an additional control for this experiment. Using this treatment strategy, all of the mice survived the initial liposome/PBS administration. Depletion efficacy was determined by flow cytometry seven days post infection. Mice treated with the Clodronate-containing

liposomes had 70% fewer F4/80$^+$ airway cells than PBS-treated controls (Figure 5.2, panel B). However, upon infection with influenza virus only 12% of the macrophage-depleted mice were able to survive infection (Figure 5.3), suggesting that alveolar macrophages are essential components of the immune response to influenza virus. Because of this, we changed our experimental approach to deplete the alveolar macrophages only during the time that excess IFNγ is being produced in the lungs of TCDD-treated mice (Figure 5.4).



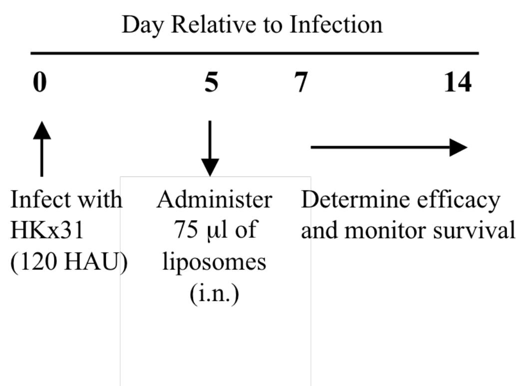
<u>Figure 5.2</u> Intranasal administration of Clodronate-containing liposomes successfully depletes F4/80$^+$ cells from the lung airways. Mice were treated as described in panel A, n=8 per treatment group. The number of F4/80$^+$ cells in lung airways was determined seven days after infection via flow cytometry (B). Depletion efficacy was determined to be > 70%. An * depicts a statistically significant difference between treatment groups ($p = 0.0036$).



<u>Figure 5.3</u> Alveolar macrophages are essential for survival from influenza virus infection. Mice were treated as described in Figure 5.2, panel A. Survival was monitored for 14 days following infection. Efficacy of macrophage depletion was 70% as determined seven days post infection. (Figure. 5.2, panel B).

To do this, mice were treated with either vehicle or TCDD (10 μg/kg) one day prior to infection with influenza A virus. Five days after infection, mice were anesthetized and given Clodronate- or PBS-containing liposomes intranasally (see Figure 5.4, panel A). Unfortunately, we discovered that intranasal (i.n.) administration of either liposome at this time during infection caused mortality, as 28% of vehicle-treated mice and 33% of TCDD-treated mice died during liposome administration (Figure 5.4, day 5 post infection). Moreover, vehicle- or TCDD-treated mice that survived the initial liposome administration had high mortality during infection, regardless of liposome content (Figure 5.4, panel B). We learned from this experiment that intranasal administration of liquid five days after infection leads to significant mortality that is likely due to an inability of the mice to clear the excess fluid from their lungs.

A. Experimental Design



B. Survival

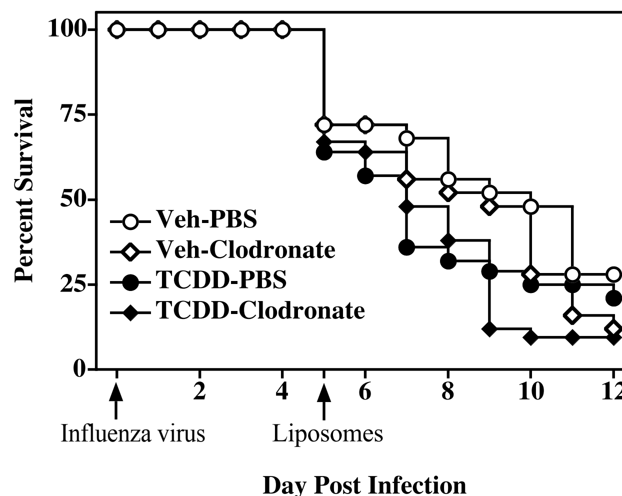


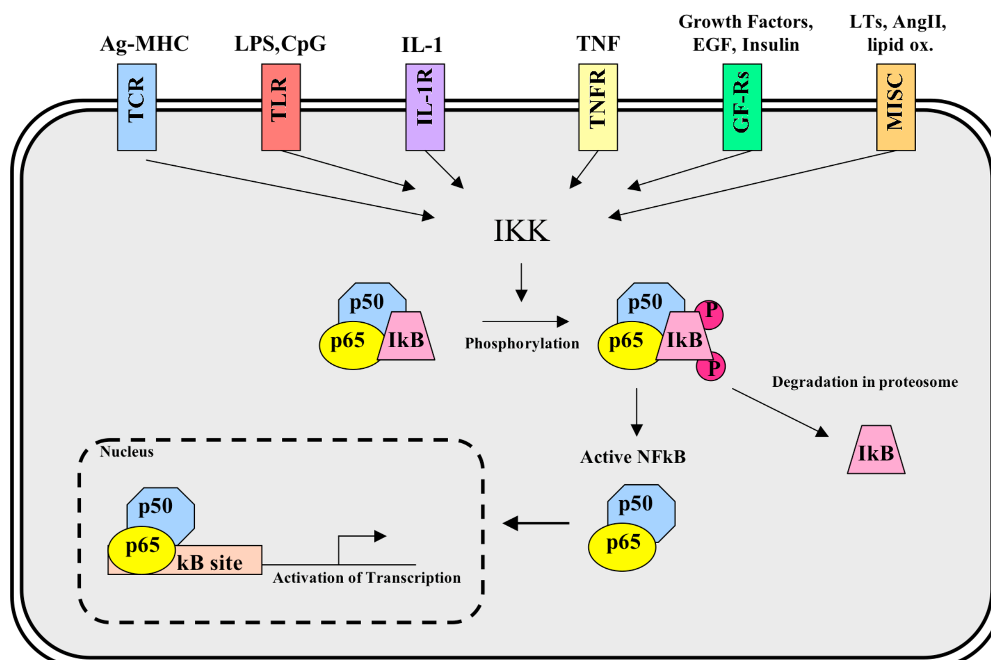
Figure 5.4 Intranasal administration of PBS- or Clodronate-containing liposomes five days after pulmonary viral infection causes significant mortality. Female C57Bl/6 mice were treated as described in panel A. (B). Over 70% of the mice that survived the initial liposome administration died during infection, regardless of liposome content or TCDD-treatment.

Thus, while the Clodronate-containing liposomes appear to be a novel and efficient tool for the depletion of macrophages, it is clear that these cells are critical for survival from influenza virus infection. Furthermore, we were unable to use this method to experimentally determine whether the excess IFNγ in the lungs is detrimental to host resistance. However, another approach we could use would be to specifically deplete the pulmonary IFNγ on the peak days of production (i.e., 5-8 days post infection) using i.n. administration of an anti-IFNγ antibody. However, this approach has two flaws. First, as we learned from the Clodronate-liposome studies, mice do not survive intranasal administration of liquid during active infection. Second, depletion of IFNγ systemically (i.e., via i.p. administration of an anti-IFNγ antibody) is unlikely to help us answer this question since IFNγ-deficient mice have difficulty surviving influenza virus infection. While it is likely that the elevated IFNγ levels in the lungs of TCDD-treated mice contribute to their decreased survival from infection, the consequences of excess IFNγ in the lung will remain unclear until an alternative experimental approach is developed.

Appendix C

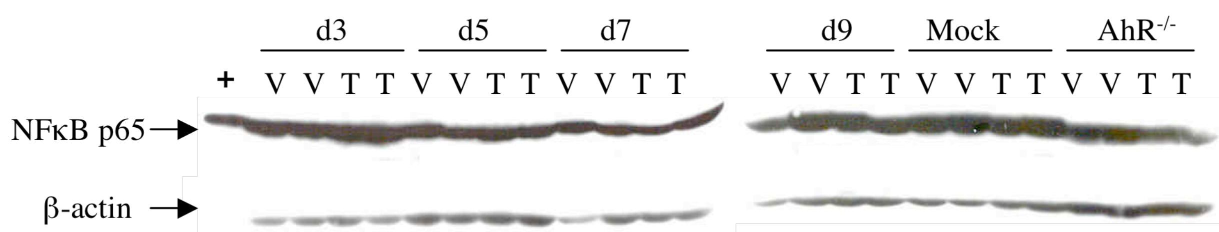
TCDD-mediated effects on NFκB levels during influenza virus infection.

NFκB is a pleiotropic transcription factor activated by multiple pathways, one of which is the regulation of many different immunoregulatory genes (Figure 5.5). NFκB also participates in many of the physiological responses that are affected by TCDD. Interestingly, both the IFNγ and iNOS genes have multiple κB sites in their upstream regulatory regions (207, 267, 268 and our unpublished data), and thus can be regulated via NFκB-mediated signaling pathways. Moreover, the AhR and p65 subunit of NFκB have been shown to associate in liver and mammary cells (28,37), making NFκB a possible target of AhR-mediated deregulation. Therefore, a mechanism whereby exposure to TCDD alters NFκB activity and the expression of NFκB-regulated genes provides a plausible explanation for AhR-mediated increased IFNγ and iNOS levels.



<u>Figure 5.5</u> The NFκB signaling pathway.

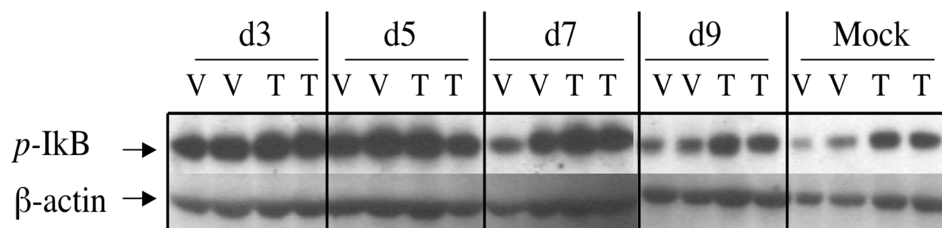
To test this idea, C57Bl/6 $AhR^{+/+}$ or $AhR^{-/-}$ mice were treated with vehicle or TCDD (10 μg/kg) one day prior to infection with influenza A virus (HKx31, 120 HAU). On the indicated days after infection, mice were sacrificed and lungs were isolated, snap frozen in liquid nitrogen and stored at -80°C. Once all lungs were collected, they were homogenized and p65 levels were determined by Western blot (Figure 5.6). These data suggest that p65 protein expression is not affected by AhR activation, as levels are similar in the lungs of wild type and $AhR^{-/-}$ mice, regardless of TCDD exposure.



<u>Figure 5.6</u> p65 levels are not affected by exposure to TCDD during influenza virus infection. See text for details. Lungs were homogenized and p65 levels were measured by Western blot using 30 μg total protein. NIH/3T3 whole cell lysates were used as a positive control as recommended by the manufacturer (+). $AhR^{-/-}$ mice were sacrificed 7 days post infection. Each band represents p65 levels in an individual animal and these results were repeated in other experiments using 3-9 animals per treatment group.

Phosphorylation of IκB is a critical step in the activation of NFκB (Figure 5.5), and thus, levels of *p*-IκB are often used as a measure of NFκB activation. We examined whether exposure to TCDD alters the levels of *p*-IκB in the lung during infection. In Figure 5.7, we show that during infection, the levels of *p*-IκB in the lungs of vehicle- and TCDD-treated mice are not different. However, it appears that exposure to TCDD in the absence of infection (i.e., mock-infected) may increase IκB phosphorylation.

A. *p*-IκB protein levels in the lung during influenza virus infection



B. Densitometry of *p*-IκB

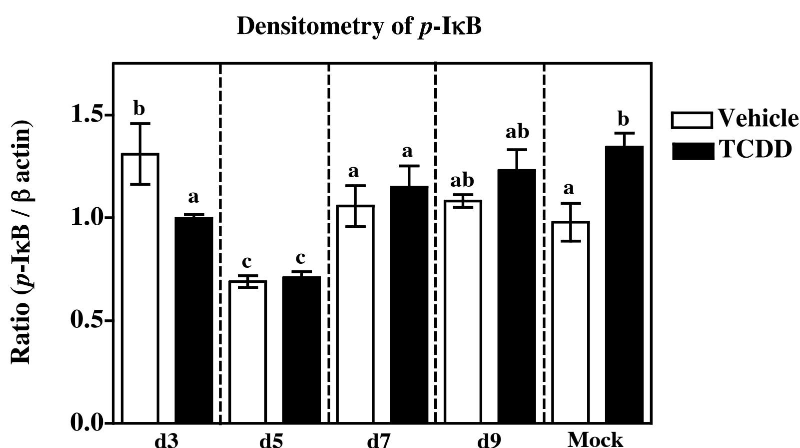
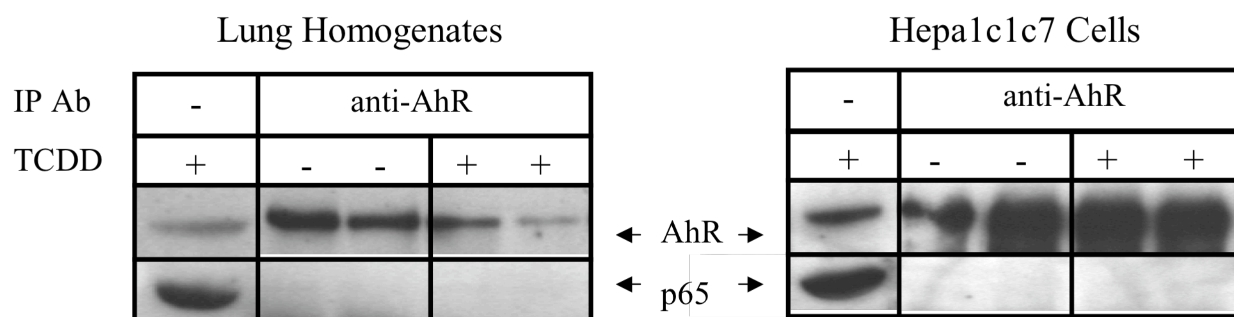


Figure 5.7 Exposure to TCDD during influenza virus infection does not alter *p*-IκB protein levels. Whole lungs were homogenized and *p*-IκB levels were measured by Western blot using 50 μg total protein (A). Each band depicts *p*-IκB levels in an individual animal and these results were repeated in other experiments using 3-9 animals per treatment group. The ratio of *p*-IκB to β-actin is shown in panel B. Error bars represent the SEM. Statistically significant ($p \leq 0.05$) differences among treatment groups are depicted by different letters.

We also examined whether the AhR and p65 subunit of NFκB associate in the lung during infection. Although others have shown that these two proteins cooperate in mammary and liver cells (28,37), our data do not corroborate their findings. The basis for this statement stems from findings using co-immunoprecipiation (Co-IP) to determine whether AhR and the

p65 subunit of NFκB associate in the lung or in Hepa1c1c7 liver cells (Figure 5.8). While these data suggest that exposure to TCDD does not affect protein levels of NFκB p65 or *p*-IκB during infection, they do not rule out the effects of AhR activation by TCDD on NFκB activity. Further assessment, using activity assays (such as EMSA) or inhibitors of NFκB-mediated signaling would be required to definitively prove that TCDD treatment does (or does not) affect this pathway. However, our current findings do not support the idea that deregulation of NF-κB activation or function underlies AhR-dependent increases in IFNγ or iNOS during infection with influenza virus.



<u>Figure 5.8</u> The AhR and p65 subunit of NFκB do not co-immunoprecipitate. *Lung homogenates:* Mice were treated with vehicle or TCDD one day prior to infection (120 HAU, HKx31) and sacrificed 7 days after infection. Whole lungs were homogenized for use in the immunoprecipiation (IP). *Liver cells:* Hepa1c1c7 cells were treated with vehicle (0.1% DMSO) or TCDD ($1x10^{-9}$ M) and harvested 2 days later. Co-IP was performed using an anti-AhR as the pull-down Ab. Following IP, 20 μl of protein was run on an SDS-PAGE gel and transferred to nitrocellulose. The membrane was probed with either an anti-AhR or anti-p65 antibody. Lungs taken from TCDD-treated mice or TCDD-treated Hepa1c1c7 cells that did not go through the IP were used as controls.

Appendix D

The role of cytokines in the stimulation of IFNγ production.

Many different cytokines have been linked with the production of IFNγ including IFNα/β, IL-18, IL-12 and IL-15 (170,172,177,200-205). Therefore, one possible way in which exposure to TCDD could elevate IFNγ production in the absence of direct AhR interaction with AhRE in the enhancer/promoter for ifng would be to increase the level of these known regulators of IFNγ production. We have already shown that exposure to TCDD does not alter the magnitude and kinetics of the infection-associated production of IFNα/β (Figure 2.2, panel C). Likewise, we measured IL-18 levels in the lung using two methods. First, we used ELISA to detect IL-18 levels in lung lavage fluid from vehicle- and TCDD-treated mice over the time course of infection (days 1-9 after infection). While the rmIL-18 standard curve developed ($p = 0.986$), no IL-18 was detected in lung lavage fluid from mice in either treatment group at any time point examined. This finding was unexpected so we repeated the IL-18-specific ELISA using kits from two separate vendors, and were unable to detect any IL-18 protein in the lung during infection (please see Chapter 2 results). For our second approach we measured IL-18 expression at the mRNA level using RT-PCR. No IL-18 mRNA was detected in lung tissues at any time point during infection, suggesting that this cytokine is not produced during this immune response. Thus, neither IFNα/β nor IL-18 appear to be responsible for the increased IFNγ levels in the lung.

IL-12 is another well-characterized stimulator of IFNγ production (200,269,270). However, we had previously determined that exposure to TCDD *decreases* IL-12 levels in the lung during infection (114,115). Nevertheless, it was of interest to further extend the

investigation of IL-12 and determine whether the decreased production of IL-12 in the lungs of TCDD-treated mice is dependent upon AhR activation. Consistent with the idea that all of the effects of TCDD are mediated by the AhR, IL-12 levels are not suppressed in TCDD-treated, infected $AhR^{-/-}$ mice (Figure 5.9). Thus, we show that the suppression of IL-12 is mediated by activation of the AhR, and thus does not likely play a role in the elevated levels of IFNγ in the lungs of TCDD-treated mice.

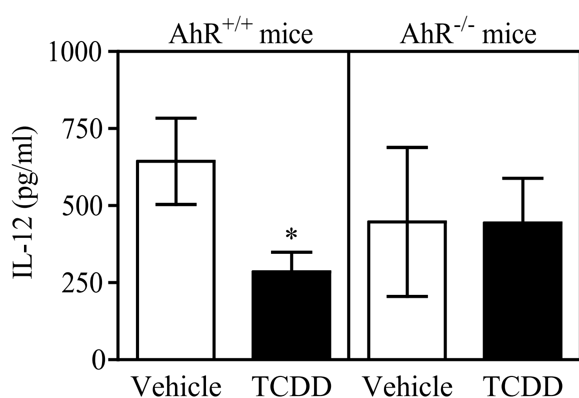
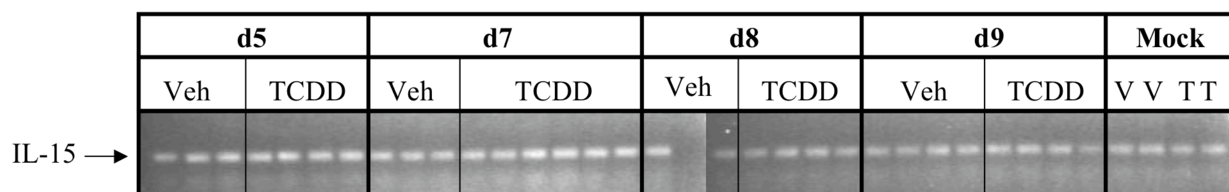


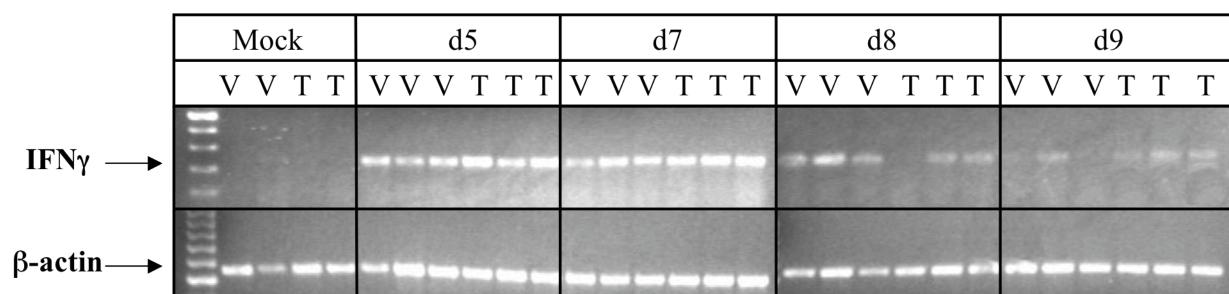
Figure 5.9 Activation of the AhR decreases IL-12 levels in the lung during viral infection. Wild-type ($AhR^{+/+}$) or $AhR^{-/-}$ mice were treated with vehicle or TCDD (10 μg/kg) one day prior to infection with 120 HAU influenza A virus. Seven days after infection, lung lavage fluid was collected and IL-12 levels were measured by ELISA. Error bars represent the SEM, n=3-4 per group. An * denotes statistically significant difference ($p \leq 0.05$).

IFNγ production by NK cells can be regulated by IL-15 (271-273). Therefore, we determined whether exposure to TCDD increased IL-15 levels in the lung during the time course of infection. In Figure 5.10, we show that IL-15 is constitutively expressed in the lung, and that exposure to TCDD does not alter the levels of this cytokine. While we did not observe a TCDD-mediated change in mRNA expression, we were unable to confirm these findings at the protein level, because at the time of these experiments anti-IL-15 antibodies for use in ELISA were commercially unavailable.



<u>Figure 5.10</u> Exposure to TCDD does not alter IL-15 levels in the lung. Wild-type mice were treated as described in Figure 5.9. On the indicated days post infection, lung tissue was isolated and RNA was extracted using Trizol. Following reverse transcription, IL-15 message was assessed using PCR, and the product (257 bp) run on an agarose gel. Vehicle- or TCDD-treated, mock-infected animals served as infection controls.

Collectively, these data imply that the elevated IFNγ levels in the lungs of TCDD-treated mice are not due to increased production of IFNα/β, IL-18, IL-12 or IL-15. Furthermore, while we see a significant increase in protein levels of IFNγ in the lungs of TCDD-treated mice, we do not see a change in mRNA expression (Figure 5.11). This suggests that a post-translational signal may be responsible for the elevated levels of IFNγ in the lungs of TCDD-treated, virus-infected mice. As we showed in Chapter 3, it is likely that a signal from the lung is responsible for the elevated levels of IFNγ in the lungs of TCDD-treated mice, and this signal is possibly regulated by NO, since it seems to be dependent upon iNOS.



<u>Figure 5.11</u> Exposure to TCDD does not alter IFNγ mRNA expression in the lung. Wild-type mice were treated as described in Figure 5.9. On the indicated days post infection, lung tissue was isolated and RNA was extracted as described in Figure 5.8. IFNγ message was assessed using PCR, and the product (239 bp) run on an agarose gel. Vehicle- or TCDD-treated, mock-infected animals served as infection controls. Analysis of β-actin was performed to confirm RNA integrity and equivalency of cDNA amounts.

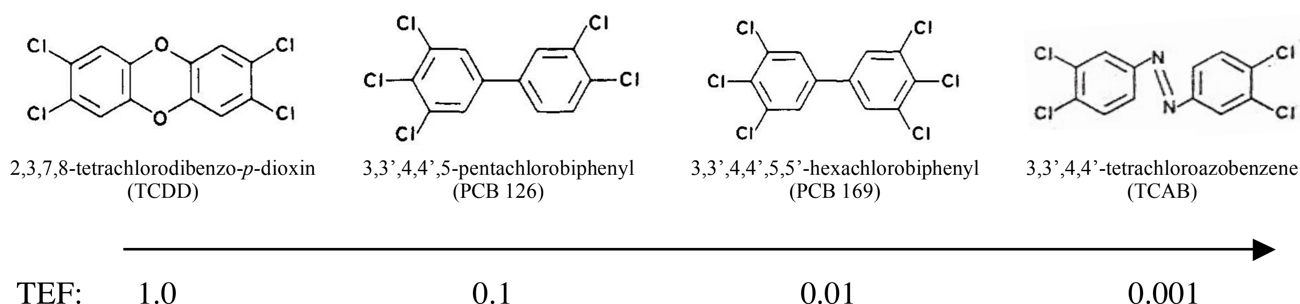
Appendix E

Characterizing the role of the AhR in deregulated immune responses to influenza virus infection: studies using multiple AhR ligands.

Our laboratory and others (107,115,121,135,147) have shown that exposure to TCDD deregulates the immune response to influenza virus infection, causing both immunosuppression and increased inflammation in the lung. For example, compared to vehicle-treated controls there are 50-80% fewer $CD8^+$ T cells in the lungs of TCDD-treated, virus-infected mice. In contrast to suppression of adaptive immune responses, mice exposed to TCDD have twice as many neutrophils and 2- to 4-fold higher levels of IFNγ in the lung as do vehicle-treated animals. Using AhR-deficient mice, we have determined that the above-listed effects of TCDD are dependent upon AhR activation (Chapter 2, and our unpublished observations).

Since TCDD is the highest affinity AhR ligand, we determined whether exposure to agonists with affinities for the AhR that are less than TCDD's would elicit the same alterations in immune function as TCDD. The dose of each AhR ligand used was based upon its toxic equivalency factor (TEF). Specifically, using TEF defined by the World Health Organization, we administered a dose of each chemical that, if it were not metabolized, would be equivalent to 10 μg/kg TCDD (Figure 5.11). In addition to considering binding affinity for the AhR, the chemicals chosen for these studies we selected because they are relatively common environmental contaminants and their TEFs have 10-fold decreasing values. All compounds were given by gavage one day prior to intranasal infection and the specific amount of each one used is as follows: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**TCDD**, 10 μg/kg), 3,3',4,4',5-

pentachlorobiphenyl (**PCB126**, 100 μg/kg), 3,3',4,4',5,5'-hexachlorobiphenyl (**PCB169**, 1 mg/kg) tetrachloroazobenzene (**TCAB**, 10 mg/kg)[2].



<u>Figure 5.12</u> Structures and WHO TEFs for TCDD and other selected AhR ligands. Structures adapted from Goldstein and Safe, 1989 (8), TEFs taken from Gilpin *et al*. 2003 (10) in Dioxins and Health, 2nd Ed.

As expected, activation of the AhR by TCDD, PCB126 and TCAB decreased host resistance to influenza virus infection (Figure 5.13). Seven days after infection, mice were sacrificed and airway immune cells were isolated by lavage. While only TCDD caused a statistically significant decrease in the number of $CD8^+$T cells in the lung, exposure to PCB 126, 169 and or TCAB caused slight suppression of $CD8^+$T cell numbers (Figure. 5.14).

[2] In one study, the AhR ligand benzo[a]pyrene was used (**BaP**; 50 mg/kg). However, since BaP is a widespread component of air pollution, we were concerned that giving this ligand orally may not be representative of human exposure. BaP is also readily metabolized, and it is likely that the parent compound had been eliminated from the body before the time of our studies (d7 post infection). Moreover, the immune response to influenza virus in the lungs of BaP-treated mice was not significantly different from vehicle-treated mice at any parameter examined. Therefore, data from these mice were not included in this appendix.

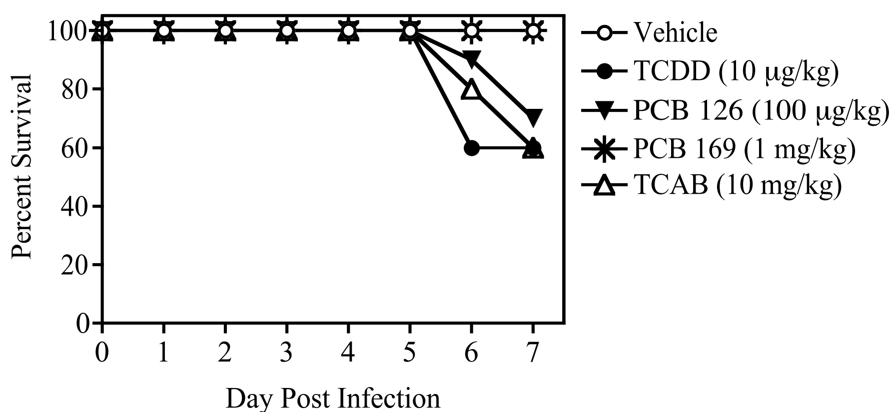


Figure 5.13 **Activation of the AhR decreases host resistance to influenza virus infection.** See text for details. Mice were treated orally with the indicated AhR ligand one day prior to infection with 120 HAU influenza A virus (HKx31). Survival was monitored for seven days after infection, n=6-7 per group.

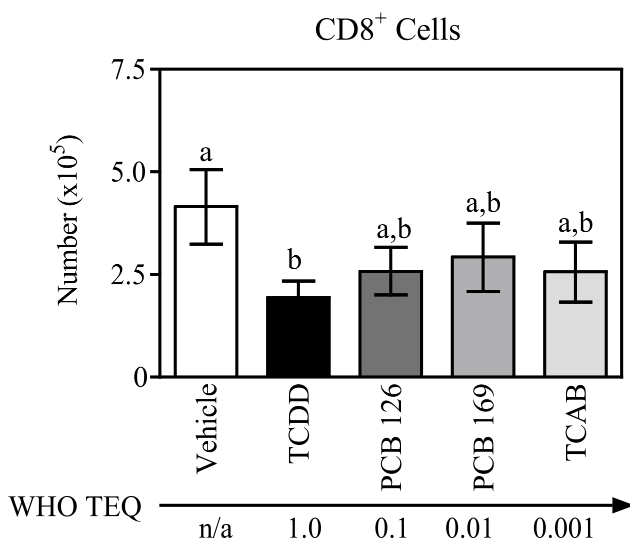


Figure 5.14 **Exposure to TCDD decreases $CD8^+$ T cell numbers in the lung.** Mice were treated as described in Figure 5.13. The number of $CD8^+$ cells in the lung was determined by flow cytometry. Error bars represent the SEM (n=7-8 per group). Statistically significant ($p \leq 0.05$) differences among treatment groups are indicated by different letters.

Another characteristic effect of exposure to TCDD is a 2-fold increase in the percent and number of $Gr1^+$ cells (neutrophils) in the lung during infection (114,115,135,139). Therefore we examined the effect of exposure to these same AhR ligands on this endpoint. As shown in Figure 5.15, exposure to TCDD or PCB 126 increased the percentage and number of neutrophils in the lung. Mice exposed to PCB 169 or TCAB had similar pulmonary neutrophil levels as the vehicle-treated controls.

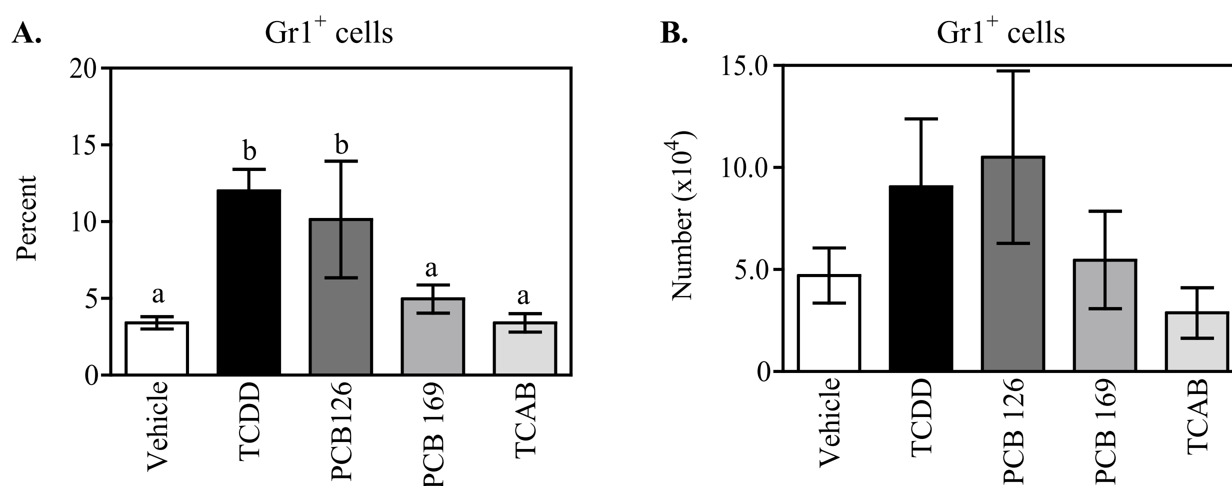
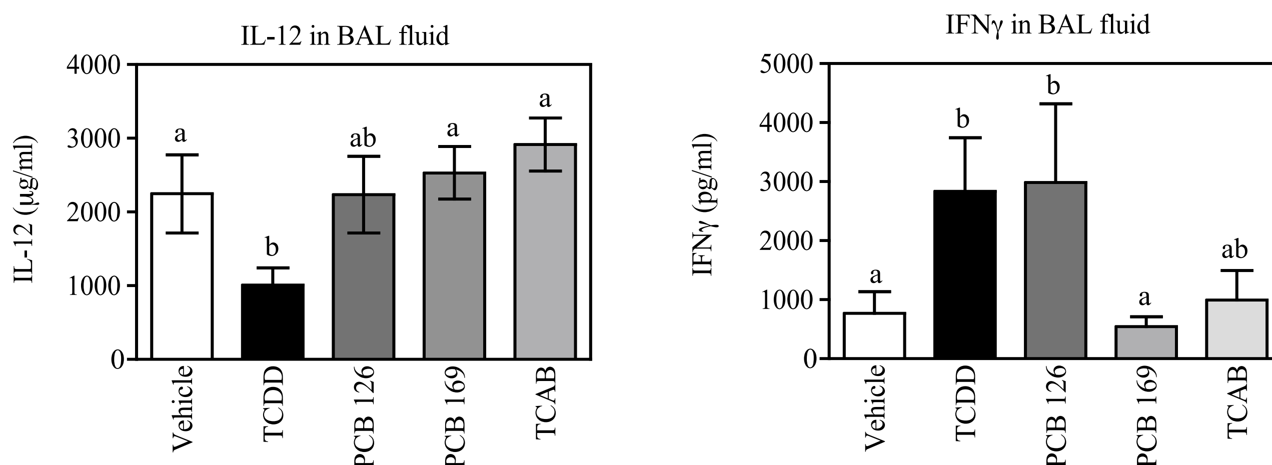


Figure 5.15 Exposure to TCDD or PCB 126 increases pulmonary neutrophil numbers during infection. Mice were treated as described in Figure 5.13. The percent (A) and number (B) of $Gr1^+$ cells in the lung was determined by flow cytometry, n=7-8 per group. Error bars represent the SEM. Statistically significant ($p \leq 0.05$) differences among treatment groups are depicted by different letters.

In addition to characterizing the effects of these chemicals on immune cell numbers, we investigated whether AhR activation by these ligands alters the production of cytokines in the lung during infection. In particular, we measured levels of IL-12 and IFNγ in the lungs of mice seven days after infection. Only TCDD was able to significantly suppress the production of IL-12 in the lung, while both TCDD and PCB 126 elevated pulmonary IFNγ levels (Figure 5.16).



<u>Figure 5.16</u> Activation of the AhR alters IL-12 and IFNγ levels in the lung during infection. Mice were treated as described in Figure 5.13. Levels of IL-12 (A) and IFNγ (B) in the lung were determined by ELISA. Error bars represent the SEM (n=7-8 per group). Statistically significant ($p \leq 0.05$) differences among treatment groups are depicted by different letters.

Although we expected all of the AhR ligands to have similar effects as TCDD at the doses used, it is clear that they do not. TCDD is poorly metabolized, and thus, has a relatively long half-life in the body (~11 days in C57Bl/6 mice, 274). However, PCB 126, PCB 169 and TCAB are metabolized faster than TCDD, and thus their concentrations in the body are not sustained as long as TCDD. Therefore, while the concentrations were adjusted to be equal at the time of administration, it is likely that the actual amount of these chemicals were quite different from each other during infection.

It is also apparent from dose-response studies using TCDD, that different immune endpoints have different sensitivities to TCDD-mediated alterations. For example, the number of lymphocytes in the lung during influenza virus infection are suppressed by as little as 2.5 μg/kg of TCDD (114), whereas higher doses of TCDD (7.5 to 10 μg/kg) are required to elevate neutrophil numbers and decrease IL-12 levels in the lung (114). These data provide a possible explanation for why exposure to PCB 126, PCB 169 and TCAB suppressed $CD8^+$T cell numbers in the lung, but only TCDD and PCB 126 increased the percentage of neutrophils and IFNγ levels in the lung. In other words, suppression of T cell numbers may require less potent activation of the AhR than the other endpoints we examined.

Few studies characterizing the immunotoxic effects of PCB 126, PCB 169 or TCAB have been performed in mice. However, Pan *et al.* (275) recently reported that exposure to 200 μg/kg of PCB 126 (2-fold higher dose than used in this study) decreases plasma IgM levels, splenic production of IL-5 and thymocyte numbers in OVA-immunized mice. Furthermore, 3 mg/kg of PCB 169 (3-fold higher dose than used in this study) suppresses the cytotoxic T cell response to P815 tumor cells (85). Our results suggest that the T cell-mediated response to influenza virus infection may be slightly more sensitive to perturbation by PCB 126 and 169, because we observed decreased $CD8^+$ T cell numbers with 2 or 3-fold lower doses of these chemicals, respectively.

Taken together, the results of these experiments were interesting because they not only show that these chemicals have immunotoxic effects, but demonstrate how metabolism likely plays a significant role in the toxicity of these chemicals. Furthermore, they suggest that in order for these chemicals to alter the immune response to infection, activation of the AhR must be sustained. For example, since TCDD is not metabolized and has a long half-life, its steady state

concentrations are higher and more prolonged than those of TCAB. Therefore, it is likely that TCDD causes a more sustained activation of the AhR than these other chemicals, which may account for why TCDD has more profound effects on the immune response to influenza virus.

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