An Abstract of the Dissertation of

Jin-Young Choi for the degree of Doctor of Philosophy in Toxicology presented on June 15, 2000. Title: Involvement of Inflammation and Associated Mac-1^+Gr-1^+ Cells in the Immune Suppression Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

Abstract approved: __

Nancy I. Kerkvliet

Exposure to TCDD suppresses the generation of immune responses through unknown mechanisms. Interestingly, TCDD enhances inflammatory responses to various stimuli. The goal of the studies presented in this thesis was to examine the role of hyperinflammation in TCDD-induced immunotoxicity. Previously we observed an increase in Mac-1^+ cells in the spleen that coincided with the suppressed CTL response in TCDD-treated C57Bl/6 mice following injection of allogeneic P815 tumor cells. We hypothesized that these Mac-1^+ cells represented a systemic inflammatory response and were an immunomodulatory population that suppressed the development of the CTL response. In order to test the hypothesis, we characterized the phenotype and function of Mac-1^+ cells in addition to investigating inflammatory components, such as TNF and SAA. Mac-1^+ cells were found to co-express Gr-1 antigen and were identified morphologically as neutrophils. The Mac-1^+ cells increased in the spleen, blood as well as bone marrow during the development of an allogeneic immune response. In mice treated with TCDD, the increase in Mac-1^+Gr-1^+ cells was enhanced. However, plasma levels of TNF and SAA were not increased in TCDD-treated mice. Mac-1^+Gr-1^+ cells from TCDD-treated mice expressed lower levels of Gr-1 and a number of surface molecules, such as ICAM-1, F4/80, LFA-1, and CD40 relative to cells from vehicle treated-mice. CD62L was shed on Mac-1^+Gr-1^+ cells in the blood from TCDD-treated mice suggesting that Mac-1^+Gr-1^+ cells were highly activated. Indeed, the oxidative burst was significantly

enhanced in Mac-1^+Gr-1^+ cells from TCDD-treated mice. When immunomodulatory functions of Mac-1^+Gr-1^+ cells were examined, splenic Mac-1^+Gr-1^+ cells from TCDD-treated mice did not inhibit cytotoxic activity of *in vivo* activated CTL, but suppressed the *in vitro* generation of allo-reactive CTL in a cell number- and cell contact-dependent manner. Although these cells appeared to have immunomodulatory properties *in vitro*, *in vivo* depletion of Mac-1^+Gr-1^+ cells did not restore the CTL response in TCDD-treated mice. Furthermore, immunohistochemical staining of spleens showed that Mac-1^+Gr-1^+ cells were located in the red pulp and separated from T cells, suggesting that direct interaction between the cells was unlikely. Therefore, although the increase in Mac-1^+Gr-1^+ cells in TCDD-treated mice coincided temporally with the failure to develop CTL activity, we conclude that Mac-1^+Gr-1^+ cells do not contribute to the immune suppression induced by TCDD in P815-injected mice and that enhanced inflammation is independent from TCDD-induced immunotoxicity.

Involvement of Inflammation and Associated Mac-1^{+}Gr-1^{+} Cells in the Immune Suppression Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

By

Jin-Young Choi

A Dissertaion
submitted to
Oregon State University

In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Presented June 15, 2000
Commencement June 2001

Doctor of Philosophy dissertation of Jin-Young Choi presented on June 15, 2000

APPROVED:

Major Professor, representing Toxicology

Head of Department of Environmental and Molecular Toxicology

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Redacted for Privacy

Jin-Young Choi, Author

ACKNOWLEDGEMENTS

I wish I could remember the names of all the individuals who have influenced me during my time in Corvallis. If I forget someone who takes the time to read this document, I apologize in advance.

This dissertation is the result of many hours of work performed by many different people. I would like to special thank to my advisor, Dr. Nancy Kerkvliet, for the wonderful opportunity she provided over the years. She has showed and taught me enthusiasm in science and life, and gave me an endless encouragement over the years whenever I confronted at dead-ends. I would like to thank to my committee members: Dr. Christopher Bayne, Dr. Jerry Heidel, Dr David Williams, Dr. Olaf Hedstrom for confidence and wisdom. I would like to thank all members in the laboratory of Dr. Kerkvliet. Linda Steppan who helped me from the designing countless experiments to technical support, Julie Oughton who taught me flow cytometer and also helped thesis writing, and Dr. Dave Shepherd, Dr. Erica Dearstyne, Dr. Beth Vorderstrasse, and Carl Ruby who provided priceless discussions and pleasant atmosphere to work with. Without their help, discussions, insights, and criticisms, this work would not have been possible. Beyond the lab members, I would like to extend thanks to Dr. Hedstrom and Kay Fisher who helped me for histopathology and immunohistochemistry works.

I will remember many friends I have met through the years in Corvallis. They listened me, gave me advises, encouraged me, and kept me moving forward. I would like to thank all the folks including many Toxicology students, faculty members and staffs in Department of Environmental and Molecular Toxicology and Environment and Health Science Center.

Last but most of all, I would like to thank my family: parents, husband, and my kids Jung-Kyu and Jung-Eun. They have always been patient, waiting, supportive, provided endless love. Thank you very much

TABLE OF CONTENTS

Page

TABLE OF CONTENTS

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

Involvement of Inflammation and Associated Mac-1$^+$Gr-1$^+$ Cells in the Immune Suppression Induced by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

CHAPTER 1

INTRODUCTION

1-1. Research problem

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a ubiquitous and persistent environmental contaminant, is the prototype and most toxic member of a large group of structurally similar chlorinated aromatic hydrocarbon compounds. TCDD is an unwanted by-product produced during chlorinated phenoxy herbicide manufacturing, pulp and paper bleaching, and municipal and hospital incineration (Safe, 1990).

TCDD produces a wide spectrum of toxic effects including teratogenicity, carcinogenicity, reproductive toxicity, immunotoxicity and death in laboratory animals. The potential human health risks resulting from exposure to TCDD and related chemicals continue to arouse widespread public concern. In 1997, the International Agency for Research on Cancer (IARC) classified TCDD as a human carcinogen based upon accumulated data showing that it contributes to a general increase in all types of cancer. This variety of toxic effects is believed to be mediated via the aromatic hydrocarbon receptor (AhR) which is present in the cytosol. Upon binding of TCDD to the AhR, the complex heterodimerizes with the aryl hydrocarbon nuclear translocator (ARNT) protein and translocates to the nucleus. The complex binds to nuclear dioxin response elements (DRE) on the target genes (Wilson and Safe, 1998; Schimidt and Bradfield, 1996). Binding of the complex to DRE sites

initiates transcription of a variety of genes including those for growth factors and cytokines.

The most well-characterized phenomenon caused by TCDD exposure is increase in the expression of drug metabolizing enzyme genes, such as cytochrome P450 1A1, 1A2 and 1B1, the glutathione-S-transferase Ya and aldehyde-3-dehydrogenase (reviewed by Lai *et al.*, 1996). These enzymes metabolize a number of xenobiotic compounds and facilitate excretion so that intracellular concentrations of parent compounds are reduced, but TCDD and related halogenated compounds are not readily oxidized by these enzymes because of their halogen atoms in certain positions of the molecules.

Hepatomegaly is a consistent finding in all of the studies in which TCDD toxicity has been evaluated (reviewed by Pohjanvirta and Tuomisto, 1994) which results in part from an increase in rough endoplasmic reticulum due to metabolic enzyme induction. Other pathologic changes frequently observed in the liver include hypertrophy of parenchymal cells, steatosis, and centrilobular hepatic necrosis with accompanying inflammatory cell infiltration.

Thymic atrophy as a result of TCDD treatment has also been reported consistently in all species examined (Pohjanvirta and Tuomisto, 1994). It consists of depletion of small immature cortical thymocytes, scattered necrotic lymphocytes and an altered differentiation in thymic epithelial cells. TCDD has been shown to alter thymocyte differentiation *in vitro* (Greenlee *et al.*, 1985) as well as *in vivo* following prenatal exposure to TCDD (Blaylock *et al.*, 1992). This effect appeared to be mediated by alterations in the ability of thymic epithelial cells to support the maturation process of T lymphocyte precursors rather than by direct effects on the thymocytes.

The immune system is recognized as one of the most sensitive targets for TCDD toxicity. TCDD-induced immune dysfunction is characterized by the suppression of acquired immunity, with both antibody and cell-mediated responses. TCDD exposure suppresses the antibody response to sheep red blood cells (SRBC). A dose of 0.65 μg/kg TCDD produces 50% suppression of the anti-SRBC response (ID_{50})

in C57Bl/6 mice (Kerkvliet *et al.*, 1985). Because the antibody response to SRBC requires $CD4^+$ T helper cells and antigen presenting cells (APCs) as well as B cell differentiation into antibody-secreting plasma cells, TCDD could affect the function of any of these cells. Several T cell-mediated immune responses also have been shown to be suppressed in animals treated with TCDD (Clark *et al.*, 1983). Studies have shown that TCDD exposure suppressed in a dose-dependent manner, in particular, cytotoxic $CD8^+$ T lymphocyte (CTL) and allo-antibody responses to allogenic P815 mastocytoma cells via an AhR-dependent mechanism (Kerkvliet et al., 1990b). The development of CTL activity and allo-antibodies are multi-step processes requiring antigen presentation, cell to cell interaction through co-stimulatory molecules and T cell receptor among $CD4^+$ or $CD8^+$ T cells, APCs and B cells.

Unfortunately, because it has been difficult to demonstrate a consistent direct effect of TCDD on T cells or B cells in vitro, mechanistic studies of TCDD-induced immune suppression have had to rely on in vivo models. Moreover, the suppressed immune responses are possibly the result of the combination of defects in several cell types involved in immune responses. Therefore, although TCDD-induced immunotoxicity has been widely studied, the cellular targets and biochemical mechanisms remain poorly understood.

In contrast to acquired immune responses, the innate immunity mediated by macrophages and natural killer cells appears to be more resistant to suppression by TCDD. Moreover, TCDD exposure enhances aspects of inflammation. For example, acute inflammatory response are enhanced following SRBC challenge as measured by an increase in recruitment of neutrophils and macrophage into the peritoneal cavity (Kerkvliet and Oughton, 1993; Moos *et al.*, 1994). In a study of the effect of TCDD exposure on paw edema produced by administration of carrageenan or dextran in rats, TCDD treatment enhanced the edemagenic activities of carrageenan or dextran (Katz *et al.*,1984). In addition, TCDD exposure enhances the sensitivity to endotoxin that is associated with hepatotoxicity and lethality. Studies have demonstrated that B6C3F1 mice administered endotoxin 7 days after TCDD treatment showed a significant increase in mortality compared to vehicle-treated controls (Rosenthal *et al.*, 1989).

Clark et al. (1991a) reported that increased sensitivity to endotoxin observed following TCDD-exposure is linked to the increased production of tumor necrosis factor (TNF), suggesting one mechanism by which TCDD may augment the inflammatory response is via enhanced production of proinflammatory mediators, such as IL-1 and TNF-α. Proinflammatory cytokines, IL-1, IL-6 and TNF-α, are mainly produced by Kupffer cells, residential macrophages in the liver, in response to endotoxin. It has been demonstrated that locally produced proinflammatory cytokines not only play a critical role in augmenting inflammation, but also may provide the signal to develop acquired immune responses. Exposure of T cells to proinflammatory cytokines likely increases the extent of clonal expansion and prevents tolerance induction (Curtsinger *et al.,* 1999). It is important to note that the imbalance in amounts or altered timing of proinflammatory cytokines release may lead to either the disruption of physiological function or the stimulation of the immune system. Specifically, TNF mediates multiple biological responses including reduced food intake, amplified inflammatory responses, and altered lipid metabolism. These responses mediated by TNF have been also observed in TCDD-treated animals. The similarity of these toxic effects has led a number of researchers to hypothesize that TCDD-mediated toxicity is associated with an enhanced production of proinflammatory cytokines. However, the hypothesis remains unproven.

One example of the organism's physiological systemic response to inflammation is the acute phase response (APR). Systemically circulating inflammatory mediators act on the liver, where hepatocytes respond to these mediators via their surface receptors to produce acute phase proteins (APPs), such as serum amyloid A, serum amyloid P, and C-reactive protein (reviewed by Steel and Whitehead, 1994). Serum amyloid A (SAA), a major APP in mice, is rapidly released into the circulation in response to injury, infection or inflammation. Although SAA is very well conserved throughout evolution, the main physiological role of SAA has not yet been established. A number of laboratories have demonstrated that chronic SAA induction produces a marked suppression of a number of humoral- and cell-mediated immunological responses. The lack of accessory function by macrophages or defects

in the interaction between macrophages and T cells have been suspected as possible mechanisms of immune suppression associated with SAA (Benson and Also-Benson, 1979; Kaminski and Holsapple, 1987).

It is important to note that the pathology associated with TCDD toxicity often includes neutrophilia and an inflammatory cell infiltration in the liver and skin characterized by activated macrophages and neutrophils. Other investigators have identified lymphocytes as a hepatic inflammatory infiltrating cell following TCDD treatment (Vos *et al.*, 1974). Interestingly, the injection of allogeneic P815 tumor cells in C57Bl/6 mice following TCDD treatment induces inflammatory Mac-1^+ cells in great frequency and number in the spleen (Prell and Kerkvliet, 1997). Mac-1 (CD11b) was originally identified as a macrophage differentiation antigen. Subsequent studies have shown that Mac-1^+ is a leukocyte-restricted integrin receptor expressed on macrophages, NK cell, neutrophils and eosinophils (Springer *et al.*, 1979; Ault and Springer, 1981). Ligands for Mac-1 have been identified including the complement component iC3b, ICAM-1, fibrinogen, factor X, zymosan, β-glucans, *E. coli* and *Leishmania* (reviewed by Petty and Todd, 1996). Interestingly, the occurrence and the frequency of Mac-1^+ cells are associated with the extent of suppression of immune response in the mice that have been injected with P815 tumor cells following TCDD treatment (Prell and Kerkvliet, 1997). This observation may simply reflect a normal inflammatory response to tissue injury, but there is suggestive evidence indicating that the presence or occurrence of these inflammatory types of cells relates to the development of an immune response (Watson and Lopez, 1995), and may cause immune suppression in mice treated with TCDD.

TCDD-induced immune-suppression may occur both through direct effects of TCDD on the immune system as well as indirectly through immunoregulatory factors from non-immunologic tissues. The studies presented in this thesis investigate the possible mechanistic linkage between hyperinflammation and immune suppression in C57Bl/6 mice treated with TCDD. The hypotheses addressed in this thesis are as follows: 1) TNF is an important proinflammatory cytokine that generates CTL activity against allogenic P815 tumor cells. 2) TCDD exposure in the presence of an

inflammatory stimulus results in over-production of SAA, and SAA is induced by enhanced TNF production. 3) Mac-1^+ inflammatory cells act as suppressor cells in the immune response to allogenic P815 tumor.

1-2. Research approach

Studies were designed to address the major hypothesis that suppressed immune responsiveness in TCDD-treated mice is linked mechanistically to TCDD-induced hyperinflammation. We used two immunogenic stimuli, SRBC and the allogeneic P815 tumor models. The advantages of using these models to address our hypotheses are as follows: 1) antigen stimulation induces inflammation, 2) TCDD treatment enhances the inflammatory response, 3) the immune responses to these antigens are suppressed in mice treated with TCDD. Several inflammatory components, such as proinflammatory cytokines, APP, and inflammatory cells were chosen as indicators of TCDD-induced inflammation and to examine how these inflammatory mediators affect immune responses. In addition, Ah receptor gene knockout mice (AhR KO mice) were used to address the question of whether TCDD-induced hyper-inflammation is AhR dependent.

We first examined the effects of TCDD on the anti-SRBC response. C57Bl/6 mice were treated with vehicle or TCDD by gavage and injected ip with SRBC. Mice develop an antibody response to SRBC while TCDD treatment suppresses this antibody production. Hyper-inflammation was observed in the peritoneal cavity in mice given TCDD. Peritoneal exudate cells were examined to measure this localized inflammation. Plasma levels of SAA and TNF were measured by ELISA and L929 bioassay, respectively, as indicators of inflammation.

We next turned our attention to TCDD effects on immune responses to allogeneic P815 tumor cells. Injection of allogeneic P815 ($H2^d$) tumor cells into C57Bl/6 mice ($H2^b$) stimulates the development of a CTL response due to differences in the major histocompatibility class I molecules (MHC class I) expressed in these two

mice strains. The sensitivity of this model to TCDD has been extensively studied in this laboratory. C57Bl/6 mice were treated with vehicle or TCDD by gavage one day prior to the injection of P815 tumor cells. Ten days after tumor injection, peak CTL activity was measured. TCDD-treated mice showed severe inflammation along with suppression of CTL development. Plasma samples were collected to measure SAA and TNF production. TNF receptor knockout mice were used to investigate the role of this proinflammatory cytokine on the development of CTL in P815- allogrfted mice. AhR knockout mice were used to examine the AhR dependency of inflammation in mice treated with TCDD. Flow cytometric analysis and immunohistochemical staining were carried out to characterize the Mac-1^+ inflammatory cells. Three studies were designed to explore the functions of these inflammatory cells in normal CTL development. The first experiments examined the effect of the Mac-1^+ cells on CTL development *in vitro*. The second experiments investigated the effect of the Mac-1^+ cells on activated $CD8^+$ cells *ex vivo*. Lastly, inflammatory neutrophils were depleted *in vivo* by using neutrophil-specific antibody to investigate the role of these cells on CTL development in the P815 tumor model.

1-3. Goals and objectives

The number and quantity of chemicals being produced daily are enormous. In the past, it has been difficult to predict which of these chemicals might cause adverse health effects in humans and animals or damage to the environment. A confounding problem is that exposure is never to one chemical at a time, but usually to a variety of chemicals that may result in synergistic effects. Risk assessment decisions which impact human health must be made with great care and utilizing all available knowledge. Risk can be over-or under-estimated if animal models are not appropriate for the type of chemical-induced toxicity being examined. Accurate information concerning the mechanisms of these toxicities is necessary to make rational decisions concerning risks of exposure and to avoid generating unnecessary fear.

TCDD, a widespread environmental contaminant, has never been manufactured previously except for scientific research intent, but is a byproduct of the manufacture of other chemicals. It exerts diverse adverse health effects including wasting syndrome, endocrine system disturbances, suppressed immune responses as well as carcinogenesis. The quantities of TCDD present in the environment and the risk to humans if exposure takes place have received extensive public attention. Without accurate risk assessment regarding TCDD, the public's fear and concerns continue to grow. Moreover, the concern extends to compounds sharing structural similarity, including halogenated aromatic hydrocarbon, polychlorinated dioxins, furans, and biphenyls. These also occur in nature and produce toxic effects similar to those of TCDD. Toxicity is believed to be mediated by AhR which is an orphan receptor in that the endogenous ligand is yet to be established. Therefore, studies of the mechanisms involved in TCDD-mediated toxicity are very important if we are to understand how TCDD as well as other prevalent environmental contaminants cause toxicity. Studying the mechanism by which TCDD exposure exerts immune system suppression will help us not only to understand TCDD-mediated toxicity, but also to predict the risk of exposure and perhaps prevent adverse health problems.

CHAPTER 2

LITERATURE REVIEW

2-1. TCDD toxicity and mode of action

2-1-1. Occurrence and chemical properties

Polychlorinated biphenyls (PCBs) and dioxin-like polychlorinated naphthalenes were produced on a large scale and used widely in the 1920s and during World War I because of their thermal and chemical stability (Figure 2-1). In contrast, TCDD has never been intentionally manufactured. Public awareness of TCDD has increased considerably, beginning with identification of TCDD as an unwanted contaminant in the manufacture of trichlorophenols and herbicides in 1957. In the mid-1970s, it was shown that Agent Orange, which had been used as a herbicide in the Vietnam War, was contaminated with large quantity of TCDD. A 1:1 mixture of n-butyl esters of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 2,4-dichlorophenoxyacetic acid (2,4-T) forms TCDD (reviewed by Webster and Commoner, 1994).

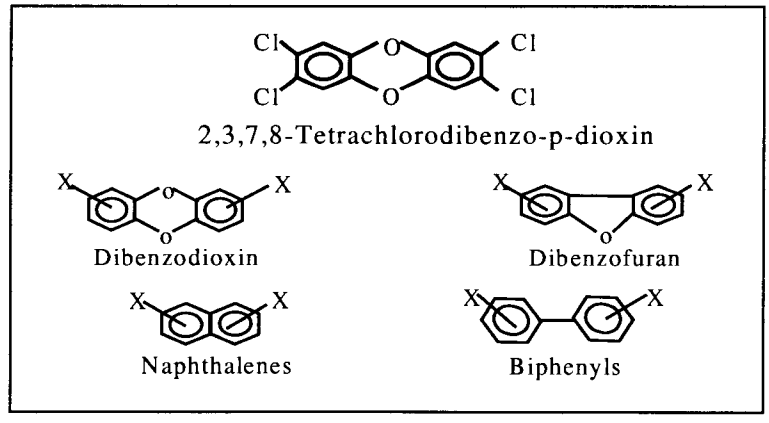


Figure 2-1. Structure of various halogenated aromatic hydrocarbons

Several accidents occurred which led to public concern of TCDD toxicity. These included the Seveso accident of 1976 in Italy in which the public was exposed to a toxic cloud containing TCDD after an explosion at a chemical plant, the Yusho rice oil poisoning incident in Japan in 1968 and the Yu-cheng rice oil poisoning incident in Taiwan in 1979 (reviewed by Masuda, 1994).

TCDD and related chemicals are produced in diverse circumstances such as incineration of chlorine-containing fuels including chemical waste, hospital waste, and sewage sludge, combustion of leaded gasoline, bleaching of pulp and paper with chlorine, and certain types of metal processing such as the coating of copper cable with PVC plastic insulation. Basically all industry processes involving the use of chlorine are suspected of generating TCDD or dioxin-like compounds to some extent (reviewed by Webster and Commoner, 1994).

TCDD consists of two benzene rings linked by oxygen bridges as shown Figure 2-1. The chlorine atoms of TCDD are spatially arranged to form a planer molecule. Physicochemical properties of TCDD include the following: 1) extremely low water solubility, 8 to 19ng/liter at room temperature; 2) low vapor pressure, 1-2 x 10^{-7} Pascal at 25^0C; 3) high lipophilicity, the octanol-water partition coefficient, approximately $K_{ow} = 9 \times 10^5$ to 4×10^6 (log $K_{ow} = 5.94$ to 6.64); and 4) high boiling point, approximately 421 to 447^0C (reviewed by Pohjanvirta and Tumoisto, 1994). Because of its physicochemical stability, TCDD has a long half-life, estimated to be a decade or more. TCDD increases in concentration from water to aquatic animals due to its high lipophilic and hydrophobic properties. Because of this bioaccumulation through the food chain, a major route of human exposure to TCDD is thought to be through the consumption of contaminated food.

Animal studies have shown that oral exposure of Sprague-Dawley rats to a single dose of TCDD, 50 μg/kg in corn oil, resulted in an average 70% absorption of the administered dose (Piper *et al.*, 1973). Other investigations using guinea pigs and hamsters, which are the species most sensitive and resistant respectively to acute lethality of TCDD, found that they absorbed 50 and 75%, respectively (Piper *et al.*, 1973; Olson *et al.*, 1980). These results indicate that TCDD is absorbed effectively

after oral exposure in mammals. Ninety % of the TCDD absorbed is associated with the chylomicron fraction in the lymph system, is readily cleared from blood ($t_{1/2}$ = 0.81 min), and is redistributed to adipose tissue and the liver (Lakshmanan *et al.*, 1986). Although significant species differences exist between rat and human, such as plasma lipid content which is low in rat and high in human, studies in humans indicate that distribution of TCDD was identical among the lipoprotein fractions (Marinovich *et al.*, 1983). Several factors must be considered regarding the distribution of TCDD, particularly time and dose. TCDD may redistribute from liver to adipose tissue over time after exposure (Abraham *et al.*, 1988), and the liver/adipose tissue concentration ratio for TCDD may increase with increasing dose. This is a very important point in risk assessment of TCDD for humans because TCDD exposure is chronic and low-level.

2-1-2. Toxicity

The acute lethality (LD_{50}) of TCDD varies widely depending on the animal species examined, ranging from 0.6 μg/kg in guinea pig to more than 3000 μg/kg in hamster. However, in most other animals such as monkey, mouse, rat, dog and rabbit, the LD_{50} is 100-300 μg/kg (reviewed by De Vito and Birnbaum, 1994). Another peculiar characteristic of TCDD-induced lethality is that fatality is delayed after exposure to TCDD compared to other toxic chemicals. In general, death occurs 1 to 6 weeks after exposure and invariably is preceded by a dramatic loss of up to >50% of body weight which is known as wasting syndrome (Gupta *et al.*, 1973). The ultimate cause of death in TCDD intoxication is not known. In regard to wasting syndrome, several working hypotheses have been suggested and examined, including exhaustion of energy sources, in particular fat, lack of nutrient by refusal of food intake, hypoglycemia and alteration of the "set point" of body weight (reviewed in Pohjanvirta and Tumoisto, 1994). None of these have been conclusively demonstrated to cause wasting syndrome and ultimately, death.

One consistent hallmark of TCDD-exposed animals is thymic atrophy. It consists of loss of small immature cortical thymocytes and alteration of differentiation

of thymic epithelial cells (Gupta *et al.,* 1973; Vos and Moore, 1974; Kerkvliet and Brauner, 1990). However, thymic atrophy is not associated with TCDD-induced immune suppression. The removal of the thymus does not alter suppression of immune function in TCDD-treated adult mice (Tucker *et al.,* 1986). In contrast, the thymus is critical to the development of immune function in immature mice. After perinatal or neonatal exposure to TCDD, the thymus is a more sensitive target in developing mice compared to adult mice and thymic atrophy does suppress immune function (Vos and Moore, 1974).

TCDD also affects liver especially in the rabbit, which displays extensive liver necrosis. In other species, such as mouse, rat, hamster and guinea pig, the predominant pathology in the liver includes hypertrophy and hyperplasia of parenchymal cells, mononuclear cell infiltration and multinucleated giant hepatocytes (Greig *et al.,* 1973; Jones and Butler, 1974; Vos *et al.,* 1974; Gasiewicz *et al.,* 1986; Pohjanvirta *et al.,* 1989). Electron microscopic examination of liver reveals an increase in smooth endoplasmic reticulum which may contribute to association with increase in microsomal enzyme induction (Jones and Butler, 1974; Olson *et al.,* 1980).

Developmental toxicity has been well-described in mice (reviewed by Theobald and Peterson, 1994). A low dose of TCDD exposure produces cleft palate and hydronephrosis. Other species such as rat, monkey, and human require a high dose of TCDD in order to display cleft palate which leads to a substantial incidence of prenatal mortality. TCDD has been reported to alter reproductive function in several animal species including mice, rats, and primates. In sexually mature rats, the effect on the reproductive system following perinatal TCDD (0.4 μg/kg) exposure consists of reduction in testis and accessory sex organ weight, abnormal testicular morphology, and decreased spermatogenesis. Despite pronounced reductions in cauda epididymal sperm reserves, perinatal TCDD exposure has little or no effect on fertility of male rats or on survival and growth of their offspring. Decreased fertility, inability to maintain pregnancy or decreased litter size has been reported as effects of TCDD exposure in female rat and monkey. Reproductive toxicity in female animals has not been as well characterized as in male animals.

The immune system, especially acquired immunity, is one of the most sensitive targets of TCDD-induced toxicity. TCDD appears to exert extensive suppression of the functions of B cells, T cells or both depending on the view of researchers. The effect of TCDD exposure on immune function is reviewed in the next section.

Every species exposed to TCDD, as low as 0.001 μg/kg body weight/day in B6C3F1 mice, exhibits tumors. TCDD exposure induces various types of tumors including lung, oral, nasal cavity, thyroid gland, adrenal gland, liver and lymphoma (Reviewed by Huff *et al*, 1994). TCDD is not considered to be genotoxic and does not form DNA adducts, however, TCDD does display promotional activity. Mechanisms such as protooncogene activation or tumor suppressor gene inactivation have been implicated in TCDD-induced carcinogenicity. However, these proposed mechanisms do not account for the rare and varied types of tumors found in TCDD-exposed experimental animals. Studies are needed to investigate the mechanism of carcinogenesis of TCDD. Accumulated evidence generated from animal experiments suggest that TCDD exposure is associated with cancers in humans. In 1997, the International Agency for Research on Cancer (IRAC) declared TCDD to be a human carcinogen based upon review of available data.

2-1-3. Molecular mechanisms of toxicity

The AhR is a ligand-dependent transcription factor that is believed to mediate the toxic effects of TCDD exposure. The AhR was originally discovered and characterized in cytosolic fractions of murine liver tissue (Nebert *et al.,* 1975). It was subsequently identified in several mammalian and non-mammalian species with a calculated molecular weight of 96, 90 and 96 kD for the human, mouse and rat receptors, respectively. It has been found in several tissues including thymus, lung, liver, kidney, placenta, tonsils, B lymphocytes, and ovary. Molecular properties of the AhR are comparable to those of steroid hormone receptors. Analysis of the amino acid sequence has revealed that it belongs to the basic helix-loop-helix (bHLH) family of proteins whose other members include the AhR nuclear translocator protein (ARNT), the Drosophila proteins SIM and PER, as well as hypoxia inducible factor 1

alpha (HIF-1α). The cytosolic AhR is found in a complex with two molecules of Hsp90 as well as an AhR-interacting protein (AIP: XAP-2). After binding of TCDD to AhR, the receptor undergoes a transformation process whereby it translocates to the nucleus, sheds Hsp90 and AIP, and forms a dimer with ARNT. The complex of AhR-ARNT binds dioxin response elements (DRE) or xenobiotic response elements (XRE), recruits several transcription activation factors or repressor factors and induces a variety of genes (Figure 2-2; reviewed by Wilson and Safe, 1998).

Induction of CYP1A1 gene expression is one of the most well described examples in which the TCDD-AhR complex interacts with specific gene sequences and enhances transcription. Besides induction of drug metabolizing enzymes such as CYP1A1, 1A2, 1B1, aldehyde 3-dehydrogenase, UDP-glucuronosyltransferase, glutathione S-transferase and quinone oxidoreductase, several other important genes have been identified as inducible genes or DRE-containing genes.

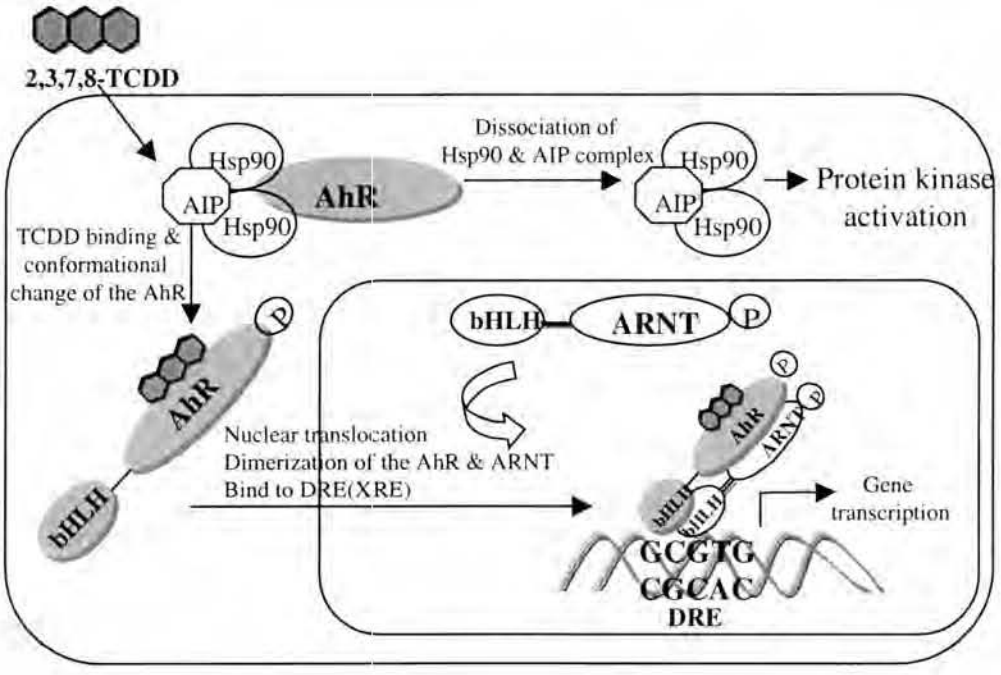


Figure 2-2. Mechanism of Ah receptor-mediated gene transcription

The following genes are known to contain DRE on their promoter regions: cytokines (Interleukin-1β (IL-1β), IL-2, IL-3, IL-5, IL-6, IL-10); kinases (protein kinase C, tyrosine kinase); growth factors (transforming growth factor, tumor necrosis factor); receptors (estrogen receptor, epidermal growth factor receptor); oncogenes (c-fos, c-jun, c-ras); plasminogen activator inhibitor 2 (PAI-2); terminal deoxynucleotidyl transferase (TdT), and others (Lai *et al.,* 1996).

Evidences exist to support the hypothesis that the AhR is the primary mediator of the toxicity of TCDD. Structure-activity studies using dioxin congener compounds indicate that toxicity correlated with binding affinity for the AhR. Secondly, sensitivity to TCDD toxicity segregates with the AhR^b and AhR^d loci, the high and low affinity forms of AhR, respectively, in mice. Possible mechanisms of TCDD toxicity have been proposed as follows: 1) TCDD-mediated toxicity may be the result of persistent transcriptional activation of genes regulated by the AhR-ARNT dimeric complex. 2) TCDD toxicity may also result from hypothetical, low-affinity AhR-ARNT binding sites in the promoters of certain genes involved in toxicity. 3) The AhR and ARNT may recognize DRE sequences involved in transcriptional repression rather than activation. 4) Alternately, the mechanism of TCDD toxicity may be unrelated to direct transcriptional regulation by the AhR-ARNT complex. Decreased concentrations of free ARNT owing to recruitment by the liganded AhR may shift the balance of the other dimeric partner away from HIF-1α or from the formation of ARNT homodimer. 5) Dramatic and sustained activation of CYP1A1 and related genes by TCDD activation of the AhR might sequester coactivators that might be needed for unrelated transcriptional processes (reviewed by Schmidt and Bradfield, 1996).

2-1-4. DRE-independent toxicity mechanism of TCDD

Clearly TCDD activates genes for a family of drug metabolizing enzyme, such as CYP1A1. The broad toxicity raises questions, however, because of a lack of correlation between induction of microsomal enzymes and lethality in experimental animals. The mechanism described in section 3) above seems insufficient to account

for some effects of TCDD that occur rapidly, such as increased Ca^{2+} uptake (Puga *et al.*, 1992), increased protein tyrosine kinase activity (Clark *et al.*, 1991b), and non-transcriptional regulation of proteins (Gaido *et al.*, 1992).

Accumulated data have demonstrated that activation of protein kinases (PKs) is observed consistently in animals after TCDD-exposure and this activation is dependent on TCDD binding to AhR (Enan and Matsumura, 1995). Subsequent studies have shown that TCDD activates PKs from murine hepatic cytosol in a cell- and nucleus-free system, and a 60kDa PK ($pp60^{src}$) is physically associated with the AhR (Blankenship and Matsumura, 1997). These results indicate that TCDD-induced toxicity is not only dependent on the transcriptional alteration of DRE responsive genes but also the activation of cytosolic PKs might be involved in toxicity.

2-1-5. AhR null mice

While it is generally understood that the AhR mediates responses to TCDD and TCDD-related compounds, basic questions remain about the endogenous function of the AhR, such what is the endogenous ligand or the physiological role of AhR. In order to determine the physiological role of the AhR, AhR gene knock out (AhR null) animals were necessary. Advanced in molecular technology have provided AhR null mice from two different laboratories using different approaches. In the laboratory of F. Gonzalez (1995), exon 1 of the Ahr locus is replaced with a neomycin resistance gene, and the translation starting site for AhR expression is deleted, as well as a stretch of basic amino acids that may play a role in DNA binding. On the other hand, the laboratory of Dr. Bradfield generated AhR null mice by replacing exon 2 of the AhR gene with a neomycin resistance gene (Schmidt *et al.*, 1996). Exon 2 encodes the bHLH domain known to be required for ARNT dimerization and DNA recognition.

Phenotypic characteristics of these two knockout mouse strains show some commonality such as no CYP1A1 inducibility, decreased liver size, decreased fertility, and some fibrosis in the liver (Gonzalez and Fernandez-Salguero, 1998). However, both mice have a few different features including neonatal lethality, degree of fibrosis in the liver, inflammation of bile duct, and splenocyte number. In general, the AhR

null mice generated from the lab of Dr. Gonzalez show severe pathologic characteristics. Regardless of the differences in characteristics, abnormal phenotypes of these mice suggest that AhR plays an important developmental and physiological role. The effect of TCDD administration on the immune system in AhR null mice will be discussed in Section 3.

2-2. Immunity and inflammation

2-2-1. Immunity

The function of the immune system is to protect the body from damage caused by invading microorganisms, including bacteria, viruses, fungi and parasites. The generation of immunity relies on the interaction of many different cells and molecules, functioning in a coordinated manner to effectively eliminate pathogens. Immunity is subdivided into two types, adaptive immunity and innate immunity, depending on the specificity of the responses to particular pathogens. The phagocytes of the innate immune system provide a first line of defense against many common microorganisms and are essential to the control of common bacterial infections. However, they cannot always eliminate infectious organisms, and there are many pathogens that they cannot recognize. The lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defense that, in addition, provides an increased level of protection to subsequent re-infection with the same pathogen.

Innate immune system has evolved to recognize pathogen-associated molecules, such as lipopolysaccharides (LPS) and teichoic acid of gram-negative and gram-positive bacteria, double-stranded RNA of RNA virus, and mannans of yeast cells walls, via several mechanisms. For example, macrophages endocytose particles by the mannose receptor, a C-type lectin, that has a broad carbohydrate specificity, and also have a receptor for LPS (CD14; Stahl, 1992; Fearon and Locksley, 1996). Innate immunity is mediated by preformed complement and other proteins (Fearson and Austen, 1980; Holmskov *et al.,* 1994) as well as numerous effector cells,

including monocytes, macrophages, neutrophils, eosinophils and natural killer cells. The responses of innate effector cells against foreign molecules include phagocytosis, pinocytosis, release of degradative enzymes, and reactive molecules. The innate immune response is rapid, and the mechanism, process, and specificity do not change with repeated exposure to a specific pathogenic agent.

In contrast, adaptive immunity has distinctive differences from innate immunity, including memory and specificity. The adaptive immune system does not respond rapidly. However, if a second contact is made with an identical antigen, the onset of the secondary response will be more rapid and intense. There are two types of adaptive immune responses. Humoral immune responses are mediated by antibodies in several ways, including neutralization, opsonization and activation of the complement system. Cell-mediated immune responses depend on direct interactions between T lymphocytes and cells bearing the antigen. Some bacterial pathogens, parasites, and all viruses replicate inside cells where they cannot be detected by antibodies. The $CD8^+$ cytotoxic T cells are specialized for recognizing infected cells and eventually destroying them. The immune system is a powerful, complex entity composed of numerous cell types and regulated by autocrine, paracrine, and hormonal mechanisms.

2-2-2. Inflammation

After injury, trauma or infection of a tissue, the host executes a complex series of reactions in an effort to prevent ongoing tissue damage, isolate and destroy the infective organism and activate the repair processes that are necessary to return the organism to normal function. This cumulative homeostatic process is called inflammation. Inflammation is traditionally defined by four characteristics, pain, redness, heat, and swelling. All these characteristics reflect the effects of cytokines and effector cells on the local blood. Dilation and increased permeability of blood vessels lead to increased local blood flow and leakage of fluid, and account for the heat, redness and swelling (reviewed by Steel and Whitehead, 1994)

The acute phase response (APR) is the organism's response to inflammation, characterized by a series of highly coordinated physiologic reactions that involve almost every major organ system. Tissue macrophages or blood monocytes are the cells most commonly associated with initiating the cascade of the APR at the site of inflammation. Other events, such as mast cell degranulation, aggregation-induced platelet activation, and bacterial products, can also result in the release of mediators that are chemotactic for macrophages and can activate macrophages. Activated macrophages release a broad spectrum of proinflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF), which act both locally and distally. At the inflammed site, these cytokines act on stromal cells to cause the release of IL-8, mononuclear cells chemoattractant protein (MCP) and IL-1, which induce adhesion molecules on endothelial cells, including intercellular adhesion molecule (ICAM), and result in recruiting inflammatory cells such as neutrophils and macrophage. Systemic responses of inflammatory mediators affect the hypothalamus altering the temperature set point and both metabolism and gene regulation in the liver (reviewed by Moshage, 1997)

The liver is the principal target of systemic inflammation and is responsible for providing the necessary components for the immediate defense at the site of tissue damage. Liver cells respond to inflammatory mediators via their cell-surface receptors and induce various kinds of acute phase proteins (APPs), including serum amyloid A (SAA), serum amyloid P (SAP), C-reactive protein (CRP), complement component C3, glucocorticoids, and several growth factors. It is believed that APPs prevent further infection and eliminate harmful agents and aid in the process of tissue repair.

SAA is a major APP in mice, produced in response to the proinflammatory cytokines IL-1, IL-6 and TNF. In response to inflammation, SAA is rapidly released into the circulation. Although SAA is very well conserved throughout evolution, the main physiological role of SAA has yet to be determined. A number of laboratories have demonstrated that chronic SAA production leads to a marked suppression of a number of humoral and cell mediated immunological responses (Miller *et al.*, 1980; Aldo-Benson and Benson, 1982; Kaminski and Holsapple, 1992). Macrophages

isolated from SAA-induced mice are unable to perform as accessory cells in T-cell dependent and T-cell independent antibody responses as well as in delayed-type hypersensitivity responses. The lack of accessory function of macrophages or defects in the interaction between macrophages and T cells have been suspected as a possible mechanism of immune suppression associated with SAA (Kaminski and Holsapple, 1987; Benson and Aldo-Benson, 1979).

2-2-3. Cells involved in immunity

All white blood cells of the immune system are derived ultimately from the same hematopoietic stem cell type in the bone marrow. The myeloid progenitor is the precursor of the granulocytes and macrophages. The granulocytes, or polymorphonuclear leukocytes, comprise three main types of cells, neutrophils, eosinophils and basophils, depending on the granules in their cytoplasm. Both macrophages and neutrophils are phagocytes and comprise the most numerous and important cellular component of the innate immune response.

Macrophages have surface receptors that have evolved to recognize and bind common constituents of many bacterial surfaces. Binding to these receptors triggers macrophages to engulf the bacterium and also induce the secretion of cytokines and other chemical mediators that augment inflammatory responses. The functions of macrophages are important not only in the inflammatory response but also in adaptive immunity. Not only do these cells actively phagocytize a variety of infectious organisms, such as bacteria and parasites, but they also internalize and process them into antigenic fragments and present them in association with MHC molecules to T cells.

Neutrophils are the earliest phagocytic cells to be recruited to the site of infection. The life span of neutrophils is relatively short in the absence of stimuli (approximately one day). Normally, neutrophils exhibit only limited microbicidal capacity. Upon stimulation, however, several changes occur in neutrophils which are all important effector tools in innate immunity. These changes include alterations in adhesion molecule expression, including up-regulation of Mac-1 and down-regulation

of CD62L, release of lysosomal enzymes by degranulation, generation of reactive oxygen species, and soluble proinflammatory mediators (Jablonska *et al.,* 1999).

Lymphoid progenitors give rise to lymphocytes, the major cell types of adaptive immunity. There are two types of lymphocytes, B lymphocytes (B cells) and T lymphocytes (T cells). B cells are responsible for humoral responses with two major functions: antibody production and antigen presentation. Upon activation, B cells differentiate into plasma cells which secrete antibodies. T cells are composed of two major subtypes, depending on their cell-surface molecules: $CD4^+$ or $CD8^+$ T cells.

The function of $CD4^+$ T cells is specialized to activate other cells and fall into two functional classes, Th1 and Th2. Activated Th1 $CD4^+$ T cells can activate macrophages to kill the bacteria-infected cells. Cytokines, soluble mediators, and/or surface associated molecules of Th2 $CD4^+$ T cells provide help to B cells in the process of antibody production or coordinate cell-mediated immune responses. $CD8^+$ T cells are also called cytotoxic T lymphocytes (CTL) due to their capability of direct killing virus-infected cells.

2-2-4. Cross-talk between inflammation and immunity

It is believed that locally produced proinflammatory cytokines can modulate the expression of adhesion molecules on endothelial cells, and while systemically released cytokines play an important role in shaping the development of the adaptive immune response (Panja *et al.,* 1998). The potential effects of inflammation on immunity are summarized in Figure 2-3.

Proinflammatory cytokines can be induced by different types of pathogens and can stimulate multiple effector functions of innate immunity. Additionally, these cytokines potentiate adaptive responses at the effector stages. Interferons (α and β) are induced by viruses and, among other effects, strongly upregulate the expression of the MHC class I molecules, thus increasing the efficiency of presentation of viral peptides to the CTL (Sen and Lengyel, 1992). IL-1, TNF-α and chemokines direct the migration of antigen-specific lymphocytes, along with other effector cells, to the site of infection either by inducing the expression of adhesion molecules on endothelial

cells (IL-1 and TNF-α; Shimizu *et al.*, 1992) or by stimulating chemotaxis (Murphy, 1994). *In vitro* studies suggest that these cytokines may activate T cells either directly by interacting with them or indirectly by up-regulating costimulatory molecules on APC (Kurt-Jones *et al.*, 1986; Liu *et al.*, 1992). IL-6 induces the terminal differentiation of B cells into immunoglubulin-producing plasma cells (Snick, 1990).

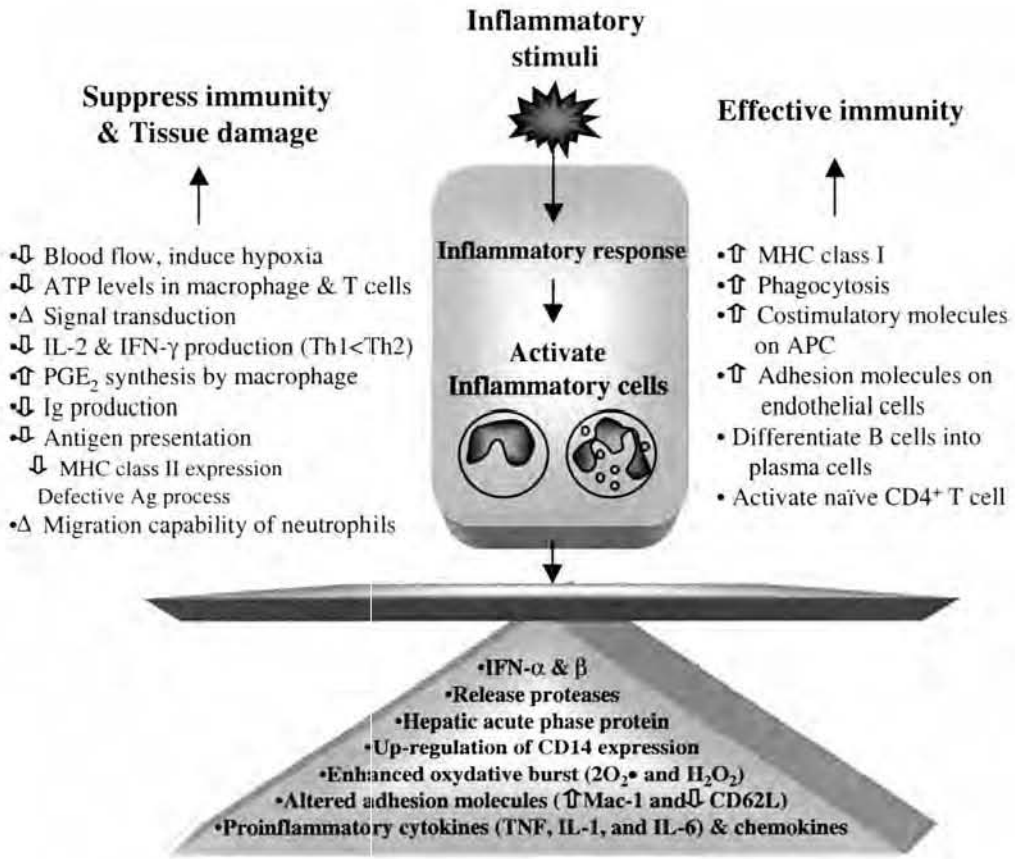


Figure 2-3. Interactions between inflammation and immunity

A number of pathogen products have also been shown to induce a set of endogenous signals, including costimulatory molecules and inflammatory cytokines and to account for the potency of the adjuvant effect. These substances are as follow; bacterial DNA (Krieg *et al.,* 1995), viral RNA (Lampson *et al.,* 1967), mycobacterial membranes (Hauschildt and Kleine, 1995), *Neisseria* porin (Liu *et al.,* 1992), and LPS (Ulevitch and Tobias, 1995; Pape *et al,* 1997). The cells of the innate immune system play a crucial role in the initiation and subsequent direction of adaptive immune responses. Recently, Curtsinger *et al.* (1999) reported that inflammatory cytokines produced by the innate immune system in response to pathogens provide the "danger signals" which are referred to as the third signal for the activation of naïve $CD4^+$ cells. Specifically, IL-1and IL-12 act directly on $CD4^+$ and $CD8^+$ T cells, respectively, along with antigen and IL-2, to activate their differentiation and clonal expansion.

On the other hand, an inappropriately activated inflammatory response can be detrimental to the host. A state of persistent inflammatory cell activation, involving monocytes and neutrophils, would be life threatening. Activated neutrophils can release reactive oxygen species and proteases, causing tissue damage (reviewed by Bellingan, 1999). In the response to diminished microcirculatory blood flow, regional hypoxia and depletion of intracellular energy stores occur, and these changes alter cellular signaling and result in the release of proinflammatory cytokines and prostanoids which mediate further suppression of immune function. Several mechanisms by which overwhelming inflammation suppresses the immune response have been suggested, such as a defective antigen presentation by macrophages and other APCs (Ayala *et al.,* 1990; Ertel *et al.,* 1991), increased production of prostagrandin E_2 (PGE_2), and/or altered cytokine production by macrophages (Kelly *et al.,* 1994; Ertel *et al.,* 1993). PGE_2 is a lipid metabolite and a potent inhibitor of lymphocyte and macrophage function (Walker *et al.,* 1983). Therefore, the ability to activate and de-activate the inflammatory process is central for homeiostasis.

2-3. TCDD-induced immune suppression

2-3-1. Overview

The immune system has been extensively studied and is recognized as the most sensitive target for TCDD-induced toxicity. A history of the studies on the immune responses in mice or rats following TCDD treatment is given in the Appendix of this thesis. It is difficult to describe 'TCDD-induced immune toxicity' in one word, due to differences in experimental designs, dose, route, frequency of TCDD administration, or experimental animal used, etc. However, generally speaking, TCDD suppresses the immune response in experimental animals.

Exposure to TCDD decreases host resistance to bacterial and viral infections. Studies have demonstrated that exposure to TCDD decreases host resistance as measured by increased mortality to infection with *Salmonella* (Thigpen *et al.,* 1975), *Listeria monocytogenes* (Luster *et al.,* 1980), or influenza virus (Burleson *et al.,* 1996; House *et al.,* 1990). In addition, TCDD exposure delays the response to *Trichinella spiralis* (Luebke *et al.,* 1994). In contrast, a few studies have reported no change in mortality in mice challenged with *Listeria monocytogenes* (Vos *et al.,* 1978; Thomas and Hinsdill, 1979; House *et al.,* 1990). These conflicting results may reflect differences in study design.

Humoral immune responses are significantly and consistently suppressed in mice treated with TCDD. SRBC, TNP-LPS and ovalbumin are historically used as antigens, and the antibody response to SRBC is the most sensitive measurement of TCDD-induced immune suppression with 0.65 μg/kg as the ID_{50} value (Kerkvliet *et al.*, 1985). In fact, the T cell-dependent humoral responses, such as the anti-SRBC response, are 10 times more sensitive than T cell-independent responses, such as the anti-TNP-LPS response. These results suggest that the T cell may be a major target for immunotoxicity caused by TCDD exposure.

The effect of TCDD exposure on cell-mediated immunity has been studied by examining delayed-type hypersensitivity (DTH) response, the graft versus host (GVH) response, and the allograft response to tumor. Studies have shown that TCDD

suppresses $CD8^+$ CTL responses in C57Bl/6 mice challenged with allogenic P815 tumor cells (Clark *et al.,* 1981; Kerkvliet *et al.,* 1990b; Hanson and Smialowicz, 1994). This suppression includes reduced splenic CTL activity, frequency in CTL effector cells and allo-antibody responses. In addition, both DTH (Clark *et al.,* 1981; Lundberg *at al.,* 1991) and GVH responses (Vos *et al.,* 1973; Vos and Moore, 1974; Vecchi *et al.,* 1983) are also suppressed by TCDD as summarized in the Appendix.

The effect of TCDD exposure on innate immune responses is less apparent. Most studies, if not all, have shown that non-specific natural killer (NK) cell activity and macrophage activity are not affected by TCDD exposure (Vos *et al.,* 1978; Luster *et al.,* 1980; Mantovani *et al.,* 1980; House *et al.,* 1990). Funseth and Ilback (1992) reported that exposure of A/J mice to TCDD weekly for four times (total TCDD dose was 9.26 μg/kg) increased NK cell activity. However, in this study, control mice exhibited extremely low NK cell activity (2%) and TCDD increased NK activity to just 6%. Thus, the effects of TCDD exposure on NK cell activity are not consistent. In general, innate immunity appears to be less sensitive to TCDD-mediated toxicity when compared to adaptive immunity.

2-3-2. Target cells for TCDD immunotoxicity

Despite a considerable number of investigations, the cells that are directly altered by TCDD exposure leading to suppressed immune function have not been unequivocally identified. There are conflicting data reported from different laboratories regarding the ability of TCDD to suppress lymphocyte functions when examined *in vitro*. The antibody response to SRBC depends on the concerted interaction among APCs, $CD4^+$ T cells, and B cells. In addition, the antibody response to SRBC can be modulated by many non-immunological factors, including hormone and nutritional variables. Therefore, it is very difficult to evaluate the cellular targets of TCDD.

There are a few studies that suggest that B cells, T cells or APCs might be sensitive targets of TCDD. Kerkvliet *et al.* (1990a) compared the sensitivity of the antibody responses in mice using different antigens including T-cell-dependent (SRBC

or DNP-Ficoll) or T cell-independent (TNP-LPS) antigens. Responses to these antigens require different cellular components. The sensitivity to suppression directly correlated with the T cell involvement in the development of the immune response. These results can be interpreted to mean that the $CD4^+$ helper T cell and/or APCs may represent the more sensitive target. In contrast, Dooley and Holsapple (1988) reported that B cells from TCDD-treated mice were functionally compromised in *in vitro* antibody responses but T cells and macrophages were not. They suggested that B cells were the cellular targets of TCDD.

Lawrence *et al.* (1996) showed that T cells contained AhR and induced 7-ethoxyresorufin-o-deethylase (EROD) activity upon TCDD treatment, but the magnitude of induction in T cells was 100-fold less than in Hepa cells. In addition, they could not detect AhR bound to a DRE, whereas the AhR derived from Hepa cells bound to a DRE. Moreover, translocation of the AhR to the nucleus occurred only in activated T cells. These findings imply that TCDD may not target T cells, but accessory cells (APCs) whose impaired function leads to aberrant T cell responses and ultimately suppresses humoral and cell-mediated immune responses. Clearly, the cellular target of TCDD and its role on immune responses is still far from understood.

2-3-3. Evidence of Ah receptor dependence in TCDD-induced immune suppression

Accumulated data indicate that the immunotoxic effects of TCDD are mediated via a Ah recepter-dependent mechanism. There is strong evidence to support this statement. First of all, approximately 10-fold lower doses of TCDD are required to suppress the response to SRBC in C57Bl/6 mice as compared to DBA/2 mice, which contain a high affinity *Ahr* (Ah^{bb}) and a low affinity *Ahr* (Ah^{dd}), respectively (Vecchi *et al.,* 1983). Secondly, the immunosuppressive potency of different dioxin or furan congeners is directly correlated with their ability to bind the *Ahr* (Silkworth and Grabstein, 1982; Kerkvliet *et al.,* 1985). In addition, the sensitivity of the antibody response to SRBC in congenic C57Bl/6 mice segregated with the expression of the *Ah* locus, Ah^{bb} or Ah^{dd} (Kerkvliet *et al.,* 1990a; Silkworth *et al.,* 1993).

The recent generation of *Ahr*-deficient ($Ahr^{-/-}$) mice provided the opportunity to assess the role of *Ahr* on normal immune response as well as on TCDD-induced immune suppression. $Ahr^{-/-}$ mice exhibit a few abnormal phenotypes in the liver, thymus, and spleen (Fernandez-Salgueto *et al.*, 1995). Age-matched $Ahr^{-/-}$ mice developed from the laboratory of Dr. Gonzalez have smaller spleens, although with normal architecture. In particular, spleens from $Ahr^{-/-}$ mice contain 75 to 85% fewer lymphocytes at 2 to 3 weeks of age as compared to wild type $Ahr^{+/+}$mice. This phenotype might be responsible for premature death as a result of opportunistic bacterial infection. However, as these mice get older, the size and the numbers of cells in lymphoid tissues appear to be normal by 10 to 12 weeks of age. The mechanisms by which *Ahr* deficiency delays the appearance of peripheral lymphocytes and subsequently decreases their number in mutant mice remain unclear.

The difference in the absolute numbers of peripheral lymphocytes between $Ahr^{+/+}$ and $Ahr^{-/-}$ mice does not result in a selective loss of any specific lymphocyte subpopulation. In fact, the ratio among B cell, $CD4^+$, or $CD8^+$ T cell is similar between age-matched wild type mice and mutant-mice. Because $Ahr^{-/-}$ mice exhibit eosinophillia in the liver and decreased splenic cellularity, AhR has been speculated to play a role in the regulation of the immune response, but that role of AhR in immune function has not been characterized (Lahvis and Bradfield, 1998). The effect of TCDD on specific immune responses, such as the P815 allograft and SRBC response, is currently under investigation in this laboratory.

2-4. TCDD-induced hyperinflammation

2-4-1. Overview

In earlier studies, the pathology associated with TCDD toxicity was found to include neutrophilia and an inflammatory response in the liver and skin as indicated the accumulation of activated macrophages and neutrophils (Vos *et al.*, 1973; Weissberg and Zinkl 1973; Vos *et al.*, 1974; Puhvel and Sakamoto, 1988). A

significant increase in the number of neutrophils in the blood was observed in mice treated with six weekly 25 μg/kg dose of TCDD. These observations may simply reflect an inflammatory response to tissue injury.

However, the results of a number of experiments have led to the speculation that exposure to TCDD may activate inflammatory cells and result in augmenting an inflammatory response (Vos *et al.*, 1974; Jones and Greig, 1975; Gasiewicz *et al.*, 1983; Rosenthal *et al.*, 1989; Shen *et al.*, 1991; Yin *et al.*, 1994). Additionally, studies (Pape *et al.*, 1997; Panja *et al.*, 1998) demonstrated that inflammatory responses could induce the expression of costimulatory molecules on APCs and release effector cytokines, suggesting that inflammatory responses can play a critical role in the initiation of the adaptive immune response as well as in the instruction of effector functions (reviewed by Medzhitov and Janeway, 1997). Therefore, it is reasonable to speculate that understanding the mechanisms of enhanced inflammatory response by TCDD may provide crucial evidence for the mechanism by which TCDD suppresses immune responses.

2-4-2. Enhanced endotoxin hypersensitivity

Treatment of Swiss mice with TCDD markedly enhanced their susceptibility to endotoxin even at doses that did not induce thymus atrophy (Vos *et al.*, 1978). Similarly, a study by Rosenthal *et al.* (1989) showed that a single dose of either 50, 100, or 200 μg/kg TCDD induced an endotoxin-hypersensitive state in B6C3F1 mice, as measured by an increase in LPS-induced mortality. TCDD-treated mice showed significantly reduced clearance of endotoxin. Administration of methylprednisolone fully protected TCDD-treated mice from endotoxin lethality. These results indicate that TCDD treatment impairs a hepatic detoxification mechanism, which may lead to the enhancement of endotoxin lethality, possibly through inflammatory mediators produced by Kupffer cells, which are hepatic residential macrophages.

On the other hand, a dose of 10 μg/kg TCDD has been shown to enhance edema formation in rats using the irritants carrageenan and dextran (Theobald *et al.*, 1983; Katz *et al.*, 1984). This enhancement was mediated by augmenting the

edemagenic activities of bradykinin and histamine and not by an increase in neutrophils or monocytes in the pleural exudates in TCDD-treated rats. In addition, TCDD did not alter granuloma formation produced by the subcutaneous implantation of cotton pellets, indicating that enhanced edema formation by TCDD exposure was not caused by the accumulation of inflammatory cells.

In order to identify the cell populations responsible for TCDD-induced inflammation and enhanced endotoxin sensitivity, the major inflammatory cells, macrophages and neutrophils, have been studied. Based on accumulated reports, one might speculate that TCDD may super-activate these inflammatory cells. Studies, however, demonstrated that TCDD did not alter macrophage function as examined by non-specific killing, phagocytosis or reduction of nitro-blue tetrazolium (Vos *et al.*, 1978).

In addition, Ackermann *et al.* (1989a) investigated the effect of TCDD on naïve neutrophils and reported that their functions were suppressed by TCDD treatment.

This study utilized two mice strains, B6C3 F1 and DBA mice, which contain a high affinity AhR and a low affinity AhR, respectively. The peritoneal neutrophils obtained from TCDD-treated mice exhibited reduced cytolytic and cytostatic activity. Supernatants collected from neutrophil cultures of B6C3F1 mice showed reduced killing capacity for L929 tumor cells, but not from DBA mice, suggesting enhanced inflammation by TCDD treatment is mediated via AhR. However, other functions of neutrophils, such as the production of superoxide and hydrogen peroxide and degranulation, were not impaired by TCDD exposure in either strain of mice.

2-4-3. Proinflammatory cytokines

TNF, a proinflammatory cytokine, has drawn a great deal of interest due to the similarity of its toxic effect to that of TCDD. TNF is an endogenous pyrogen (Dinarello *et al.*, 1986) and mediator in host resistance (Rouzer and Cerami, 1980). TNF is very toxic to rats as evidenced by a LD_{50} value of 0.7 mg/kg (Tracey *et al.*, 1986). TNF also induces cachexia and disrupts lipid metabolism. A number of

studies hypothesized that TCDD-mediated hyperinflammation may be associated with TNF. Clark *et al.* (1991a) reported that TCDD exposure resulted in a dose-dependent increase in the concentration of TNF in the serum of endotoxin-exposed mice. Subsequently, Taylor *et al.* (1992) investigated the role of TNF in TCDD acute toxicity. Their results demonstrated that anti-TNF antibody treatment reduced TCDD-mediated mortality by 54%, suggesting that TCDD-mediated changes in the TNF pathway may be an important mechanism for acute TCDD toxicity.

Kerkvliet and Oughton (1993) reported that TCDD exposure resulted in an enhanced inflammatory response following SRBC challenge as measured by a two to four fold increase in the number of neutrophils and macrophages in the peritoneal cavity. A subsequent study by Moos *et al.* (1994) demonstrated that IL-1β or TNF-α activity played a role in mediating this TCDD-induced hyperinflammatory response to SRBC. In addition, they found that inhibition of TNF activity by blocking soluble TNF receptor, but not inhibition of IL-1 activity, dramatically reduced the inflammatory cell influx into the peritoneal cavity (Moos and Kerkvliet, 1995). Moreover, they evaluated the effect of TCDD on TNF production *ex vivo* and *in vitro* and observed that TCDD treatment increased the levels of bioactive TNF when cells were stimulated with LPS (Moos *et al.,* 1997). However, the reduction in the inflammatory response failed to restore TCDD-induced antibody suppression.

On the other hand, studies by Sutter *et al.* (1991) and Yin *et al.* (1994) demonstrated a direct effect of TCDD on IL-1 expression *in vitro*. Their data showed that the genes encoding cytokine IL-1 and plasminogen activator inhibitor-2 were induced by TCDD treatment in human keratinocytes. These results indicate that the IL-1 gene may be transcriptionally regulated by TCDD. Likewise, Steppan and Kerkvliet (1991) found that, under some conditions, TCDD increased the level of mRNA for IL-1 in the IC21 macrophage cell line.

On the contrary, Lang *et al.* (1998) reported that the production and/or expression of the proinflammatory cytokines IL-6, IL-8 and IL-1 were not changed by TCDD in a number of other tissues, such as human airway epithelial cells, alveolar macrophages, peripheral blood monocytes and lymphocytes. These results indicated

that the inducibility of proinflammatory cytokines by TCDD may vary depending on the cell type. In spite of the large body of relevant scientific research, the effects of TCDD on proinflammatory cytokines remain to be clearly elucidated.

2-4-4. Ah receptor involvement in inflammation

Recent advances in recombinant technology have provided the experimental approach necessary to assess the role of the AhR on TCDD-induced inflammation and hepatotoxicity. Currently, two strains of Ah receptor deficient ($AhR^{-/-}$) mice have been developed in two independent laboratories as described in Section 2-1 (Fernandez-Salguero *et al.,* 1995; Schmidt *et al.,* 1996). The pathology of the $AhR^{-/-}$ mice involves decreased liver size and subtle hepatic portal fibrosis which increases with age. By 11 to 13 months, adenomas and carcinomas are sometimes found in these mice (Fernalndez-Salguero *et al.,* 1995; Schmidt *et al.,* 1996; Gonzalez and Fernandez-Salguero, 1998; Lahvis and Bradfield, 1998).

$AhR^{-/-}$ mice were compared with wild-type mice in their susceptibility to acute TCDD-induced toxicity (Fernandez-Salguero *et al.,* 1996). Their results demonstrated that $AhR^{-/-}$ mice were relatively unaffected by TCDD, even at a dose that is ten-fold higher that the dose induced severe toxic effects in wild type mice, including wasting syndrome and lipid accumulation, and thymic atrophy. Their results indicate that $AhR^{-/-}$ mice are relatively resistant to acute TCDD toxicity and that the pathological changes induced by TCDD treatment in the liver appear to be mediated entirely via the AhR.

Recently, an $AhR^{-/-}$ chimeric model was used to investigate the effect of AhR in hematopoietic and/or parenchymal cells on TCDD-induced hepatotoxicity. Thurmond *et al.* (1999) evaluated the necrotic and inflammatory changes in liver sections from both control and chimeric mice following treatment with 30 μg/kg TCDD. TCDD produced moderate inflammation and necrosis in mice containing wild type $AhR^{+/+}$ hepatic parenchyma but not in mice containing $AhR^{-/-}$ hepatic parenchyma. These data indicate that the presence of AhR in hepatic parenchyma alone is sufficient for TCDD induction of hepatic necrosis, and its presence in

hematopoietic cells is necessary for the inflammatory response in the liver following TCDD treatment. The lack of an inflammatory response in AhR-deficient mice in hematopoietic cells may indicate that the hematopoietic components of the bone marrow are TCDD-sensitive.

CHAPTER 3

MATERIALS AND METHODS

3-1. Animals

Six weeks old female DBA/2 (H-2^d) and male C57Bl/6 (H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 7-8 weeks of age.

Breeding pairs of TNF receptor knockout mice, originally from Immunex (Seattle, WA), but generously donated by Tony Vella (National Jewish Hospital, Denver, CO), were bred at Laboratory Animal Resources at Oregon State University. TNF receptor 1&2 knockout mice were back-crossed onto C57Bl/6 mice for three generations. TNF receptor 2 knockout mice were back-crossed to C57Bl/6 mice for five generations. AhR-deficient mice created by the deletion of exon 2 of the AhR gene (Schmidt *et al.,* 1996) were obtained as breeders from The Jackson Laboratory. These mice were backcrossed for ten generations onto C57Bl/6 and bred as homozygotes at the OSU facility. Knock out mice were used in experiments at 7-12 weeks of age.

For all studies, male mice were housed individually whereas female mice were caged in group of 2-6 in polycarbonate cages in front of a sterile laminar flow device and acclimated for a minimum of 7 days prior to experiments. Animal rooms were maintained with a 12-hour light/dark cycle and at a constant temperature of 72^0F and 50% humidity. Animals were provided with food and tap water *ad libitum* and maintained in accordance with National Research Council Guidelines.

3-2. Reagents and antibodies

TCDD (≥99% purity, Cambridge Isotopes, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil. A vehicle control solution consisting of peanut oil

and anisole was similarly prepared. *Escherichia coli* endotoxin (055:B5, Sigma Chemical Co.) was used as a source of LPS. SRBC were obtained from Colorado Serum Co. (Denver, CO). PAB consisted Dulbecco's Phosphate-Buffered Saline containing 5% Sodium Azide and 1% Bovine Serum Albumin (pH 7.2) and was used for washing media for flow cytometric staining.

Monoclonal antibodies (mAbs) were titrated for optimal concentration and used for flow cytometric analysis. CD44 (IM7.8.1) and CD11b (Mac-1; M1/70.15) were purchased from Caltag (South San Francisco, CA). CD8α (clone 53-6.7), CD40 (3/23), CD62L (MEL-14), CD11a (LFA-1; M17/4), B7-1 (16-10A1), B7-2 (GL1), I-A^b (AF6-120.1), H-$2K^b$ (AF6-88.5), CD45 (30-F11), CD54 (ICAM; 3E2), and Gr-1 (RB6-8C5) were purchased from Pharmingen (San Diego, CA). F4/80 (A3-1) was purchased from Serotec Ltd. (Kidlington, England). PE-labeled streptavidin (Becton Dickinson Immunocytometry Systems, San Jose, CA) or RED613-labeled streptavidin (GIBCO BRL, Grand Island, NY) was used as a second-step reagent. Purified rat IgG (ICN Biomedicals) was used to block FcR-mediated binding of cytophilic Ig.

For T cell depletion, mAbs against CD4 (clone GK1.5) and CD8 (clone 53-6.72) were purified by HPLC from ascites and generously provided by Dr. Randy Noelle, Dartmouth Medical School (Lebanon, NH). Goat anti-rat IgG (ICN Biomedicals) and rat anti-mouse IgG (Jackson ImmunoResearch Lab. Inc.,West Grove, PA) was purchased. For depleting Mac-1^+Gr-1^+ cells *in vivo,* the hybridoma RB6-8C5, which produces a rat anti-mouse granulocyte mAb (Gr-1), was kindly provided from R. Coffman, DNAX Research Institute (Palo Alto, CA).

3-3. P815 mastocytoma cells

P815 tumor cells, derived from a methylcholanthrene-induced mastocytoma of DBA/2 origin, were maintained *in vivo* as an ascites tumor by weekly ip transfer in DBA/2 mice. In order to evaluate the effect of P815 tumor cell-secreted factors on Mac-1^+Gr-1^+ cells, ascites was collected from DBA mice 6-8 days after tumor injection using syringes prefilled with 12 U sodium heparin. Ascites was centrifuged at 1000 rpm, and supernatant was stored at -80^0C. DNAX-P815 tumor cells were

maintained *in vitro* in RPMI 1640 medium supplemented with 10% FBS and 1 mM gentamycin at 37^0C in 5% CO_2.

3-4. Animal treatment

3-4-1. P815 model

Male C57Bl/6 mice were treated with vehicle or TCDD (15 μg/kg body weight) by gavage one day prior to ip injection of 1 x 10^7 P815 tumor cells. At various times after P815 injection, mice were sacrificed by CO_2 asphyxiation. Spleens and blood samples were collected and processed.

3-4-2. SRBC model

Mice were given a single dose of vehicle or TCDD (15 μg/kg body weight) by gavage two days prior to ip injection of 2.5×10^8 SRBC. Mice were sacrificed by CO_2 asphyxiation at various times after SRBC injection. Peritoneal exudate cells and blood samples were collected.

3-5. Cell preparation

3-5-1. Spleen

Animals were sacrificed by asphyxiation using CO_2. Spleens were removed, weighed and placed in a 60 x 15 mm culture dish with 4 ml HBSS supplemented with 5% FBS. Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Cell suspensions were centrifuged at 1000 rpm (Beckman TJ6, 200 x G) for 10 minutes. The cell pellet was resuspended and RBCs were lysed by hypotonic shock. Cell concentrations were determined using the Coulter Counter (Beckman Coulter, Hialeah FL).

3-5-2. Peritoneal exudate cells

Peritoneal exudate cells were harvested immediately after euthanasia by injecting 900 μl cold PBS containing 10% FBS and 5 U heparin/ml into the peritoneal cavity and withdrawing 500-600 μl volume from each mouse. Cells were counted on a Coulter Counter and 5×10^3 cells were deposited on slides by cytospin centrifugation (Shandon). The slides were stained using a Wright-Giemsa stain (Gugol stain Co., Long Island, NY) for differential cell counts.

3-5-3. Blood

Blood was collected by heart puncture using syringes prefilled with 12 U sodium heparin. Plasma was separated by centrifugation and stored at -70^0C until assay. For blood differential, blood smear slides were prepared and stained with Wright-Giemsa stain, and neutrophils and lymphocytes were enumerated through a light microscope. The number of neutrophils and lymphocytes in peripheral blood was calculated using the WBC count and differential count.

3-5-4. Bone marrow cells

Bone marrow cells were obtained by aspirating the femurs of mice under sterile conditions with RPMI media supplemented with 50% FBS. Cells were pelleted and washed with RPMI media with 10% FBS.

3-6. TNF-α assay

Serum TNF levels were determined using the L-929 fibroblast cytolytic assay. A confluent layer of L929 cells was prepared in a 96 well flat-bottomed microplate and incubated with 100 μl of actinomycin D (6 μg/ml; Calbiochem, San Diego, CA) for 30 minutes. Recombinant mouse TNF (Genzyme) or diluted serum samples were then added and incubated for an additional 18 hrs in 5% CO_2 and 37^0C with humidity. After incubation, the media was removed, and the plates were stained with 200 μl

0.2% crystal violet for 10-15 minutes. Crystal violet was removed, and plates were washed five times and subsequently dried. The remaining cells were solubilized with 1% SDS. Absorbance was detected at at 595 nm using a microplate reader, SPECTRA MAX250 (Molecular Devices Corp. Menlo Park, CA). TNF-α concentrations were determined by plotting a standard curve using SOFTmax®PRO V1.2.0 program (Molecular Devices Corp. Menlo Park, CA). The lower limit of detection for TNF in serum was 5 pg/ml.

3-7. Enrichment of Mac-1$^+$Gr-1$^+$cells

3-7-1. Spleen

A negative panning technique was used to deplete splenic CD4$^+$ and CD8$^+$ T cells and B cells. Spleen cell suspensions were incubated on ice for 30 min with the primary mAbs specific for CD4, CD8, and B cells, at a concentration of 20 μg/10^7 cells for T cells and 10 μg/10^7 cells for B cells. Cells were centrifuged, washed twice, resuspended in ice-cold 5% FBS-DPBS at a concentration of 1 x 10^{7}cells/ml, transferred to plates coated with goat anti-rat IgG, and incubated for 40 min at 4^{0}C. Non-adherent cells were transferred to another set of rat IgG-coated plates for a second panning. Following the second panning, non-adherent cells were suspended in 10% FBS-RPMI media for further functional studies. To verify the enrichment for Mac-1$^+$Gr-1$^+$ cells, cells were stained for flow analysis. Contamination of CD8$^+$, CD4$^+$ or CD19$^+$ cells was less than 3% in both vehicle-or TCDD-treated mice. Cells stained with 50 μl of Propidium Iodide (PI, 500 μg/ml in PBS) indicated viability that was >95%.

3-7-2. Blood

A modified Ficol-gradient centrifugation method was used to enrich Mac-1$^+$Gr-1$^+$ cells from the blood. Blood samples from three vehicle or TCDD treated mice were pooled. An aliquot of 3 ml blood was layered on Histopaque-1077 (3 ml; Sigma Co.) in a 15 ml conical plastic centrifuge tube and centrifuged 30 minutes at

1350 rpm (400 g) at room temperature. The pellet containing neutrophils and RBC was washed once with PBS and RBC were lysed by hypotonic shock. Cells were washed twice in RPMI media containing10% FBS, and cell concentrations were determined using a Coulter Counter. The purity of Mac-1^+Gr-1^+ cells was assessed by flow cytometric analysis and >95% found to be Mac-1^+Gr-1^+ cells. The enriched cell (5-10 x 10^3) were deposited on slides by cytospin centrifugation, and stained with Wright-Giemsa for morphological identification. Viability was assessed using PI staining (>95%).

3-8. Histopathology and immunohistochemistry

Whole spleens were collected, snap-frozen in liquid nitrogen, and stored at -80^0C until staining. Serial sections, cut at 8 μm on a cryostat at -20^0C and placed on glass microscope slides, were fixed in freshly prepared cold acetone containing 30% H_2O_2 for 10 min and allowed to air dry before storage at -20^0C. Sections were stained with H&E for histopathology. For immunohistopathology, sections were re-hydrated with PBS for 10 min and blocked for 10 min with PBS containing 0.1% BSA to prevent nonspecific binding from immunoglobulin. Biotin-labeled mAbs to CD4, CD8, CD19, Mac-1, or Gr-1 were added for 60 min at 37^0C. Following six washes, sections were treated with streptavidin conjugated to alkaline phosphatase (Zymed) for 10 min. Zymed AP-blue was added as substrate. In order to investigate whether Mac-1^+ cells co-express Gr-1 antigen, a second mAb staining procedure was performed using biotin-labeled anti Gr-1 antibody sequentially followed by incubation with avidin-conjugated horseradish peroxidase (Zymed) for 20 min, and Aminoethyl carbazole as a substrate for horseradish peroxidase. Sections were then washed in distilled water, counter stained with hematoxylin for 5 sec and mounted with an aqueous mounting solution. For negative control staining, rat anti-mouse IgG2b and IgG2a were used at same concentrations.

3-9. Flow cytometric analysis

3-9-1. Spleen

Spleen cells (2-4 x 10^6 cells/well) were incubated on ice with 30 μg of rat IgG in 50 μl PAB to prevent non-specific binding through the FcR. Cells then were incubated with various mAbs in 96-well V-shape bottom microplate on ice for 15min. Appropriately-labeled isotype matched immunoglobulins were used to assess nonspecific staining. Data were collected by listmode acquisition using an Epics XL flow cytometer (Coulter Electronics). Subsequently listmode data were analyzed using WinList software (Verity Software House, Inc., Topsham, MA).

3-9-2. Blood

An aliquot of 100 μl blood in 15 x 75 mm disposable tubes was washed twice with 3 ml PAB by centrifugation at 4^0C at 2700 rpm for 5 minutes. Blood cells were resuspend in 50 μl of Rat IgG (600 μg/ml) and incubated on ice for 10 minutes to prevent non-specific binding through the FcR. Cells were then incubated with various mAbs on ice for 15 min and appropriately-labeled isotype matched immunoglobulins were used to assess nonspecific staining. Cells were washed once, RBC were lysed by adding FACS lysing solution (Becton-Dickinson), incubated for 10 minutes at room temperature, followed by one wash. Data were collected by listmode acquisition using a XL Flow Cytometer (Coulter). Subsequently, listmode data were analyzed using WinList software.

3-9-3. Bone marrow cells

Bone marrow cells (1-2 x 10^6) were stained with mAbs in a similar manner as for blood cell.

3-10. Measurement of SAA

The concentration of plasma SAA was measured using an ELISA kit specific for murine SAA according to the manufacturer's directions (Biosource International, Wetlake Village, CA). Each sample was assayed in duplicate. Absorbance was detected at 405 nm using a microplate reader, SPECTRA MAX250. SAA concentrations were determined by plotting a standard curve using SOFTmax®PRO V1.2.0 program.

3-11. CTL assay

The cytolytic activity of spleen cells to P815 tumor cells was measured in a standard 4-hr ^{51}Cr-release assay as previously described (DeKrey and Kerkvliet, 1995). Effector to target (E:T) ratios from 100:1 to 3.7:1 were tested in duplicate to quadruplicate, depending on experiments. The amount of ^{51}Cr released into the supermatant was measured and the percent cytotoxicity was calculated for each E:T ratio as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release - naïve release}}{\text{maximum release - spontaneous release}} \times 100$$

where experimental release was obtained with splenocytes from P815-injected mice, naïve release was obtained using splenocytes from uninjected mice, maximum release was obtained by incubating ^{51}Cr-labeled tumor cells in SDS (0.5%), and spontaneous release was obtained by incubating tumor cells in culture medium.

In the experiments to assess the effect of Mac-1$^+$Gr-1$^+$ cells on *in vivo* activated CTL activity, responders (5×10^5), spleen cells from 9 days after P815-challenged vehicle-treated mice, were co-cultured with the enriched Mac-1$^+$Gr-1$^+$ cells that had been obtained from TCDD-treated mice 9 days after tumor injection. Mac-1$^+$Gr-1$^+$ cells were added at various ratios (1:1, 1:0.5, or 1:0.25) prior to a standard

CTL assay for either 3 hr or 1.5 hr. In order to maintain a constant cell concentration in each well, naïve splenocytes from C57Bl/6 were added at an appropriate number. At the end of co-culture, cytolytic activity of spleen cells to P815 tumor cells was measured in a standard ^{51}Cr release assay.

3-12. Labeling of P815 or Yac-1 cells with ^{51}Chromium (^{51}Cr)

P815 tumor cells were collected from DBA mice using RPMI media supplemented with 5% FBS and washed once. P815 cells (3 x 10^6) were resuspended in 100 µl tris-phosphate buffer before incubation with 200 µCi ^{51}Cr for one hour in a 37°C water bath. Cells were washed twice, resuspended in 10 ml media, and incubated for an additional hour at 37°C. Cells were subsequently washed, and cell concentration was determined using a hematocytometer. Labeled cells were used within 30 minutes.

Yac-1 cells were cultured *in vitro* in RPMI media supplemented with10% FBS. Yac-1 cells (5 x 10^6) were labeled with in a similar fashion as for P815 tumor cells.

3-13. *In vitro* CTL development

A modification of the mixed lymphocyte tumor cell culture method (MLTC) was used for induction of allogenic CTL activity in a 5-day culture (DeKrey and Kerkvliet, 1995). Responders, spleen cells from naïve C57Bl/6 mice, were incubated with stimulators ($P815_M$) which were P815 tumor cells treated with mitomycin C (Sigma Co.) at an E:T ratio of 50:1 in a flat bottom 24-well tissue culture plate (Corning, Corning, NY). Splenic Mac-1^+Gr-1^+ cells, enriched from TCDD-treated mice nine days post P815 injection, were added into wells at a ratio of 30%, 10%, or 5% relative to responders. In order to maintain a constant cell concentration in each well, mitomycin C-treated naïve splenocytes (NI_M) from C57Bl/6 mice were added at an appropriate number. Each well contained 2.5 x 10^6 spleen cells, 5 x 10^4 $P815_M$ and 7.5 x 10^5 cells of either the enriched Mac-1^+Gr-1^+cells or NI_M in a final volume of 1.0

ml of RPMI medium. Some wells contained spleen cells and NI_M only and served as negative controls. Plates were incubated in 5% CO_2 at 37^0C for 5 days. Cytolytic activity of spleen cells to P815 tumor cells was measured in a standard ^{51}Cr-release assay. ^{51}Cr-labelled P815 tumor cells (5×10^4) were added to each well in 100 µl medium and the plates were incubated for 4 hr. A volume of 200 µl culture medium was harvested from each well and counted for γ emission.

In separate experiments, Mac-1^+Gr-1^+ cells were separated from naïve splenocytes by a culture chamber insert containing a semi-permeable membrane (Millipore, Bedford, MA). Responders and stimulators were similarly prepared as described above, and inserts were placed in each well. The enriched Mac-1^+Gr-1^+ cells (7.5×10^5 or 2.5×10^5) were added into insert in a 250 µl media following adding media (150 µl). As a control, some inserts containing a corresponding number of NI_M or media itself were prepared. After five-days of culture, the inserts were removed, and fresh RPMI media (400 µl) were added. The cytolytic activity of spleen cells to P815 tumor cells was measured in the same way as described above.

3-14. Cytotoxic antibody assay

Cytotoxic alloantibody titers were determined using a complement-dependent ^{51}Cr-release assay. Plasma samples were heat inactivated for 30 minutes at 56^0C and centrifuged prior to assay. Serial dilutions of plasma (1:10 to 1:5120) were incubated with 1×10^4 ^{51}Cr-labeled P815 tumor cells for 20 min at 37^0C and 5% CO_2 in 96-well round bottomed plates. After the cells were washed, 100 µl of Low-Tox-M rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY, diluted 1:12) were added to each well and plates were incubated for an additional 45 minutes at 37^0C. Supernatant (50 µl) was collected and the amount of ^{51}Cr released was measured by γ-counter. Specific cytotoxicity was calculated using the following equation:

$$\% \text{ specific cytotoxicity} = \frac{\text{experimental release - complement only}}{\text{maximum release - spontaneous release}} \times 100$$

where experimental release was obtained from ^{51}Cr-labeled P815 cells incubated with plasma and complement, ^{51}Cr release from complement only was obtained by incubating the ^{51}Cr-labeled P815 cells with complement, maximum release was obtained by incubating ^{51}Cr-labeled P815 tumor cells in SDS (0.5%), and spontaneous release was obtained by incubating ^{51}Cr-labeled P815 tumor cells in medium. All plasma samples were tested in duplicate on separate plates.

3-15. Measurement of superoxide anion production

Superoxide anion production was assayed spectrophotometrically by the reduction of ferricytochrome C (Current Protocols in Immunology, 1996; You-Li Zu *et al.,* 1998). Reactions involved in the formation of superoxide are as shown below.

1) Superoxide formation

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + H^+ + 2O_2\bullet$$

2) Cytochrome c reduction

$$O_2\bullet + \text{cytochrome c } (Fe^{3+}) \longrightarrow O_2 + \text{cytochrome c } (Fe^{2+})$$

The enriched Mac-1$^+$Gr-1$^+$ cells (1 x 10^{7}cells/ml) were suspended in HBSS containing 10 mM HEPES (pH 7.5) and 1mM calcium and then resuspended in HBSS containing 150 μM cytochrome C and equilibrated at 37°C for10 minutes. Cells (100 μl) were dispensed into a pre-warmed 96-well plate, and superoxide anion production was initiated by stimulating the cells with 100 μM N-Formyl-Met-Leu-Phe (FMLP, Sigma Co.) or 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Calbiochem). Superoxide anion production was measured kinetically at 37°C for 40 minutes by the reduction of cytochrome C at 550 nM using a microplate reader, SPECTRA MAX250. Data were analyzed using SOFTmax®PRO V1.2.0 program.

3-16. Yac-1 cell cytolysis assay

The cytolytic activity of Mac-1^+Gr-1^+ cells to Yac-1 tumor cells was measured in a 6 hr ^{51}Cr-release assay. ^{51}Cr-labeled Yac1 cells (2×10^4) were incubated with Mac-1^+Gr-1^+cells enriched from the spleen or blood from P815-injected mice in 96-well round-bottom plates. After 6 hr incubation, 100 µl culture medium was harvested from each well and the amount of radioactivity was determined by γ-counter. Specific cytotolysis was calculated using the formula:

$$\% \text{ specific cytotoxicity} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

where experimental release was obtained from ^{51}Cr-labeled Yac-1 cells incubated with Mac-1^+Gr-1^+cells from either spleen or blood, maximum release was obtained by incubating ^{51}Cr-labeled Yac-1 cells in SDS (0.5%), and spontaneous release was obtained by incubating ^{51}Cr-labeled Yac-1 cells in culture medium.

3-17. RB6-8C5 purification

The hybridoma RB6-8C5, which produces a rat anti-mouse granulocyte mAb, was kindly provided by R. Coffman, DNAX Research Institute (Palo Alto, CA). These cells were cultured in RPMI media supplemented with 10% FBS. Culture supernatant was collected at the time when cell viability was <20%, as assessed by trypan blue exclusion, and stored at -20^0C until purification. The antibody was purified by affinity chromatography on protein G-Sepharose Fast Flow (Pharmacia, Piscataway, N.J.), according to the manufacturer's directions. In brief, culture supernatant was loaded slowly on to the column, and the column was washed with buffer (0.1 M Na_2HPO_4, pH 7.4) to remove media. Antibodies were then eluted with 0.1 M glycine (pH 3.0). Fractions were collected in 15 ml conical centrifuge tubes, and pH was neutralized immediately with 1 M Tris to avoid loss of RB6-8C5 antibody

activity. Pooled fractions containing antibody were dialyzed against PBS buffer overnight at 4^0C. The concentration of antibody was determined using the Pierce protein assay.

3-18. Data analysis

The student's t-test was used to analyze data for statistical significance. A p value<0.05 was considered to be statistically significant.

CHAPTER 4

RESULTS

4-1. Effect of TCDD on inflammatory cells

Enhancement of inflammatory cells by TCDD treatment has been previously reported in both the SRBC and P815 allograft tumor models (Kerkvliet and Oughton, 1993; Prell and Kerkvliet, 1997). Studies were initiated to follow the kinetics of appearance of these inflammatory cells in both models.

4-1-1. TCDD enhances an acute inflammatory response induced by SRBC injection

Mice were given a single dose of vehicle or TCDD two days prior to ip injection of SRBC. Mice were sacrificed by CO_2 asphyxiation at 24 and 48 hours after SRBC injection. Peritoneal exudate cells were collected by lavage, and stained with Wright-Giemsa for differential enumeration.

As previously reported by Kerkvliet and Oughton (1993), injection of SRBC induced a localized acute inflammatory response in the peritoneal cavity as shown in Figure 4-1-1. This response was characterized by the influx of macrophages and neutrophils, which resulted in a 2-3-fold increase in the total number of peritoneal exudate cells as compared to non-immune levels. The increase in macrophages and neutrophils was first observed at 24 hrs. By 48 hrs, the number of neutrophils had greatly diminished while macrophages remained elevated. Exposure to TCDD significantly enhanced the influx of both macrophages and neutrophils. In particular, TCDD treatment resulted in a transient four-fold increase in neutrophils at 24 hour post SRBC injection when compared to vehicle-treated mice. These recruited neutrophils also disappeared in TCDD-treated mice by 48 hours. The number of

macrophages was significantly higher in TCDD-treated mice at 24 and 48 hr when compared to vehicle-treated mice.

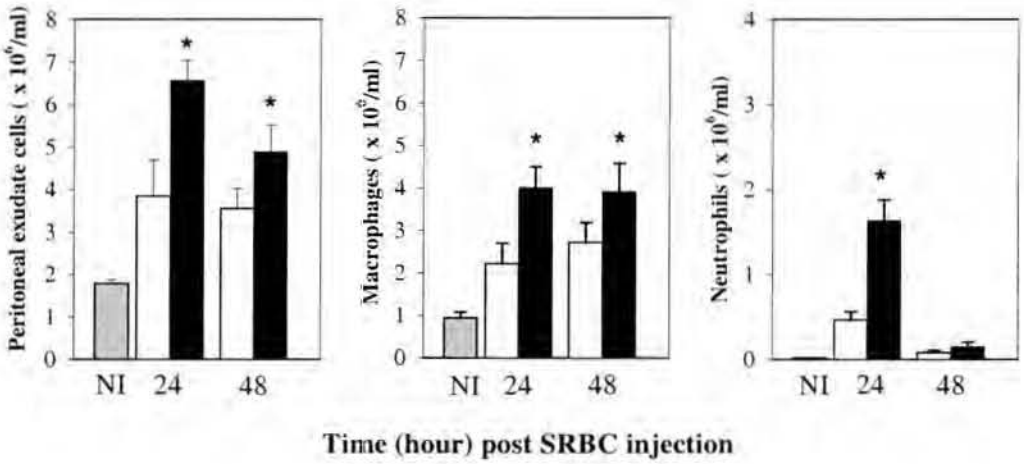


Figure 4-1-1. Effect of TCDD on the recruitment of neutrophils and macrophages to the peritoneal cavity of SRBC-injected mice.
Mice were treated with a single dose of vehicle (□) or 15μg/kg of TCDD (■) by gavage two days prior to injection of 2.5 x 10^8 SRBC. Peritoneal exudate cells were collected and stained with Wright-Giemsa stain, and enumerated by light microscopy. Non-immune group (4 mice) represented as ■. Bars represent the mean ± SEM for 4-6 mice per treatment group. * indicates a statistically significant difference ($p<0.05$) when compared to vehicle control.

4-1-2. Injection of P815 induces Mac-1$^+$Gr-1$^+$ cells in the spleen and blood

Exposure to TCDD dose-dependently suppresses cytotoxic T lymphocyte (CTL) activity and the development of cytotoxic T lymphocyte effector cells (CTL_E) following the injection of allogeneic P815 tumor cells (Kerkvliet *et al.*, 1990b). In addition, an increase in Mac-1$^+$ cells was observed in the spleens of TCDD-treated mice at the time when CTLe failed to develop (Kerkvliet *et al.*, 1996; Prell and Kerkvliet, 1997; Oughton and Kerkvliet, 1999).

A time course study was conducted to further characterize Mac-1^+ cells during the allograft response to P815 tumor cells. Mice were treated with vehicle or TCDD by gavage one day prior to the injection of P815 tumor cells. Mice were sacrificed on day 1 through day 9. Spleen and blood samples were collected and stained with antibodies to Mac-1 and Gr-1 for flow cytometric analysis. For blood samples, a mAb against CD45, a pan leukocyte markers, was included to exclude residual RBCs and platelets from analysis. Mac-1 (CD11b) was originally identified as a macrophage differentiation antigen (Springer *et al.,* 1979) but is also expressed at varying levels on granulocytes, NK cells, dendritic cells, and B-1 cells (Ault and Springer, 1981; Kantor *et al.,* 1992; Vremec *et al.,* 1992). Gr-1 is a granulocyte differentiation marker. Expression of Gr-1 in the bone marrow is directly correlated with granulocyte differentiation and maturation (Hestdal *et al.,* 1991). Gr-1 is also expressed transiently on the monocyte lineage in the bone marrow.

Figure 4-1-2 shows the flow cytometric profiles and gating parameters used to identify Mac-1^+ cells. The majority of Mac-1^+ cells in the spleen and the blood also express Gr-1 antigen, suggesting that these cells are granulocytes, not macrophages. Gr-1^+ cells without Mac-1 expression were only <1 % of cells. As shown in Figure 4-1-3 A, Mac-1^+Gr-1^+ cells were first detected above NI level in the spleens of vehicle-treated mice on day 3 after P815 tumor injection. These cells continued to increase through day 9. TCDD treatment caused a significant increase in the number and percentage of Mac-1^+Gr-1^+ cells on days 7 and 9 in the spleen.

Similar findings were also observed in the blood as shown in Figure 4-1–3 B. Mac-1^+Gr-1^+ cells were already elevated in the blood one day after tumor injection in vehicle-treated mice. The number and percentage of Mac-1^+Gr-1^+ cells reached maximal levels on day 7 and began to decline on day 9. TCDD treatment caused a significant increase in the number and the percentage of Mac-1^+Gr-1^+ cells on days 5-9 when compared to vehicle-treated mice. By day 9, Mac-1^+Gr-1^+ cells represented >70 % of whole WBC peripheral leukocytes in TCDD-treated mice.

Differential counting of white blood cells (WBC) showed a temporal increase in neutrophils. The kinetics of increase in neutrophils was identical to the results of

Mac-1$^+$Gr-1$^+$ cells obtained from flow cytometric analysis. Therefore, these observations strongly suggest that Mac-1$^+$Gr-1$^+$ cells are neutrophils.

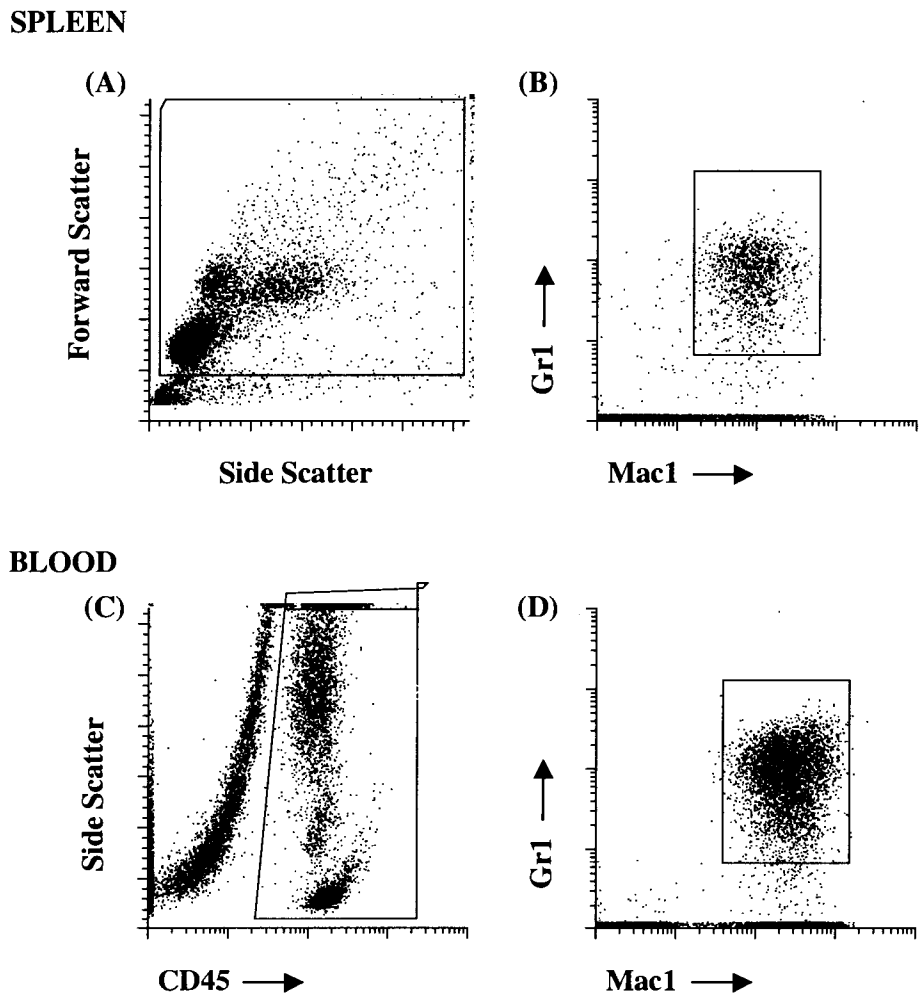


Figure 4-1-2. Flow cytometric profiles and gating used to identify Mac-1$^+$cells in the spleen (A & B) and blood (C & D).
Mice were treated with TCDD by gavage one day prior to P815 tumor injection and sacrificed nine days after tumor injection. Spleen and blood cells were stained with mAbs to Mac-1 and Gr-1 (B and D, respectively). Viable spleen cells were identified on the basis of forward and side scatter profiles (A) while viable WBC were identified on the basis of side scatter and CD45 profiles (C).

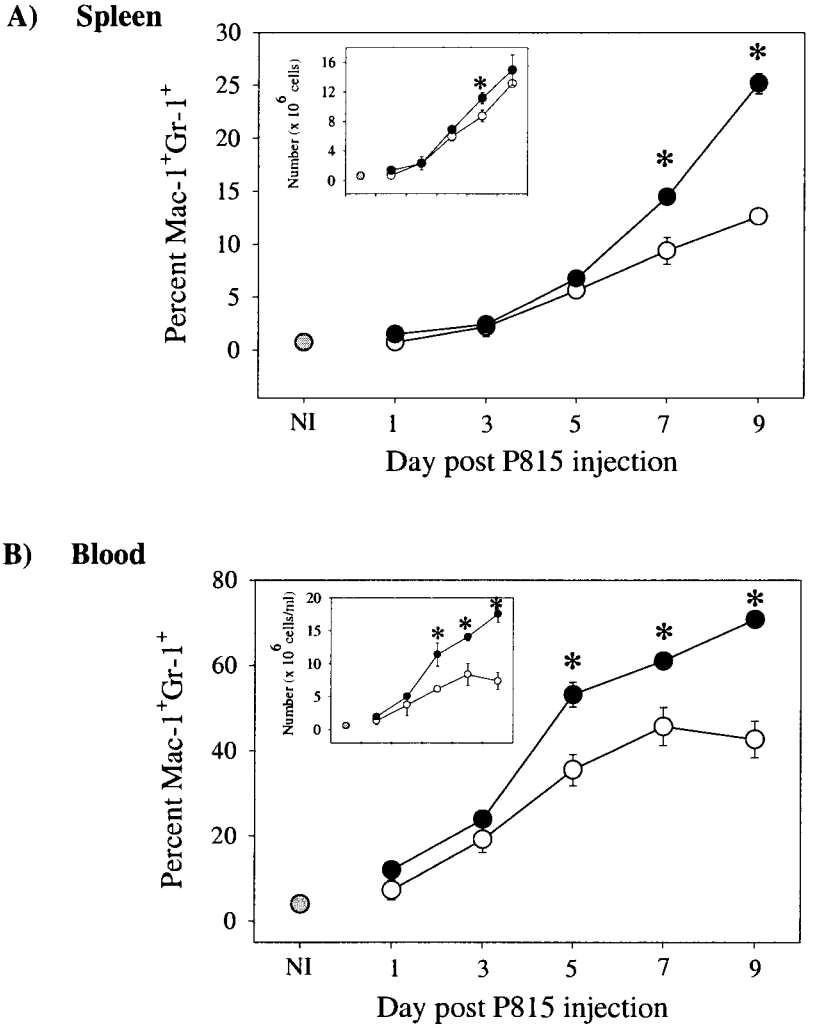


Figure 4-1-3. Effect of TCDD treatment on Mac-1$^+$Gr-1$^+$ cells in the spleen and blood in P815 tumor-injected mice.

Mice were treated with a single dose of vehicle (O) or 15 μg/kg of TCDD (●) by gavage one day prior to injection of P815 tumor cells. Mac-1$^+$Gr-1$^+$ cells were enumerated in the spleen and the blood at various times after P815 injection as described in Figure 4-1-2. NI represents 10 non-injected mice (●) sacrificed in groups of two on each test day. Data points represent the mean ± SEM for 3 mice per treatment group. * indicates a statistically significant difference ($p<0.05$) when compared to vehicle control.

4-2. Effect of TCDD on acute phase response

An acute inflammatory response is accompanied by increased production of various acute phase proteins (APPs; Baumann and Gauldie, 1994). Serum amyloid A (SAA), a major APP in mice, is an apolipoprotein, known to be induced by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor (TNF). Chronic production of SAA has been shown to suppress immune responses (Aldo-Benson and Benson, 1982; Kaminski and Holsapple, 1992). Since TCDD enhances the number of inflammatory cells by TCDD, it raised the question whether TCDD induces an acute phase response and reflected in elevated levels of SAA protein.

Based on prior studies which showed that TCDD treatment was associated with enhanced TNF production (Clark *et al.,* 1991a; Moos *et al.,* 1994), we hypothesized that enhanced TNF production would lead to the over-production of SAA and an increase in the number of inflammatory cells in TCDD-treated mice. In order to induce inflammation, mice were treated with a number of stimulants including LPS, SRBC, and P815 tumor cells. Plasma levels of SAA were measured by ELISA methods. Circulating TNF-α levels were also measured using a L929 bioassay as an indicator of systemic inflammation

4-2-1. TCDD effect on SAA induction in response to LPS

Initial experiments were conducted using LPS, a strong inflammatory stimulator, to determine the kinetics and dose response in the induction of SAA. Mice were gavaged with vehicle or 15 μg/kg TCDD one day prior to ip injection of LPS (*E. coli* 055:B5). Blood samples were collected at various times after injection. The concentration of SAA in the plasma was determined as described in Materials and Methods. Peritoneal exudate cells were harvested and stained using a Wright-Giemsa stain for differential cell counts.

As shown in Figure 4-2-1, SAA was transiently induced by LPS treatment in a dose-dependent manner. The basal level of SAA in naive mice was < 20 μg/ml

plasma throughout the experiment. In mice treated with either 50 μg or 5 μg doses of LPS, a maximal level of SAA was observed at 24 hours, followed by a rapid decline by 48 hr. As shown in Figure 4-2-1 A, TCDD did not affect SAA induction in mice injected with 50 μg LPS. When mice were injected with 5 μg LPS, TCDD treatment significantly increased SAA levels only at 24 hr (Figure 4-2-1 B). The kinetics of SAA production was not altered by TCDD treatment. When mice were given a single dose of TCDD in the absence of LPS, there was no evidence of SAA induction at 5, 24, 48 or 96 hr following the TCDD treatment (Figure 4-2-1 A).

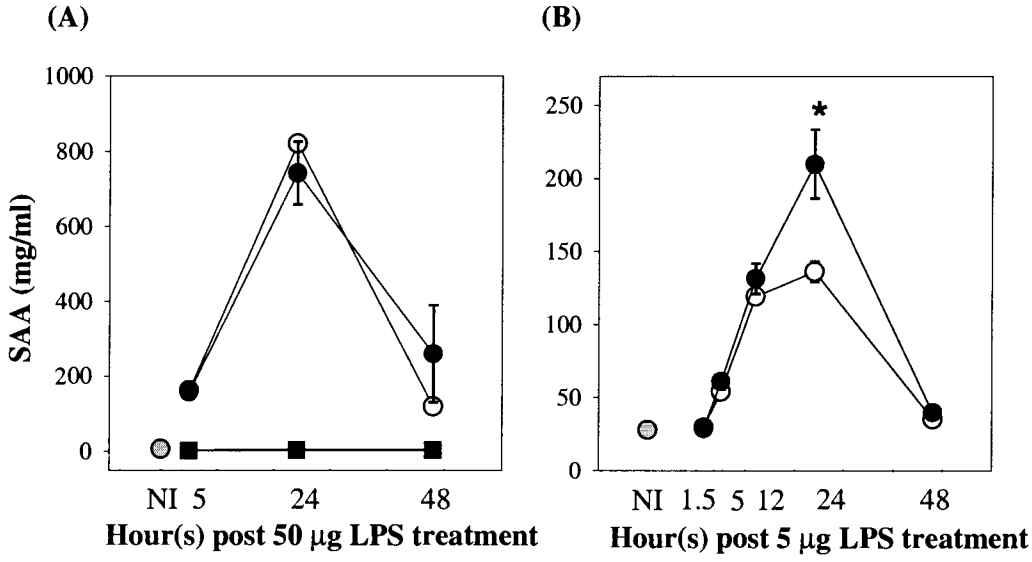


Figure 4-2-1. Effect of TCDD treatment on SAA production following LPS injection.
Mice were treated with vehicle (O) or TCDD (15 μg/kg, ●) by gavage one day prior to LPS injection. ■ represents single dose of TCDD-treated mice without LPS injection. SAA levels were measured in the plasma by ELISA method at indicated time points. Data points represent the mean ± SEM for 4-10 mice per group. Non-immune group (NI; ●) represents 4 mice. * indicates statistically significant difference ($p<0.05$) when compared to vehicle control.

4-2-2. TCDD effect on SAA induction in the response to SRBC

Mice were treated with vehicle or TCDD by gavage two days prior to the injection of SRBC. Blood samples were collected at various time points after SRBC injection to measure plasma SAA levels. As shown in Table 4-2-1, plasma levels of SAA were not detected in vehicle-treated mice above the level in naïve mice at any time tested. TCDD did not affect SAA production, suggesting that the localized hyperinflammatory response to SRBC does not reflect a systemic inflammatory response.

Table 4-2-1. TCDD effect on SAA induction in the response to SRBC injection

	Plasma SAA (μg/ml)					
	Hour(s) post SRBC injection					
	0	1.5	5	12	24	48
Vehicle	8.5 ± 2.0[a]	1.74 ± 1.1	2.96 ± 1.6	3.66 ± 2.5	4.18 ± 1.5	4.46 ± 1.1
TCDD		0.56 ± 0.6	0.76 ± 0.2	2.44 ± 1.6	16.5 ± 7.6	5.69 ± 1.0

Mice were given a single dose of vehicle or TCDD (15 μg/kg) two days prior to ip injection of SRBC. Blood samples were collected at various times after SRBC injection and plasma samples were prepared. SAA levels were measured by ELISA methods. Values represent the mean ± SEM for 4-10 mice per group. [a] NI mice (4) were represented as 0 hr.

4-2-3. TCDD effect on SAA induction in the response to P815 allogenic tumor

Mice were treated with vehicle or TCDD one day prior to the injection of P815 tumor cells. Blood samples were collected 4-7 days later. As shown in Table 4-2-2, P815 allogenic tumor cells induced an acute phase response as measured by plasma SAA levels. The concentration of SAA correlated temporally with tumor growth in the peritoneal cavity. Similar SAA induction was observed in TCDD-treated mice,

indicating that TCDD itself did not induce an acute phase response. Furthermore, TCDD did not enhance a SAA production.

Table 4-2-2. TCDD effect on SAA induction in the response to allogeneic P815 tumor

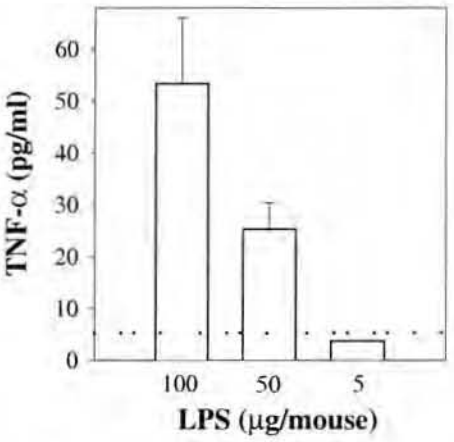
	Plasma SAA (μg/ml)			
	Day post P815 tumor injection			
	Day 0	Day 4	Day 5	Day 7
Vehicle	17.4 [a]	72.4 ± 20.5	88.3 ± 16.7	182.9 ± 13.4
TCDD		79.2 ± 17.4	73.6 ± 10.0	173.2 ± 19.7

C57Bl/6 mice were treated with vehicle or TCDD (15 μg/kg) by gavage one day prior to ip injection of 1 x 10^7 P815 tumor cells. At indicated days after P815 injection, mice were sacrificed by CO_2 asphyxiation. Blood samples were collected by heart puncture and plasma SAA levels were measured by ELISA. Values represent the mean ± SEM for 4-5 mice per group. [a] NI mice (2) were represented as day 0.

4-2-4. Influence of TNF-α on SAA induction

TNF, a major proinflammatory cytokine, induces SAA and amplifies the inflammatory response by inducing IL-1, IL-6 and TNF itself. Figure 4-2-2 A shows plasma TNF-α levels measured at 5 hr after LPS injection. LPS induced plasma TNF-α levels in a dose-dependent manner. Plasma TNF-α level in mice treated with TCDD alone were below the level of detection. In addition, exposure to TCDD did not affect TNF-α levels in LPS-treated mice (Figure 4-2-2 B). These results suggest that enhanced SAA production seen at 24 hr in TCDD-treated mice is not mediated by increased TNF-α production.

(A) LPS dose response



(B) Effect of TCDD on LPS-induced TNF-α

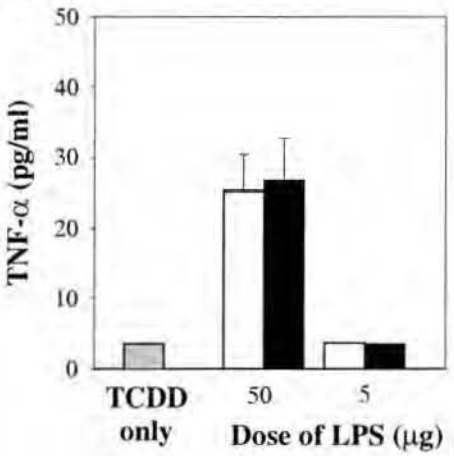


Figure 4-2-2. TCDD treatment does not affect plasma TNF-α production in LPS-injected mice.

Mice were injected ip with various doses of LPS (*E. coli* 055:B5) and sacrificed 5 hr later. Blood samples were collected, and TNF-α levels in plasma were determined using L929 bioassay. Lower limit of detection represented by -----. (A) LPS dose response. (B) Mice were treated with vehicle (□) or TCDD (■) by gavage one day prior to 50 or 5 μg/ml LPS and sacrificed 5 hr later. TCDD only-treated group (3 mice) represented by ■. Bars represent the mean ± SEM for 4 mice per group.

Figure 4-2-3 shows the kinetics of TNF-α induction and SAA production in response to LPS. Transient induction of TNF-α occurred shortly after LPS stimulation (1.5 hr) while maximal SAA production occurred at 24 hr. These data indicate that TNF-α production precedes SAA induction.

Following SRBC injection, plasma TNF levels were below the level of detection in both vehicle and TCDD-treated mice when examined at various times (1.5-48 hr; data not shown). This result agrees with the fact that we did not see increase in SAA levels either and further supports that SRBC induces only a local inflammatory response.

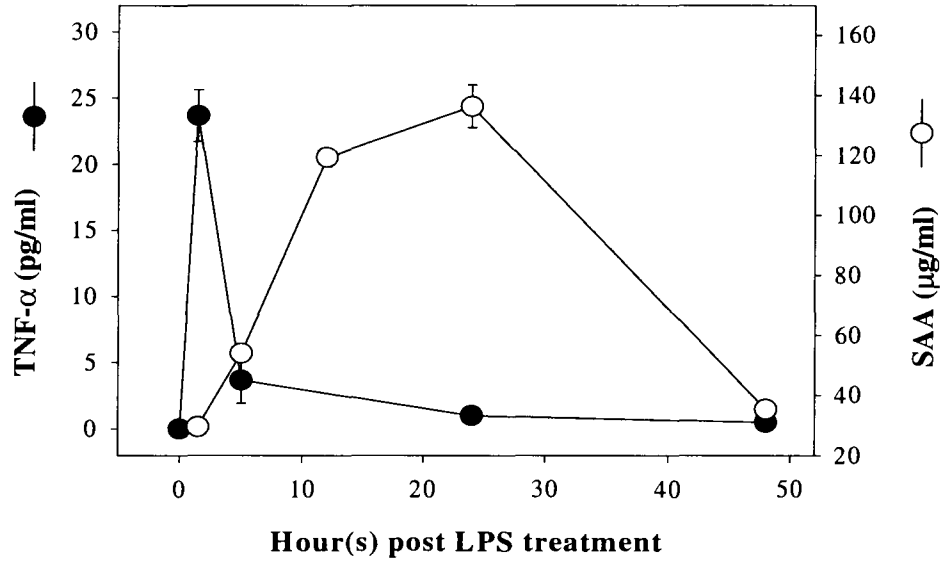


Figure 4-2-3. Kinetics of TNF-α induction and SAA production.
The induction of TNF-α (●) is rapid and the transient increase in TNF-α precedes SAA production (O). Mice were injected ip with 5 μg dose of LPS (*E coli* 055:B5) and sacrificed at the indicated time points and blood samples were collected. TNF-α and SAA levels in the plasma were determined using L929 bioassay and ELISA, respectively. SAA data represented are the same as those in Figure 4-2-1 B. Data points represent the mean ± SEM for 4-5 mice per group.

Furthermore, in P815 tumor-injected mice, TNF-α levels in the blood and peritoneal exudate were below the level of detection in both vehicle- and TCDD-treated mice 1-5 days after tumor injection. Taken together, these results suggest that TNF and SAA are not induced/enhanced by TCDD in SRBC-injected mice or modulated by TCDD in P815 tumor-injected mice.

These results conflict with those of Moos *et al.* (1994) who reported a dose-dependent increase in circulating levels of TNF-α in TCDD-treated mice. The ELISA method was used to detect TNF-α levels in Moos' study but we used a L929 bioassay. We conducted L929 bioassay as well as ELISA using the same standard, and found that the bioassay was more sensitive than the ELISA method. Interestingly, circulating TNF-α levels may not reflect TNF activity (Moos *et al.* 1994), although these investigators observed much lower but similar patterns of TNF levels by bioassay using the WEHI-164 cell line when compared to ELISA method.

The lack of a TCDD effect on the levels of TNF induced by LPS also contradicts other previously published data. Clark *et al.* (1991a) reported that TCDD exposure resulted in a dose-dependent increase of TNF-α in the serum of LPS-injected mice. Taylor *et al.,* (1992) showed that treatment with anti-TNF antibody or dexamethasone resulted in a reduction of LPS-mediated mortality, suggesting a possible involvement of TNF in TCDD-induced toxicity and. The dose of TCDD, however, used in those studies was 2-6 times higher, approaching the LD_{75} for C57Bl/6 mice. In addition, Clark *et al.* used a 3-fold higher dose of LPS. Nevertheless, even at these high doses of TCDD, TNF was not detected in the absence of LPS injection, in agreement with our results.

4-3. The role of the proinflammatory cytokine, TNF, on CTL generation in the allogeneic P815 tumor model

Even though we were unable to detect TNF-α in P815 tumor cell-injected mice, there was a possibility that a small quantity of TNF could still suppress CTL. Many cytokines are difficult to detect *ex vivo* since their production can be highly

localized and tightly regulated. This is especially relevant to TNF production since systemic production of TNF can result in tissue damage.

In order to further investigate the role of TNF in TCDD-induced immune suppression in response to P815 allogenic tumor cells, two TNF receptor knock out (TNF-R1 KO and TNF-R1&2 KO) mouse strains were utilized. TNF-α activities are elicited by binding to at least two distinct surface receptors of TNF-R1 (55-kDa) and TNF-R2 (75-kDa) with a similar binding affinity. These two receptors are ubiquitously coexpressed on almost all cell types (Vassalli, 1992). Both TNF receptors have similar extracellular domains, but their intracellular domains are entirely unrelated suggesting distinct functions of these two receptors *in vivo* (Bazzoni and Beutler, 1996). Studies in TNF-R1 KO mice revealed a decisive role for TNF-R1 in the host defense against intracellular pathogens, whereas studies of TNF-R2 KO mice indicated a role for TNF-R2 in TNF-induced necrosis (Pfeffer *et al.,* 1993; Rothe *et al.,* 1994; Erickson *et al.,* 1994).

KO and wild type mice were treated with vehicle or TCDD (15 μg/kg) by gavage one day prior to P815 tumor injection. Ten days after tumor injection, CTL activity was measured and CTL effector phenotype (CTL_E) was analyzed. Figure 4-3-1 shows CTL activities in two experiments using either TNF-R1&2 KO (A) or TNF-R2 KO mice (B). As expected, TCDD suppressed CTL activity in wild-type mice. Interestingly, the responses of both TNF-R KO mice to P815 tumor cells were not different from vehicle-treated wild type mice. Moreover, CTL activity in KO mice treated with TCDD was not as suppressed as TCDD-treated wild type mice.

Our laboratory has reported that CTL activity in P815 allografted mice was attributed to a population of $CD8^+$ cells that express the characteristic CTL_E phenotypes, high levels of CD44 and low levels of CD62L ($CD44^{high}CD62L^{low}$; Oughton and Kerkvliet, 1999). CTL_E phenotype was examined in spleen cells from the two TNF-R KO experiments described above. Table 4-3-1 shows the percentage and number of CTL_E in TNF receptor KO mice in response to P815 tumor cells.

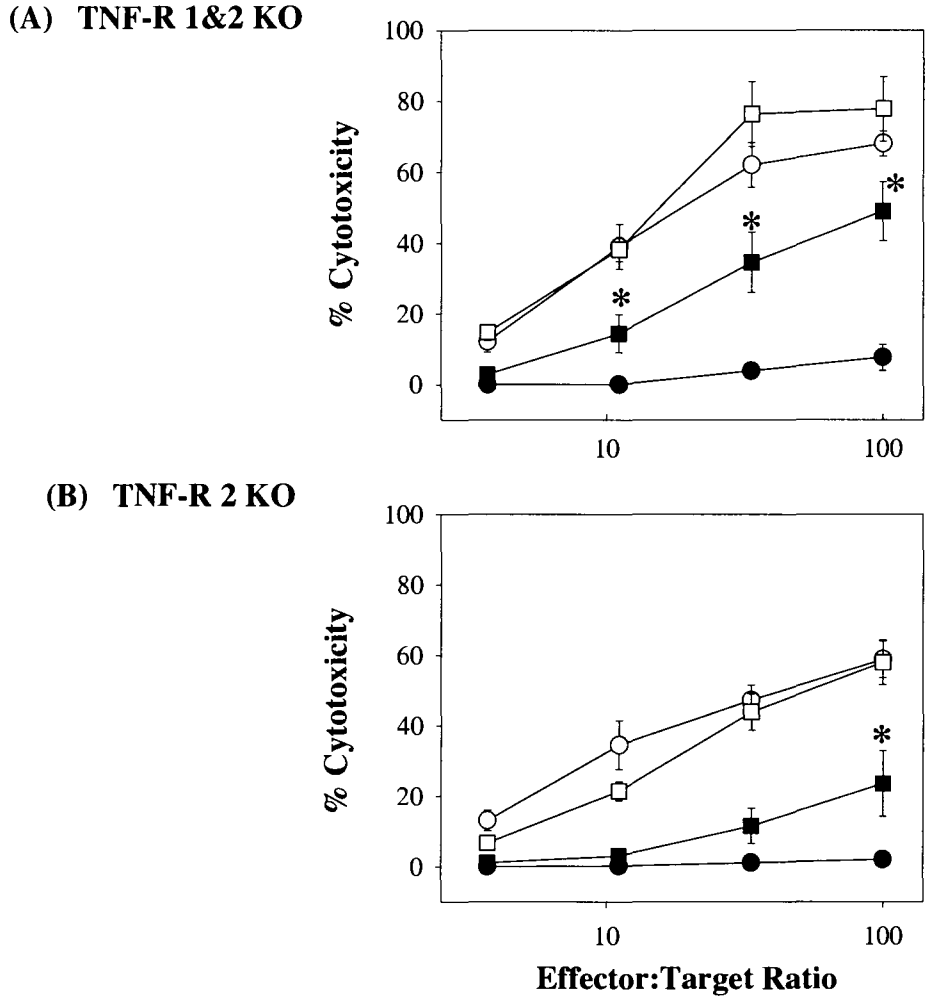


Figure 4-3-1. CTL activity in response to P815 allogenic tumor cells in TNF-R KO mice.

Mice were treated with vehicle or TCDD by gavage one day prior to P815 tumor injection. Ten days after tumor injection, CTL activity was measure in a standard ^{51}Cr-release assay. Panel A and B represent TNF-R 1&2 KO and TNF-R 2 KO experiments, respectively. Data points represent the mean ± SEM for 5-9 mice per group. Symbols represent wild type-vehicle (O), wild type-TCDD (●), KO-vehicle (□), and KO-TCDD (■). * indicates a statistically significant difference ($p<0.05$) when compared to TCDD-treated wild type mice.

Table 4-3-1. The percent and number of CTL_E developed in TNF receptor knockout mice response to P815 injection

		Percent CTL_E		Number CTL_E[1]	
		Wild type	KO mice	Wild type	KO mice
Exp. I[2]	Vehicle	60.72 ± 5.3	67.14 ± 6.0	32.72 ± 5.7	49.42 ± 12.5
	TCDD	6.88 ± 0.6	28.96 ± 6.9*	0.67 ± 0.1	8.35 ± 2.5*
Exp. II[3]	Vehicle	64.38 ± 3.7	56.11 ± 4.3	25.93 ± 3.8	27.10 ± 6.6
	TCDD	7.55 ± 0.7	17.68 ± 4.0*	0.62 ± 0.1	3.84 ± 1.9*

Wild type (C57Bl/6) and TNF-R KO mice were treated with vehicle or TCDD by gavage one day prior to the injection of P815 tumor cells. Mice were sacrificed ten days after tumor injection. Spleen cells were prepared and stained with mAbs against CD8, CD62L, and CD44 for flow cytometric analysis. Value represents the mean ± SEM of 5-9 mice per group. * indicates significant difference ($p<0.05$) between wild type mice and KO mice. [1] Number of CTL_E is $x10^6$ per spleen [2] TNF-R 1&2 KO experiment
[3] TNF-R2 KO experiment

As expected, in wild type mice, there were very few CTL_E detected in TCDD-treated mice when compared to vehicle-treated mice. In vehicle- treated KO mice, the development of CTL_E was similar to vehicle-treated wild type mice. In KO mice treated with TCDD, the number and percentage of CTL_E were significantly lower than vehicle-treated KO mice, however, the magnitude of reduction in CTL_E was not as remarkable as wild type mice. These results suggest that the development of CTL activity and CTL_E in TNF-R KO mice is not different from wild type mice, and TCDD treatment in these KO mice does not suppress the response as seen in wild type mice.

The role of TNF in T cell-mediated immunity has been controversial. TNF has been shown to enhance proliferation of naïve T cells (Joseph *et al.*, 1998) and clonal expansion of Th1 and Th2 effector subsets. In contrast, TNF has also been hypothesized as a major contributing factor of UVB-induced immune suppression via inhibiting the migration of Langerhans cells to the draining lymph nodes (Kurimoto and Streilein, 1992; Moodycliffe *et al.*, 1994). However, when TNF-R KO mice were evaluated, there was no difference in local and systemic immune responses to UVB

irradiation compared to control mice (Hart *et al.,* 1998; Kondo *et al.,* 1995). Therefore, the role of TNF in inflammation and immunologic reaction appeared to range from protective to pathologic, depending on the timing, target cell, and magnitude of the inflammatory reaction (Jacob, 1992).

Interestingly, we observed normal CTL activity response to P815 tumor in both TNF-R1&2 KO and TNF-R2 KO mice. These results suggest that TNF may not be required in the P815 response or that the absence of TNF may be beneficial.

Moreover, we also observed that TCDD-mediated immune suppression was ameliorated, to some extent, in TNF-R KO mice. These results suggest a possible mechanistic linkage between TNF and TCDD-mediated immune toxicity. However, there are several concerns that should not be overlooked and which may confound the interpretation of our findings. First, there was substantial variation within the KO groups, suggesting heterogeneity in the genetic background of these mice due to the lower number of back-crosses (3 times in TNF-R1&2 KO and 5 times in TNF-R2 KO). Secondly, Kerkvliet *et al.,* (1990b) reported that TCDD-induced immune suppression was segregated by the type of AhR ($Ah^{b/b}$ vs. $Ah^{d/d}$), suggesting a role for the AhR in TCDD-mediated immunotoxicity. The AhR phenotype of the KO mice is not known. Currently, we have no evidence to help us describe whether partial restoration or less suppression of CTL responses in TNF-R KO mice is due to a suppressive role of TNF in CTL generation or is confounded by the heterogeneous genetic background.

4-4. Effect of P815 tumor cell-secreted factors on Mac-1^+Gr-1^+ cells

We demonstrated previously in Result 4-1 that mice injected with P815 tumor cells induced Mac-1^+Gr-1^+ cells in the spleen and blood, and TCDD treatment caused an increase in these cells. We were interested in investigating the factors that might cause this large increase in Mac-1^+Gr-1^+ cells in the P815 allograft tumor model. We

hypothesized that P815 tumor cells secreted inflammatory factors that were responsible for this induction.

In order to determine the presence of P815 tumor-secreted components, ascites was collected from peritoneal cavity of DBA mice 6 to 8 days post P815 tumor injection and centrifuged to collect cell-free supernatant as described in Materials and Methods. This supernatant was injected into vehicle or TCDD-treated mice and will be referred to simply as 'P815-sup'. Mice were treated with vehicle or TCDD by gavage one day prior to the first daily injection of peritoneal sup and sacrificed one day after the 9^{th} injection. Blood samples and spleen cells were prepared and stained with mAbs against Mac-1 and Gr-1 for flow cytometric analysis. In order to evaluate the potential effect of peritoneal sup on Mac-1^+Gr-1^+ cell induction and detect any confounding effect due to multiple ip injections, two negative control groups were included. One group received only vehicle or TCDD without ip injection (None in Figure 4-4-1) and a second group received multiple ip injections of PBS in addition to vehicle or TCDD (PBS in Figure 4-4-1).

As shown in Figure 4-4-1, in NI mice, Mac-1^+Gr-1^+ cells comprised <1 % of the leukocytes in the spleen and about 4 % of the WBC in the blood. Nine injections of PBS had no effect on the number and the percentage of Mac-1^+Gr-1^+ cells in vehicle-treated groups (PBS vs. None). Injection of P815-sup induced a 4-5-fold increase in the number and the percentage of Mac-1^+Gr-1^+ cells in the spleen and the blood. However, we observed a large variation within the P815-sup-injected group. Interestingly, TCDD did not further enhance the induction of these cells. Furthermore TCDD showed a trend lowering the induction of these cells compared to vehicle-treated mice, although significant difference was observed between these two groups due to large variation.

Interestingly, treatment with TCDD alone, in the absence of stimulation, led to a small but statistically significant increase in Mac-1^+Gr-1^+ cells in the spleen and the blood as shown in control groups (None).

These results demonstrate that P815 ascites fluid contains factors that lead to the induction of Mac-1^+Gr-1^+ cells. However, this induction is an order of magnitude

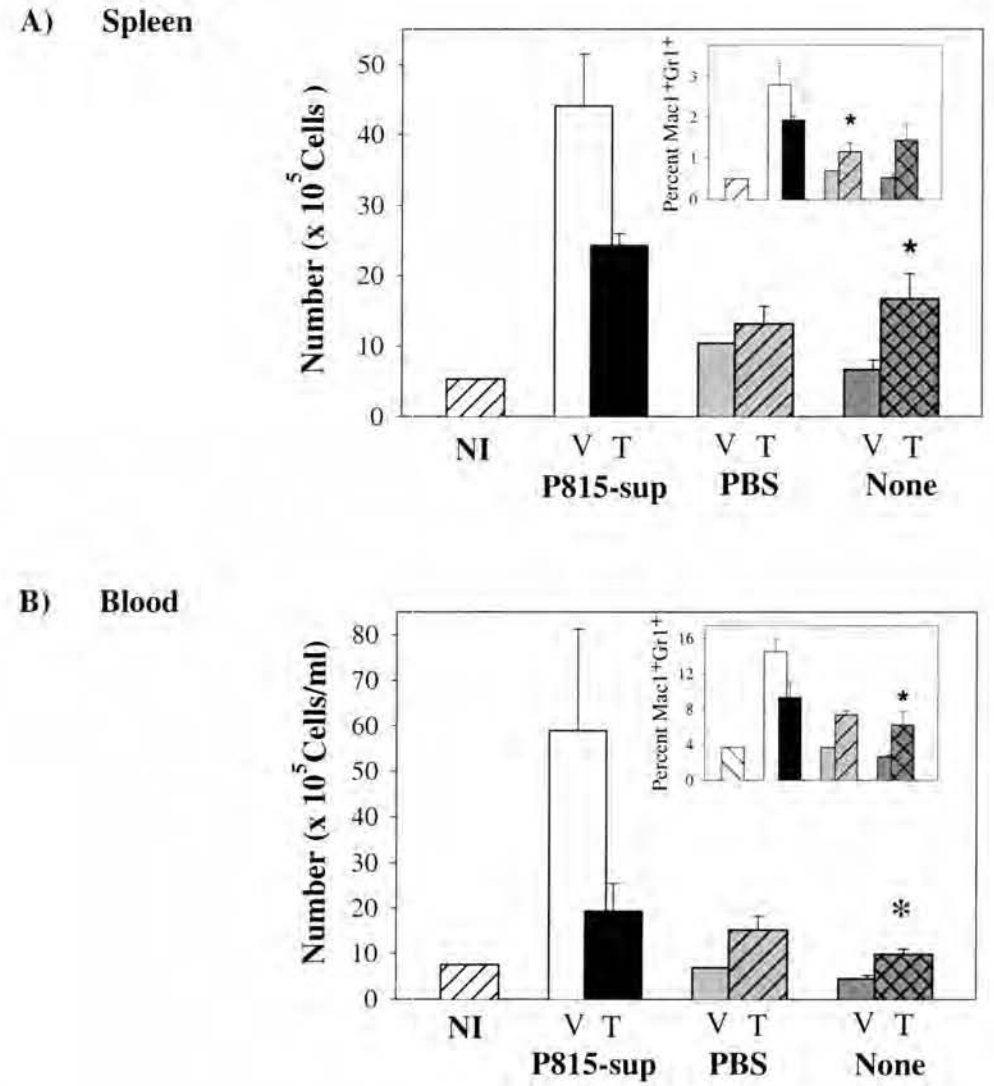


Figure 4-4-1. Effect of P815 tumor cell-secreted factors on the induction of Mac-1$^+$Gr-1$^+$ cells in the spleen and blood.
Ascites was collected from P815-bearing DBA mice and centrifuged to collect cell-free supernatant (P815-sup). Mice were treated with vehicle or TCDD one day prior to the first ip injection of P815-sup daily. Mice were sacrificed one day after the ninth injection. Spleen and blood samples were collected and stained with mAbs against Mac-1 and Gr-1 for flow cytometric analysis. NI represents non-immune control mice. P815-sup represents the treatment group. PBS represents the PBS-injected in addition to vehicle or TCDD treatment and None represents only vehicle or TCDD-treated group. V and T represent vehicle-treated and TCDD-treated group, respectively. Bars represent the mean ± SEM for 3 mice per group. * indicates statistically significant difference (p<0.05) when compared to control.

lower than observed in P815 tumor cell-injected mice. Therefore, it is apparent that there are other factors that are responsible for inducing Mac-1^+Gr-1^+ cells. Rather, a combination of factors, such as tumor burden, failure in CTL development, and perhaps the inflammatory potential of TCDD, are possibly responsible for the increase in Mac-1^+Gr-1^+ cells observed in TCDD-treated mice injected with P815 cells. Indeed, a small but significant increase in Mac-1^+Gr-1^+ cells observed in mice treated with TCDD alone suggests an inflammatory potential of TCDD. However, when P815-sup was injected, this TCDD effect was no longer observed. Therefore, it appears that the enhancement of inflammation by TCDD can not be simply explained. It is, however, possible to speculate that TCDD primes an inflammatory potential which augments the inflammatory response, depending on the type of additional stimulators.

4-5. Mac-1^+Gr-1^+ cells in the bone marrow in P815 allograft model

The bone marrow is a major hematopoietic organ where stem cells proliferate and differentiate into various types of mature lymphocytes, macrophages and granulocytes. Experiments were conducted to determine the contribution of the bone marrow in producing Mac-1^+Gr-1^+ cells that were detected in high numbers in the blood and spleen. A cell population that was positive for CD19, a B cell-specific transmembrane protein and a B cell differentiation antigen, was also examined to define if cellular components are altered in the bone marrow. Not only could we gain insight into the origin of Mac-1^+Gr-1^+ cells, but these experiments might also help us understand the mechanisms by which TCDD suppresses allo-specific responses.

4-5-1. P815 tumor cells cause an increase in Mac-1^+Gr-1^+ cells in the bone marrow and TCDD enhances this increase

In order to characterize Mac-1^+Gr-1^+ cells in the bone marrow, vehicle or TCDD-treated mice were injected with P815 tumor cells and sacrificed seven days later. Bone marrow cells (BMC) were prepared and stained with mAbs against Mac-1,

Gr-1, and CD45 for flow cytometric analysis. TCDD did not affect BMC recovery. Therefore, results are reported in terms of the frequency of cells expressing the Mac-1^+Gr-1^+ phenotype.

Figure 4-5-1 shows that Mac-1^+Gr-1^+ cells comprised 38% BMC in NI mice. Injection of P815 tumor cells resulted in a 1.6-fold increase in the frequency of Mac-1^+Gr-1^+ cells (58%) in vehicle-treated mice. There were even more Mac-1^+Gr-1^+ cells in TCDD-treated mice (70%).

As shown in column Figure 4-5-1 panel C, Gr-1 was constitutively expressed at high levels in NI mice. In response to P815 tumor cells, Gr-1 was expressed at significantly lower levels on Mac-1^+Gr-1^+ cells from vehicle-treated mice. Interestingly, there was evidence for two subpopulations of Mac-1^+Gr-1^+ cells in TCDD-treated mice based on the differential expression of Gr-1, a population expressing high levels of Gr-1 (Gr-1^{hi}) and a population expressing low levels of Gr-1 (Gr-1^{low}). The Gr-1^{low} population was much smaller in vehicle-treated mice.

These results suggest that P815 tumor cell challenge promotes the production of Mac-1^+Gr-1^+ cells in the bone marrow without altering overall cell population number. Based on the differential expression of Gr-1 in TCDD-treated mice, the Mac-1^+Gr-1^+ cells that express Gr-1^{low} may represent immature cells or cells that could further differentiate into other types of cells including myelocytes. Hestdal *et al.* (1991) reported that neutrophils in the bone marrow exclusively express Gr-1^{high} while myelocytes or immature progenitor cells express low to intermediate levels of Gr-1.

Our results are not consistent with the study of Luster *et al.* (1985) who demonstrated that progenitor cells, specifically CFU-GM, were suppressed following an acute exposure to TCDD at doses as low as 1 μg/kg. They hypothesized that this suppression occurred by direct inhibition of proliferating stem cells. However, this is contrary to the increase in Mac-1^+Gr-1^+ cells in TCDD-treated mice following P815 injection, because Mac-1^+Gr-1^+ cells probably derived from granulocyte progenitor cells in the bone marrow unless they are recruited from circulation. However, a direct comparison of these two different studies is inappropriate, because the mice in this

study were injected with P815 tumor cells in addition to TCDD. It is yet to be resolved whether progenitor cells are affected by TCDD in P815-allografted mice.

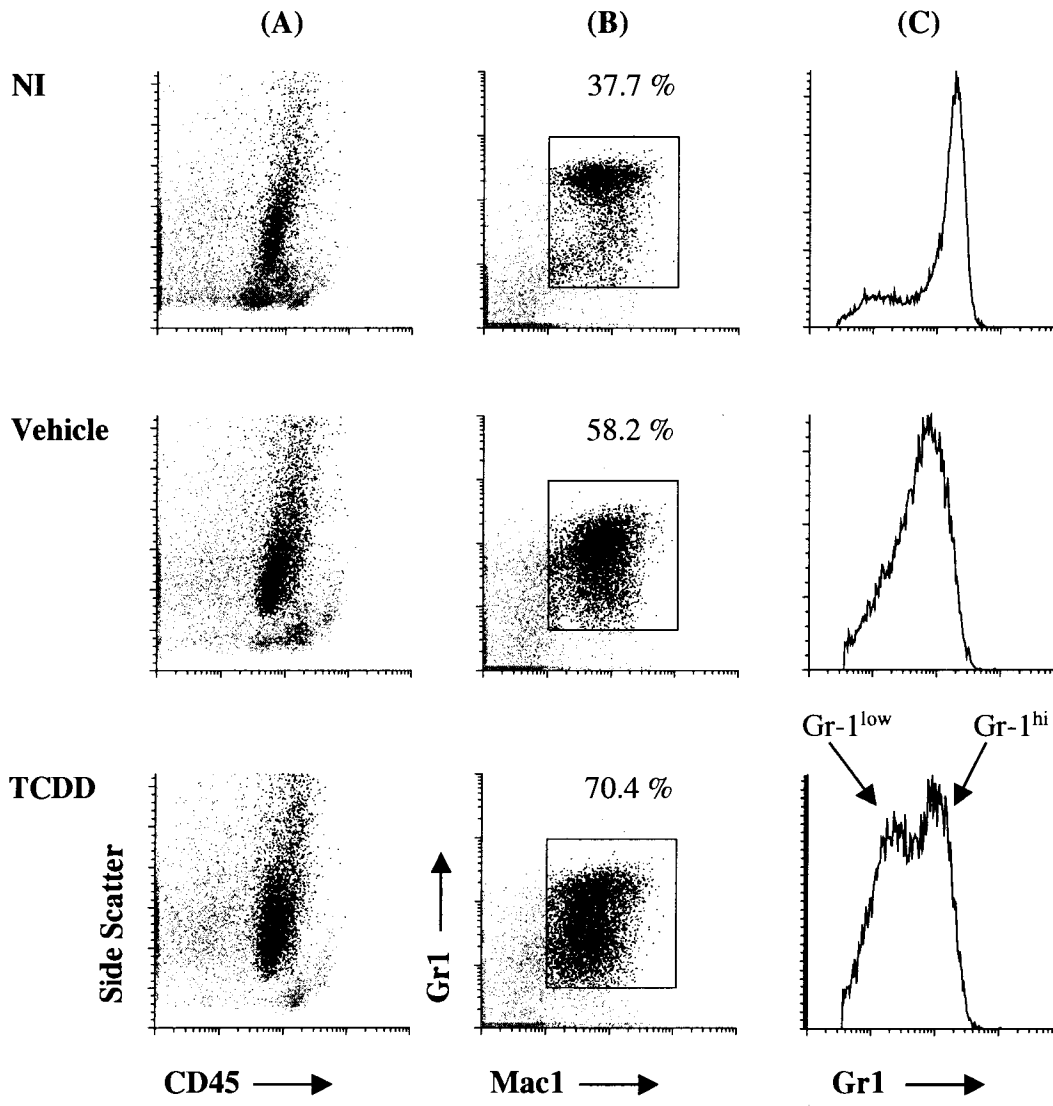


Figure 4-5-1. Mac-1 and Gr-1 expression on bone marrow cells.
Mice were treated with vehicle or TCDD by gavage one day prior to P815 tumor injection and sacrificed seven days later. Bone marrow cells were prepared and stained with mAbs against CD45, Gr-1 and Mac-1 for flow cytometric analysis. The number above the boxed region represents the percentage of $CD45^+$ cells expressing $Mac\text{-}1^+Gr\text{-}1^+$ phenotype (illustrated as black dots). Graphs in column C represent the levels of Gr-1 expression on $Mac\text{-}1^+Gr\text{-}1^+$cells from each group.

4-5-2. $CD19^{low}CD45^{low}$ cells are absent in bone marrow of TCDD-treated mice

CD19, a B cell-specific transmembrane protein and a B cell differentiation antigen, is expressed on all B cells, from the pro-B cell stage through the mature B cell stages (Krop *et al.,* 1996), but not on antibody-producing cells. It has been demonstrated that CD19 is essential for murine B cell development and T cell-dependent B cell immune responses (Krop *et al.,* 1996).

CD19 expression was also evaluated on BMC prepared from P815-injected mice (Figure 4-5-2). In NI mice, $CD19^+$ cells comprised approximately 52% of the $CD45^+$ leukocytes in the bone marrow. In response to P815 tumor cells, there were 3-fold fewer $CD19^+$ cells in vehicle-treated mice (16.7%) and another 2-fold reduction in TCDD-treated mice (7.5%).

As shown in Figure 4-5-2, at least two distinct $CD19^+$ populations can be distinguished in the BM, based on the level of CD45 expression ($CD19^+/CD45^{low}$ and $CD19^+/CD45^{hi}$). Both of these populations were clearly evident in vehicle-treated mice, but only one $CD19^+$ population was present in TCDD-treated mice ($CD19^+/CD45^{hi}$). This is a novel finding. It is tempting to speculate that the absence of the $CD19^+/CD45^{low}$ population in TCDD-treated mice may account for the suppression in the allo-antibody response.

Others have proposed that small changes in CD19 expression on pre-B cells may have an impact on their functional responses as they progress through different stages of maturation (Sato *et al.,* 1996; Martensson *et al.,* 1997). Studies using CD19-deficient mice indicated that CD19 may not be required for normal generation and maturation of B cells in the bone marrow (Rickert *et al.,* 1995; Sato *et al.,* 1996). However, CD19-deficient mice show a profound deficiency in responding to protein antigens that require T-cell help. These studies indicate that CD19 is crucial for the initiation of B cell activation by T cell-dependent antigens as well as the maturation of activated cells into memory cells. Therefore, alterations in $CD19^+$ populations could result in dramatic changes in their functional capacity.

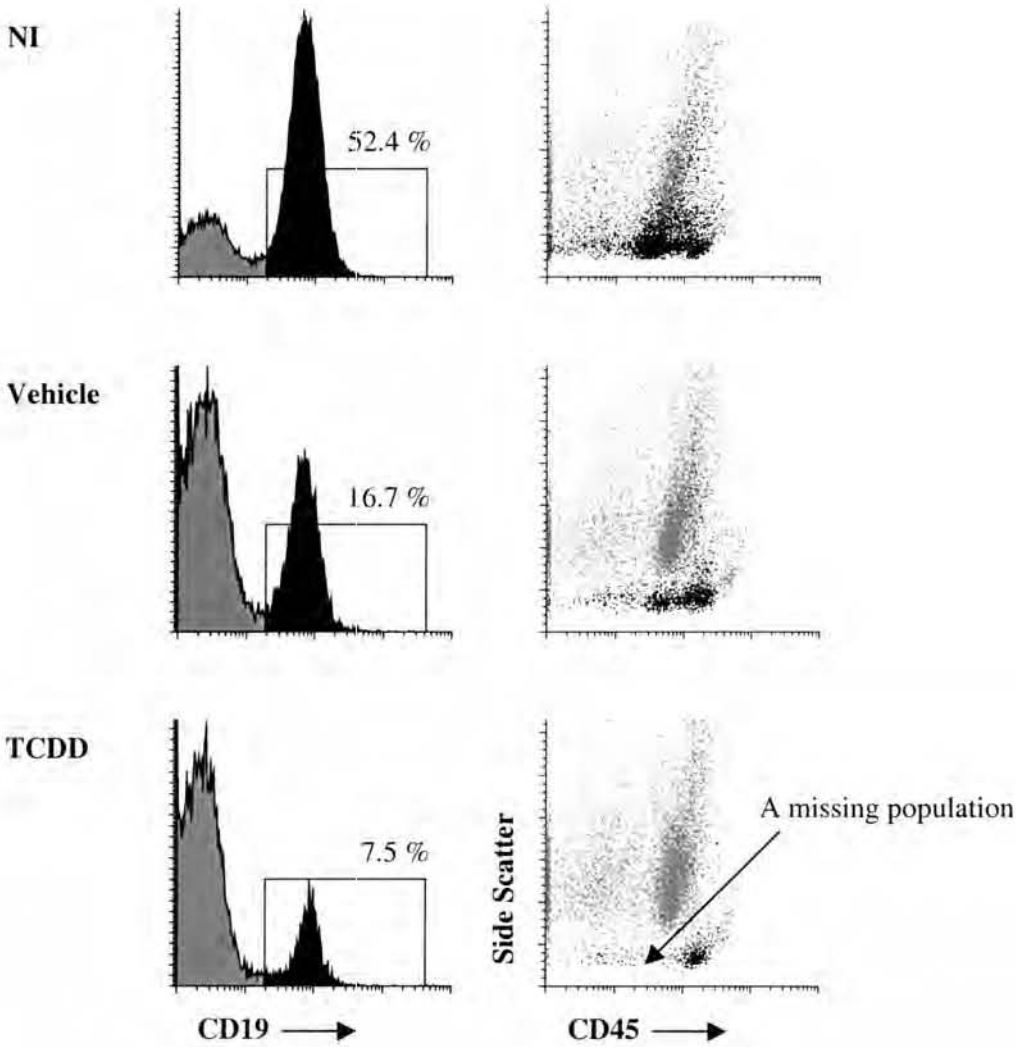


Figure 4-5-2. CD19 expression on bone marrow cells.
Vehicle- or TCDD-treated mice were injected with P815 tumor cells and sacrificed seven days later. Bone marrow cells were prepared and stained with mAbs against CD45 and CD19 for flow cytometric analysis. The numbers above the boxed regions represent the percentage of $CD19^+$ cells. The black dots on the right panels are the corresponding $CD19^+$ cells for each group. The total number of cells recovered from bone marrow was not different between vehicle- or TCDD-treated mice.

TCDD has been shown to suppress humoral immune responses by perturbing B cell activation and differentiation in the periphery (Morris and Holsapple 1991; Tucker *et al.*, 1986). The AhR complex has been shown to recognize a DNA binding site for the B lymphocyte transcription factor, B cell lineage-specific activator protein (BSAP), and to cause the reduction in CD19 gene transcription (Masten and Shiverick, 1995). Based on these results, the authors hypothesized that CD19 signaling plays a role in the reduction of immunoglobulin production by TCDD. Bone marrow B cell function also has been reported and immature B cells are particularly sensitive to TCDD toxicity (Chastain & Pazdernik, 1985). These studies, along with our finding, might explain, at least in part, the mechanisms by which TCDD suppresses immune responses.

4-6. Phenotypic characteristics of Mac-1$^+$Gr-1$^+$ cells in P815 tumor injected mice

Previous studies showed that, in the P815 allograft model, the CTL response increased over time to a maximum of CTL activity on day 10, whereas TCDD-treated mice did not develop CTL activity (Kerkvliet *et al.*, 1996). At the same time, we observed a large increase in Mac-1$^+$Gr-1$^+$ cells in the spleen and blood. The temporal correlation between the increase in Mac-1$^+$Gr-1$^+$ cells and suppressed CTL development in TCDD-treated mice led to the hypothesis that Mac-1$^+$Gr-1$^+$ cells suppress the differentiation of CTL precursor cells into CTL effector cells during the development of P815 allo-specific immune responses.

We hypothesized that Mac-1$^+$Gr-1$^+$ cells provide co-stimulatory signals that promote CTL_E development, while in TCDD-treated mice, they act as suppressor cells to inhibit the progression of CD8$^+$ cells. The alteration in Mac-1$^+$Gr-1$^+$ cell function results in TCDD-induced suppression in CTL development. This hypothesis is supported by the studies of Bronte *et al.* (1998; 1999) which showed that Mac-1$^+$Gr-1$^+$ cells induced apoptotic death in activated CD8$^+$cells, suggesting that these cells are immuno-suppressive.

Initial studies were conducted to characterize Mac-1^+Gr-1^+ cells using flow cytometry to evaluate the expression of various surface molecules involved in immunologic responses. Vehicle- or TCDD-treated mice were injected with P815 tumor cells and sacrificed nine days later. Splenic Mac-1^+Gr-1^+ cells were analyzed to assess their expression of lymphocyte markers CD4, CD8 or B220, or the dendritic cell marker CD11c. None of these lineage-specific markers were expressed on Mac-1^+Gr-1^+ cells (data not shown). These results indicate that Mac-1^+Gr-1^+ cells are not lymphocytes or dendritic cells.

In the spleen, there are two distinct populations of Mac-1^+Gr-1^+ cells, based on the differential expression of Gr-1. The majority of cells express high levels of Gr-1 (Gr-1^{hi}), while a smaller population expresses lower levels of Gr-1 (Gr-1^{low}) as shown in Figure 4-6-1, A. In addition, we also observed a small but consistent decrease in the level of Gr-1 expression in TCDD-treated mice. This reduction was even more evident in the blood (Figure 4-6-1, A and C). Similar levels of Mac-1 were expressed on splenic Mac-1^+Gr-1^+ cells. In the blood, Mac-1 was down-regulated in TCDD-treated mice but this decrease was not consistently observed in all studies (Figure 4-6-1, B and D). Since Mac-1^+Gr-1^+ cells comprise <1% in NI mice, these results indicate that P815 injection induces a large number of immature Mac-1^+Gr-1^+ cells, suggesting that Mac-1^+Gr-1^+ cells either originate in the spleen, a hematopoietic organ in mice, or are actively recruited from circulation. Moreover, the further reduction in Gr-1 expression in TCDD-treated mice suggests a possible change in function.

The expression of several cell surface molecules important in the generation of antigen-specific immune responses was also examined on day 5, 7, and 9 after tumor injection. These included costimulatory molecules (B7-1, B7-2 and CD40), adhesion molecules (ICAM-1, LFA-1, and CD62L), activation molecules (F4/80), and MHC molecules ($H2K^b$ and IA^b). Figure 4-6-2 shows representative histograms illustrating the expression of these molecules on Mac-1^+Gr-1^+ cells obtained from P815-injected mice. The expression of B7-1, B7-2, IA^b, and $H2K^b$ was similar in vehicle- and TCDD-treated mice.

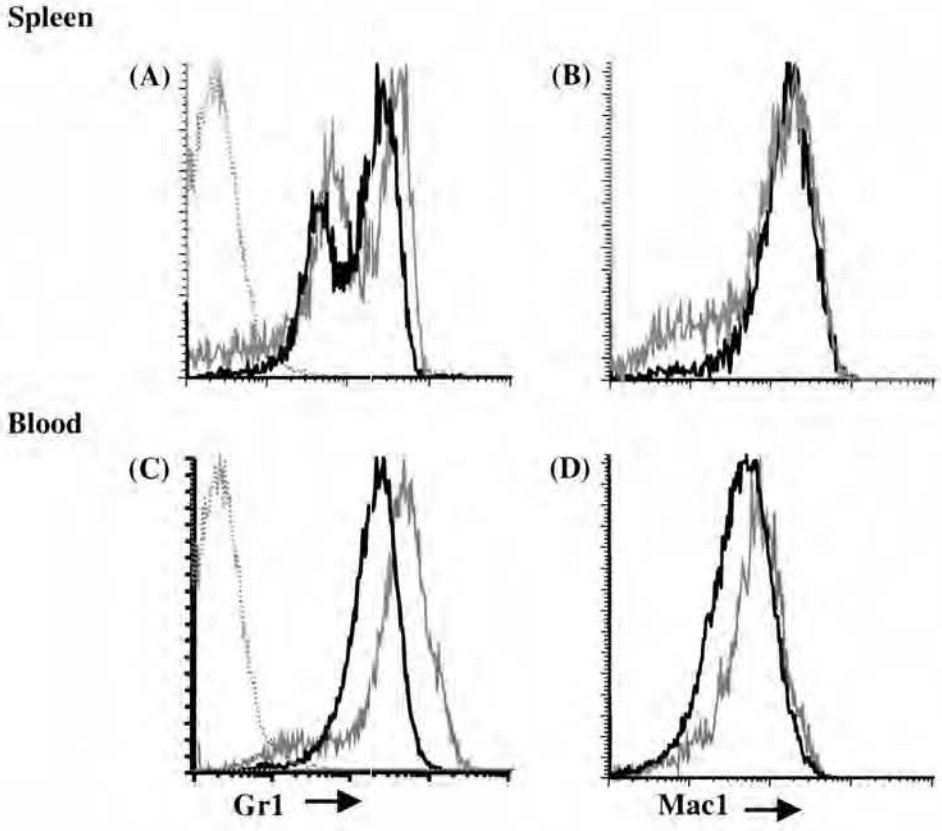


Figure 4-6-1. Gr-1 and Mac-1 expression on Mac-1$^+$Gr-1$^+$ cells in the spleen and the blood. Vehicle ( ) or TCDD ( )-treated mice were injected with P815 tumor cells and sacrificed nine days later. Spleen (A and B) and blood (C and D) samples were prepared and stained with mAbs against Mac-1 and Gr-1 for flow cytometric analysis. Values represent mean channel fluorescence (MCF) that indicates fluorescent intensity of the surface marker. Isotype control (-----) is indicated in A and C.

In contrast, we observed very small decreases in the expression of LFA-1 and CD40 in TCDD-treated mice. Both ICAM-1 and F4/80 were significantly down-regulated in TCDD-treated mice. These results indicate that TCDD treatment modulates the expression of some critical molecules on Mac-1$^+$Gr-1$^+$ cells, suggesting that these

reductions might be a mechanism by which Mac-1$^+$Gr-1$^+$ cells suppress an immune response.

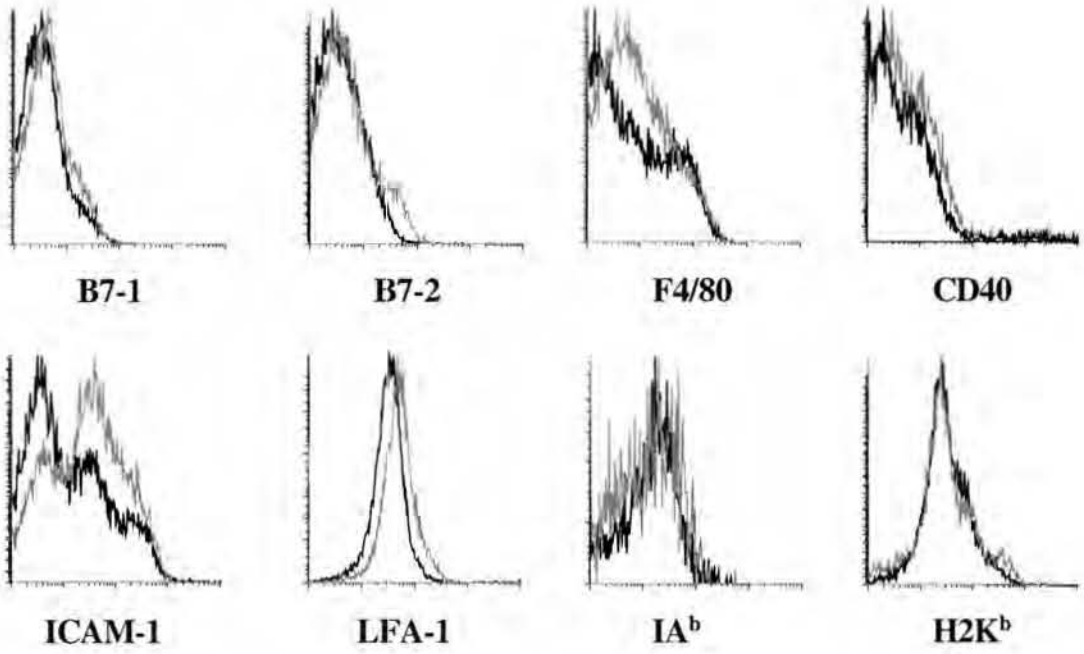


Figure 4-6-2. Costimulatory and adhesion molecule expression on Mac-1$^+$Gr-1$^+$ cells in the spleen.
Figures shown are representiatives of three mice from each group. Mice were gavaged with vehicle (▬) or TCDD (■) one day prior to P815 injection and sacrificed nine days later. Spleen cells were stained with mAbs againstMac-1 and Gr-1 and the indicated surface markers for flow cytometric analysis.

CD62L, a homing molecule, is important in the trafficking of leukocytes to sites of inflammation. Upon activation, the level of CD62L expression has been shown to be down-regulated on neutrophils (Kishimoto *et al.*, 1989; Soler-Rodriguez *et al.*, 2000). Figure 4-6-3 compares the level of CD62L expression on Mac-1$^+$Gr-1$^+$

cells from the spleen, blood, and bone marrow seven days after tumor injection. CD62L was expressed at high levels on BMC and at lower levels in the spleen and blood in vehicle-treated mice. TCDD did not alter the expression of CD62L in the bone marrow but it did down-regulate CD62L expression in the spleen and completely down-regulated it in the blood. These results suggest that Mac-1^+Gr-1^+ cells from TCDD-treated mice are more activated compared to vehicle-treated mice.

The reduction of CD62L expression should be interpreted with great caution, because several mechanisms could be involved in the modulation of CD62L expression on circulating neutrophils. Neutrophils lose CD62L as they age in the circulation (van Eeden *et al.,* 1997). *In vitro* studies have also demonstrated that the life span of neutrophils can be modulated via enhancing survival mechanisms by mediators derived from epithelial cells (Daffern *et al.,* 1999) or inhibiting apoptosis by glucocorticoid treatment (Cox, 1995). Alternatively, steroid and non-steroid anti-inflammatory drugs have been shown to reduce CD62L on circulating neutrophils (Burton *et al.,* 1995; Nakagawa *et al.,* 1999; Diaz-Gonzalez *et al.,* 1995). TCDD treatment has been shown to induce plasma corticosterone levels (DeKrey *et al.,* 1995; 1993). However, this plasma corticosterone was induced at only higher doses of TCDD (>40 μg/kg). Reduced levels of CD62L expression may be explained by any one of these mechanisms.

4-7. Effect of Mac-1^+Gr-1^+ cells on *in vivo* activated CTL activity

Previous studies by Bronte *et al.* (1998; 1999) reported that Mac-1^+Gr-1^+ cells mediated the elimination of activated $CD8^+$ T cells in vivo by inducing apoptosis through a cell contact-dependent mechanism. These reports supported our hypothesis that Mac-1^+Gr-1^+ cells were immunomodulatory cells. In order to evaluate the immuno-modulatory function of Mac-1^+Gr-1^+ cells, we assessed the ability of Mac-1^+Gr-1^+ cells to directly inhibit CTL activity.

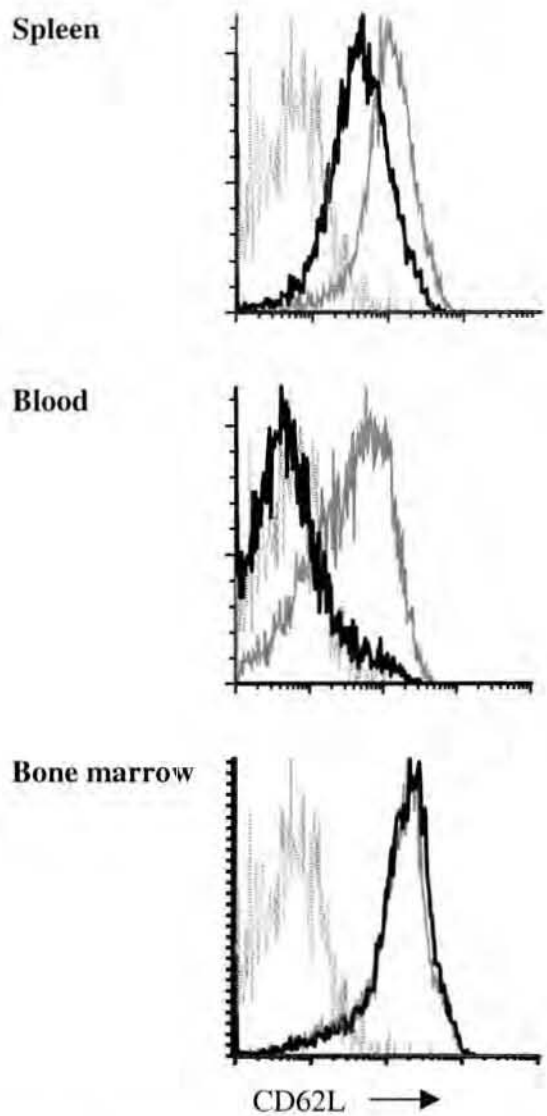


Figure 4-6-3. CD62L expression on Mac-1$^+$Gr-1$^+$ cells obtained from spleen, blood, and bone marrow. Mice were gavaged with vehicle ( ) or TCDD ( ) one day prior to P815 tumor cell injection and sacrificed seven (for bone marrow) and nine days later (for blood and spleen). Cells were prepared and stained with mAbs against Mac-1, Gr-1 and CD62L for flow-cytometric analysis. Mac-1$^+$Gr-1$^+$ cells from the spleen and the blood were gated as described in Figure1-2. For bone marrow cells, same gating protocol used in blood cells was utilized. ----- indicates isotype control.

Spleen cells from P815-injected mice containing a population of cytotoxic $CD8^+$ lymphocytes were utilized as responder cells (responders). Mac-1^+Gr-1^+ cells were enriched from TCDD-treated mice by panning as described in Materials and Methods. In order to detect the ability of Mac-1^+Gr-1^+ cells to influence CTL activity, responders were pre-incubated with Mac-1^+Gr-1^+ cells for 1.5-3 hr before the standard ^{51}Cr release assay. Responders without Mac-1^+Gr-1^+ cells were prepared as a positive control.

As shown in Table 4-7-1, Mac-1^+Gr-1^+ cells from TCDD-treated mice did not suppress the ability of *in vivo*–sensitized CTL to lyse P815 tumor cells in a ^{51}Cr release assay. This result suggests that killing of CTL cells or inhibition of CTL activity by Mac-1^+Gr-1^+ cells is not the mechanism of suppression of CTL activity in TCDD-treated mice.

Table 4-7-1. Presence of Mac-1^+Gr-1^+ cells does not affect the cytolytic activity of CTL *ex vivo*.

CTL Activity (%)			
Responder : Mac-1^+Gr-1^+	Pre-incubation period with Mac-1^+Gr-1^+		
	0 hr	1.5 hr	3 hr
1 : 1	29.15 ± 0.17	28.77 ± 1.25	28.69 ± 0.15
1 : 0.5	28.27 ± 1.14	27.96 ± 0.84	31.79 ± 2.89
1 : 0.25	26.42 ± 1.95	22.07 ± 3.29	21.42 ± 1.01
Responder only		25.78 ± 1.26	

Mice were treated with vehicle or TCDD by gavage one day prior to P815 tumor cell injection. On day 9 post tumor injection, spleen cells (responders) were prepared from a pool of three vehicle-treated mice. Splenic Mac-1^+Gr-1^+ cells were enriched from a pool of three TCDD-treated mice by panning, as described in Materials and Methods. Flow cytometric analysis revealed that 85% of the spleen cells expressed the Mac-1^+Gr-1^+ phenotype following enrichment. Responders (5×10^5) were pre-incubated with Mac-1^+Gr-1^+ cells for 1.5 or 3 hr at various ratios of responder to Mac-1^+Gr-1^+ cells in a flat bottom 96-well tissue culture plate. In order to adjust the cell concentration difference, naive spleen cells from C57Bl/6 mice were added at the appropriate number. At the end of co-culture, cytolytic activity against P815 tumor cells was measured in a standard ^{51}Cr-release assay as described in Materials and Methods. 'Responder only' represents a positive control without Mac-1^+Gr-1^+ cells. Values represent the mean ± SEM of 4 wells.

4-8. Effect of Mac-1$^+$Gr-1$^+$ cells on the development of CTL activity *in vitro.*

In a continued effort to evaluate the immunomodulatory function of Mac-1$^+$Gr-1$^+$ cells, we next investigated the ability of Mac-1$^+$Gr-1$^+$ cells to suppress the *in vitro* development of cytotoxic CD8$^+$ lymphocyte responses. Phenotypic differences in Mac-1$^+$Gr-1$^+$ cells from vehicle- or TCDD-treated mice suggested that their function might also be different. Therefore, we compared the inhibitory effect of Mac-1$^+$Gr-1$^+$ cells from vehicle- or TCDD-treated mice on CTL development.

CTL activity against P815 tumor was generated *in vitro* for 5 days by mixing C57Bl/6 mice spleen cells (H-2^b; responder) with mitomycin C-treated P815 tumor cells (P815$_M$; H-2^d; stimulator). In order to examine inhibitory function of Mac-1$^+$Gr-1$^+$ cells on *in vitro* CTL development, Mac-1$^+$Gr-1$^+$ cells were directly added to these cultures (Figure 4-8-1 A). To determine if soluble factors secreted from Mac-1$^+$Gr-1$^+$ cells were responsible for the suppression, we set up another experiment in which Mac-1$^+$Gr-1$^+$ cells were separated from the responders and stimulators by a permeable membrane (Figure 4-8-1 B). At the end of the five day culture, allogenic targets (^{51}Cr-labelled P815 tumor cells) were added and cytotoxic activity against P815 cells was measured in a standard ^{51}Cr-release assay, as described in Materials and Methods.

4-8-1. Effect of Mac-1$^+$Gr-1$^+$ cells on the development of cytotoxic CD8$^+$ lymphocyte response in vitro

As shown in Figure 4-8-2 A, the addition of Mac-1$^+$Gr-1$^+$ cells from vehicle-treated mice to culture did not alter the development of CTL activity. In contrast, addition of Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice significantly suppressed CTL activity in a number-dependent manner. These results indicate that Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice, but not from vehicle-treated mice, can inhibit the *in vitro* CTL development, suggesting functional differences in Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice.

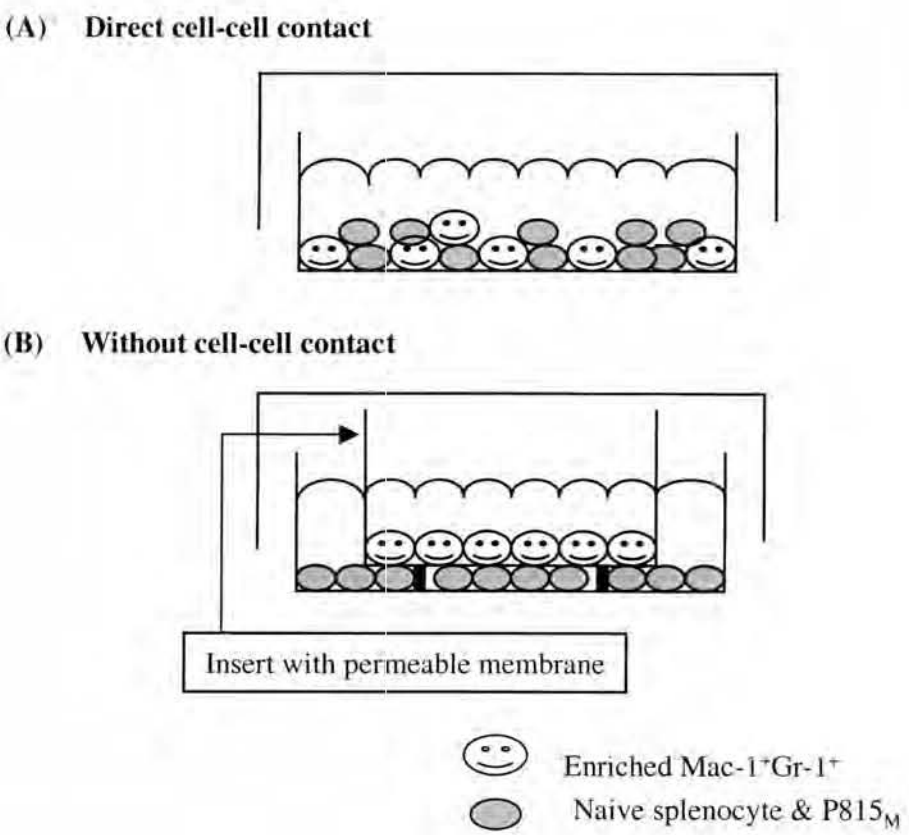


Figure 4-8-1. Culture conditions used to investigate the mechanism by which Mac-1$^+$Gr-1$^+$cells suppress the development of CTL activity *in vitro*
CTL activity against P815 tumor was generated *in vitro* 5-day culture by incubating C57Bl/6 mice naive spleen cells (H-2^b; responder) with mitomycin C-treated P815 tumor cells ($P815_M$; H-2^d; stimulator). Enriched Mac-1$^+$Gr-1$^+$cells were directly admixed to culture (A), or separated by permeable membrane (B), during *in vitro* CTL development. Mac-1$^+$Gr-1$^+$cells were enriched by panning from P815-injected mice following either vehicle- or TCDD-treatment. Enriched Mac-1$^+$Gr-1$^+$cells were identified Mac-1$^+$Gr-1$^+$ (>85%) by flow cytometry. In order to adjust cell concentration differences, mitomycin C-treated naive splenocytes from C57Bl/6 were added at appropriate numbers.

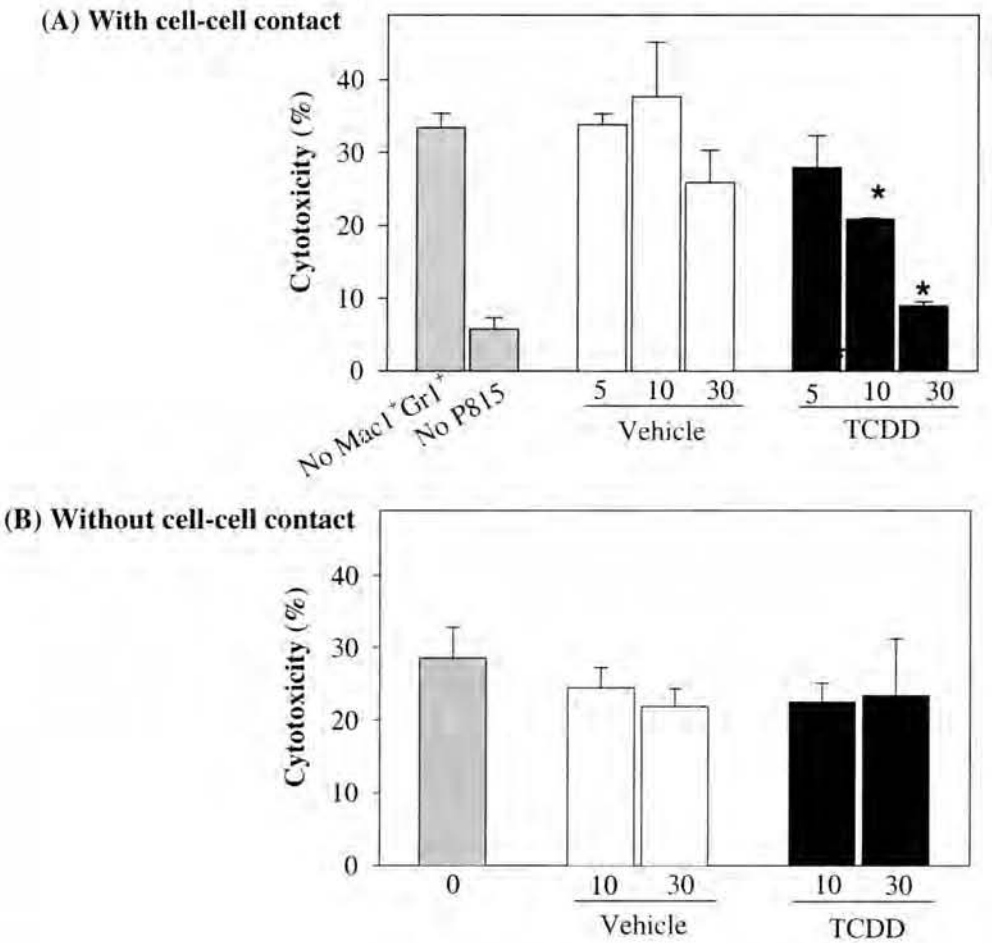


Figure 4-8-2. Effect of Mac-1$^+$Gr-1$^+$ cells on *in vitro* CTL development.
Splenic Mac-1$^+$Gr-1$^+$ cells were enriched from P815 tumor cell-injected mice following vehicle or TCDD treatment by panning as described in Materials and Methods. *In vitro* CTL culture was prepared as illustrated in Figure 4-8-1. Responders were cultured at a 50:1 ratio with $P815_M$ in wells of a flat bottom 24-well tissue culture plate. Mac-1$^+$Gr-1$^+$ cells were either admixed in the same well (A) or separated by semi-permeable membrane (B) at various percentage relative the number of responders. Each well contained responders, stimulators and Mac-1$^+$Gr-1$^+$ cells in a final volume of 1 ml of medium as described in Materials and Methods and then incubated in 5% CO_2 at 37^{0}C for 5 days. Numbers represent the percentage of Mac-1$^+$Gr-1$^+$ cells. Vehicle or TCDD represents treatment group that Mac-1$^+$Gr-1$^+$ cells were enriched. No Mac-1$^+$Gr-1$^+$ and No P815 in panel A represent positive and negative controls, respectively. * indicates statistically significant difference (p<0.05) when compared to the positive control group (No Mac-1$^+$Gr-1$^+$ cells).

4-8-2. Suppressive effects of the Mac-1$^+$Gr-1$^+$ cells on in vitro CTL development requires cell to cell contact.

Soluble factors, such as inhibitory cytokines or reactive oxygen species produced by Mac-1$^+$Gr-1$^+$ cells, may be responsible for suppressed CTL activity. As shown in Figure 4-8-2 B, when Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice were separated from responders by a semi-permeable membrane, CTL activity was not suppressed by soluble factors. This result indicates that cell to cell contact is required for inhibiting development of CTL activity, or alternatively, short-lived soluble factors need to be secreted in a close proximity to the responding cells in order to be effective. These results clearly indicate that Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice are an immunomodulatory population (suppressor cells) and their inhibitory function can be effective when they are present over a long-term and are close in proximity.

4-9. Oxidative burst of Mac-1$^+$Gr-1$^+$ cells

During phagocytosis or upon stimulation, neutrophils exhibit a marked increase in oxidative metabolism, which provides the host with a first line of defense against invading microorganisms. This response, the respiratory burst, is mediated by activation of NADPH oxidase and results in the formation of superoxide anion ($O_2\cdot$) as well as hydrogen peroxide (H_2O_2) and other reactive species. These reactive oxygen species are important defensive tools that protect the host from invading foreign microorganisms, and generation of these molecules is an important function of activated neutrophils in the innate immune system.

The oxidative burst function of Mac-1$^+$Gr-1$^+$ cells was evaluated by measuring superoxide production. C57Bl/6 mice were injected with P815 tumor cells one day following vehicle or TCDD treatment. Seven days later, Mac-1$^+$Gr-1$^+$cells were enriched from the spleen and blood by negative panning and ficol-gradient centrifugation, respectively. As described in Materials and Method, Mac-1$^+$Gr-1$^+$ cells were stimulated by the addition of phorbol 12-myristate 13-acetate (PMA). The kinetics of superoxide production was assessed by the reduction of cytochrome C.

As shown Figure 4-9-1 A, upon PMA stimulation, Mac-1^+Gr-1^+cells from TCDD-treated mice produced 5.5-fold higher levels of superoxide in the spleen (1.798 nmol superoxide/10^6 cells/10 min) when compared to those from vehicle-treated mice (0.327 nmol superoxide/10^6 cells/10 min). We did not detect spontaneous superoxide production in the absence of PMA stimulation (data are not shown). Interestingly, blood cells produced higher levels of superoxide than spleen cells. Mac-1^+Gr-1^+cells from the blood of TCDD-treated mice produced nearly 2.5-fold higher levels of superoxide (2.942 nmol superoxide/10^6 cells/10 min) compared to those from vehicle-treated mice (1.144 nmol superoxide/10^6 cells/10 min).

These results show that Mac-1^+Gr-1^+ cells generate superoxide anion and that TCDD augments superoxide production. It is not known how much superoxide is produced *in vivo* or the relevant amount that will affect normal physiology. However, it is important to point out that, in the P815 allograft model, TCDD-exposed mice contain an excessive number of Mac-1^+Gr-1^+ cells and these P815 tumor cells might provide additional inflammatory stimuli. Therefore, it is reasonable to assume that over-production of reactive oxygen species by Mac-1^+Gr-1^+ cells is associated with TCDD-induced immune suppression.

Ackermann *et al.* (1989a) showed that TCDD did not affect superoxide production or hydrogen peroxide release by neutrophils upon stimulation with either PMA or FMLP. It should be noted, however, that PMNs in Ackermann's study were recruited to the peritoneal cavity by injecting 6 % sodium caseinate into mice treated with TCDD. Therefore, it might not be relevant to compare these conflicting results because the activation status of the neutrophils may be different. There is no evidence that Mac-1^+Gr-1^+ cells directly affect the development of allo-specific immune response. However, it is apparent that Mac-1^+Gr-1^+ cells can be over-activated upon stimulation. In mice injected with P815 tumor cells, it needs to be determined whether Mac-1^+Gr-1^+ cells are over-activated inappropriately by TCDD.

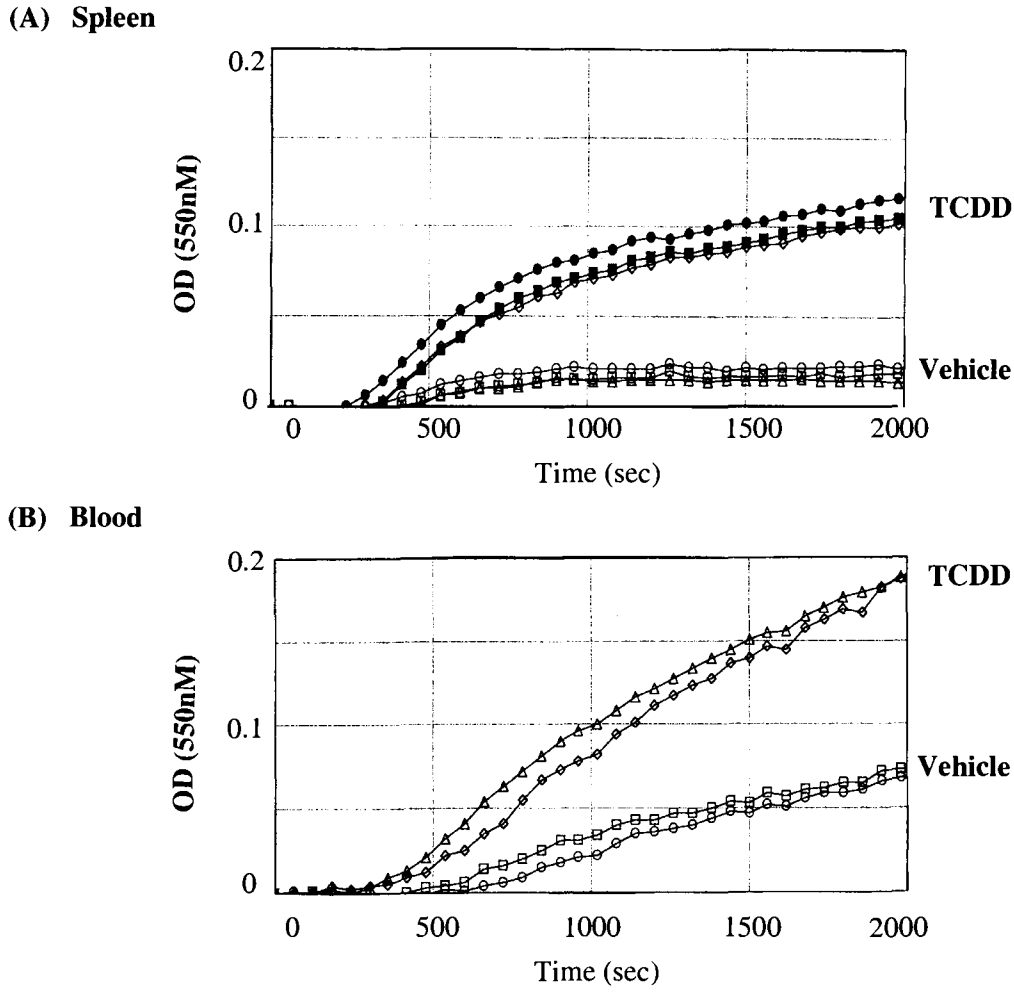


Figure 4-9-1. Superoxide production by Mac-1$^+$Gr-1$^+$ cells from the spleen and blood

Vehicle or TCDD-treated mice were injected with P815 tumor cells and sacrificed seven days after injection. Mac-1$^+$Gr-1$^+$ cells were enriched from the spleen (A) and blood (B) by panning and ficol-gradient centrifugation, respectively. Mac-1$^+$Gr-1$^+$ cells were stimulated with PMA (100 ng/ml), and superoxide anion production was measured kinetically at 37^{0}C for 40 minutes by the reduction of cytochrome *c* at 550 nm (OD550) using a SPECTRA MAX250 microplate reader, as described in Materials and Methods. The data shown illustrate response of individual samples, and are representative of three similar experiments.

4-10. Effect of TCDD exposure on tumor lysis mediated by Mac-1$^+$Gr-1$^+$ cells

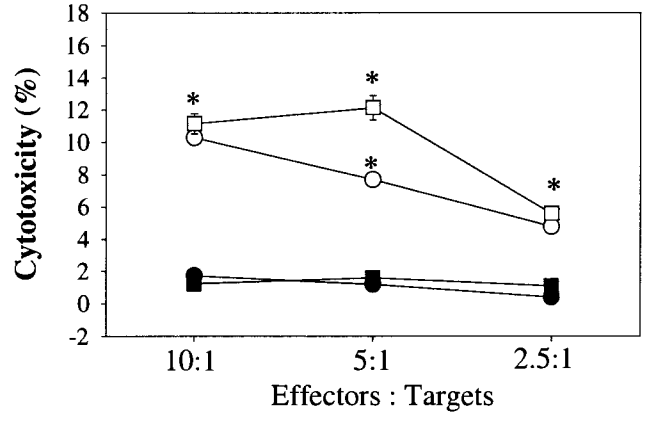
The increased oxidataive burst capacity of Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice indicated that these cells were highly activated. We evaluated the effect of TCDD on the ability of Mac-1$^+$Gr-1$^+$ cells to lyse tumor cells. Previous studies have demonstrated that neutrophils activated by inflammatory stimuli can exert rapid lysis of a variety of murine or human tumor cells (Lichtenstein *et al.,* 1984a and b; Lichtenstein, 1986). Tumor-lytic activity of Mac-1$^+$Gr-1$^+$ cells was assessed by using the Yac-1 tumor cell line, a lymphoma that has been shown to be sensitive to PMN-mediated tumor cytotoxicity (Inoue and Sendo, 1983; Morikawa *et al.,* 1985).

Vehicle or TCDD-treated mice were injected with P815 tumor cells and sacrificed nine days later. Mac-1$^+$Gr-1$^+$cells were enriched from the spleen and blood by negative panning and Ficol-gradient centrifugation, respectively, as described in Materials and Methods. Mac-1$^+$Gr-1$^+$cells and ^{51}Cr-labeled Yac-1 tumor cells were cocultured at different effector : target ratios in a 6-hour ^{51}Cr-release assay.

As shown in Figure 4-10-1, TCDD completely suppressed the cytolytic ability of Mac-1$^+$Gr-1$^+$ cells from the spleen and blood to lyse Yac-1 tumor cells. Surprisingly, stimulation with PMA did not further enhance the level of cytolytic activity in vehicle-treated mice. The suppressed cytolytic function of Mac-1$^+$Gr-1$^+$ cells from TCDD-treated mice was not restored by treatment with PMA. These results indicate that TCDD treatment alters the cytotoxic function of Mac-1$^+$Gr-1$^+$ cells.

Previous studies demonstrated that naive peripheral neutrophils require *in vitro* activation to exhibit tumor-lysis whereas peritoneal inflammatory neutrophils can lyse tumor cells spontaneously (Clark and Klebanoff, 1975; Arkermann *et al.,* 1989b). For example, tumor lytic neutrophils were detected in the peritoneal cavity 6 hours after ip injection of *Corynebacterium parvum* in mice following a challenge with the murine ovarian teratocarcinoma (Lichtenstein *et al.,* 1984a). These studies suggested that neutrophil-mediated tumor lysis was dependent on the activation status of neutrophils, and that the active oxygen products, lysosomal enzymes, and the functional myeloperoxidase-hydrogen peroxide-halide system were responsible for the PMNs-mediated tumor lysis (Clark and Klebanoff, 1975; Lichtenstein, 1986).

(A) No stimulation



(B) With PMA (30ng/ml) Stimulation

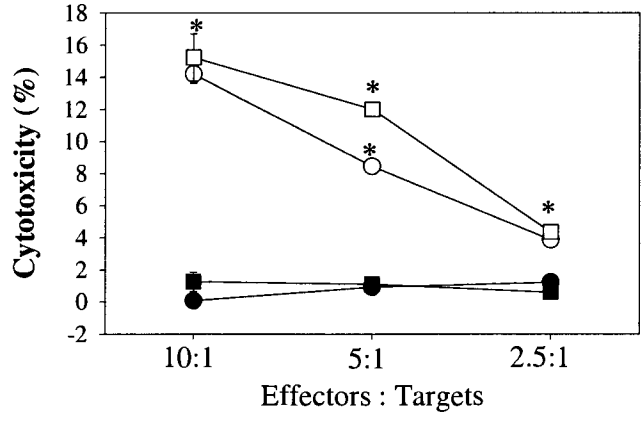


Figure 4-10-1. TCDD suppresses Mac-1$^+$Gr-1$^+$ cell-mediated Yac-1 tumor cell cytolytic activity.

Mac-1$^+$Gr-1$^+$cells were enriched from the spleen and blood from vehicle- or TCDD-treated mice nine days after P815 injection as described in Materials and Methods. Mac-1$^+$Gr-1$^+$ cells were cocultured with ^{51}Cr-labeled Yac-1 tumor cells in a 96 well round-bottom plate at various E : T ratios in the absence (A) or presence (B) of 30 ng/ml of PMA. Cytolytic activity was measured in a 6 hr ^{51}Cr-release assay. Symbols represent cells from spleens of vehicle (O)-or TCDD (●)-treated mice or cells from the blood of vehicle (□)- or TCDD (■)-treated mice. Data points represent the mean ± SEM of 4 wells. * represents statistically significant difference (p<0.05) when compared to TCDD-treated mice.

In addition, Lichtenstein (1986) pointed out two distinct phases on PMN-mediated tumor lysis, tumor cell binding and a post-binding lytic event. They suggested that the ability to bind target cells was as important as the production of reactive oxygen species.

These results demonstrate that Mac-1^+Gr-1^+ cells from vehicle-treated mice can lyse Yac-1 tumor cells, indicating *in vivo* activation. Interestingly, TCDD treatment abolished the Mac-1^+Gr-1^+ cell-mediated cytolytic activity. These results might be explained either by an altered binding capability of Mac-1^+Gr-1^+ cells to tumor cells or reduced production of reactive oxygen species by Mac-1^+Gr-1^+ cells. Based on the result shown in the previous section, the deficiency in production of reactive oxygen metabolites is unlikely because TCDD causes an increase in superoxide production by Mac-1^+Gr-1^+ cells. However, the down-regulation in the level of surface molecule expression on Mac-1^+Gr-1^+ cells by TCDD, specifically ICAM-1 and CD62L, may be a mechanism by which the cytolytic activity of Mac-1^+Gr-1^+ cells was reduced.

4-11. Histopathology and Immunohistochemisty

Inhibition of *in vitro* CTL development by Mac-1^+Gr-1^+ cells indicated that physical proximity between Mac-1^+Gr-1^+ cells and T cells in the spleen may be critical. We hypothesized that the presence of a large number of Mac-1^+Gr-1^+ cells in TCDD-treated P815 tumor-injected mice suppressed CTL development by accumulating in the white pulp of the spleen, thereby disrupting cell-cell communication between T cells-APCs. This disruption would ultimately interfere with the differentiation and clonal expansion of P815-specific CTL_E. In order to test the hypothesis, the localization of Mac-1^+Gr-1^+ cells was examined in the spleen by immunohistochemical staining.

Mice were treated with vehicle or TCDD by gavage one day prior to P815 tumor cell injection. Mice were sacrificed on days 5, 7, and 9 after P815 tumor

injection and spleen samples were snap frozen and then sectioned with a cryostat (8 μm) for immunohistochemical staining or routine H&E staining for histopathology. As described in Materials and Methods, spleen sections were fixed with acetone and stained with mAbs against CD19 and Mac-1, or Mac-1 and Gr-1. Slides were counter-stained with hematoxylin (1:10, Gills stocks) for 30 seconds.

For morphologic identification of Mac-1^+Gr-1^+ cells on day 9 after P815 injection, Mac-1^+Gr-1^+ cells were enriched from spleen by negative panning and from the blood using ficol-gradient centrifugation, as described in the Materials and Methods section. Cytospin slides were prepared and stained by Wrights-Giemsa. Flow cytometric analysis revealed that 85% of spleen cells and >95% of peripheral blood cells expressed Mac-1^+Gr-1^+ following enrichment.

Spleen weight was smaller in TCDD-treated mice (81 ± 7.2 mg) when compared to vehicle-treated mice (130.3 ± 11.2 mg). Moreover, when spleens were sectioned for histopathology and immunohistochemistry, we noticed that spleens from TCDD-treated mice on day 9 were fragile compared to spleens from vehicle-treated mice. Figure 4-11-1 shows H&E staining of spleens from vehicle- and TCDD-treated mice following P815 treatment as well as from NI mice. The spleen of NI mice revealed large areas of white pulp and thin cords of red pulp interspersed between the white pulp regions. The white pulp regions contained prominent areas of periarteriolar lymphoid sheaths and lymphoid nodules with germinal centers. The red pulp regions contain cords of mononuclear cells and vascular sinusoids. Megakaryocytes were routinely found scattered throughout the red pulp regions.

In vehicle-treated mice,nine days after P815 tumor injection, the white pulp regions were reduced in size when compared to NI mice and the red pulp areas were expanded with abundant numbers of neutrophils, activated macrophages, mononuclear cells and megakaryocytes. The white pulp regions contained distinct periarteriolar lymphoid sheaths and lymphoid nodules but germinal centers were not frequently observed. In mice treated with TCDD on day 9 after tumor injection, the white pulp regions were even more decreased when compared with vehicle-treated mice, and the red pulp regions were markedly expanded.

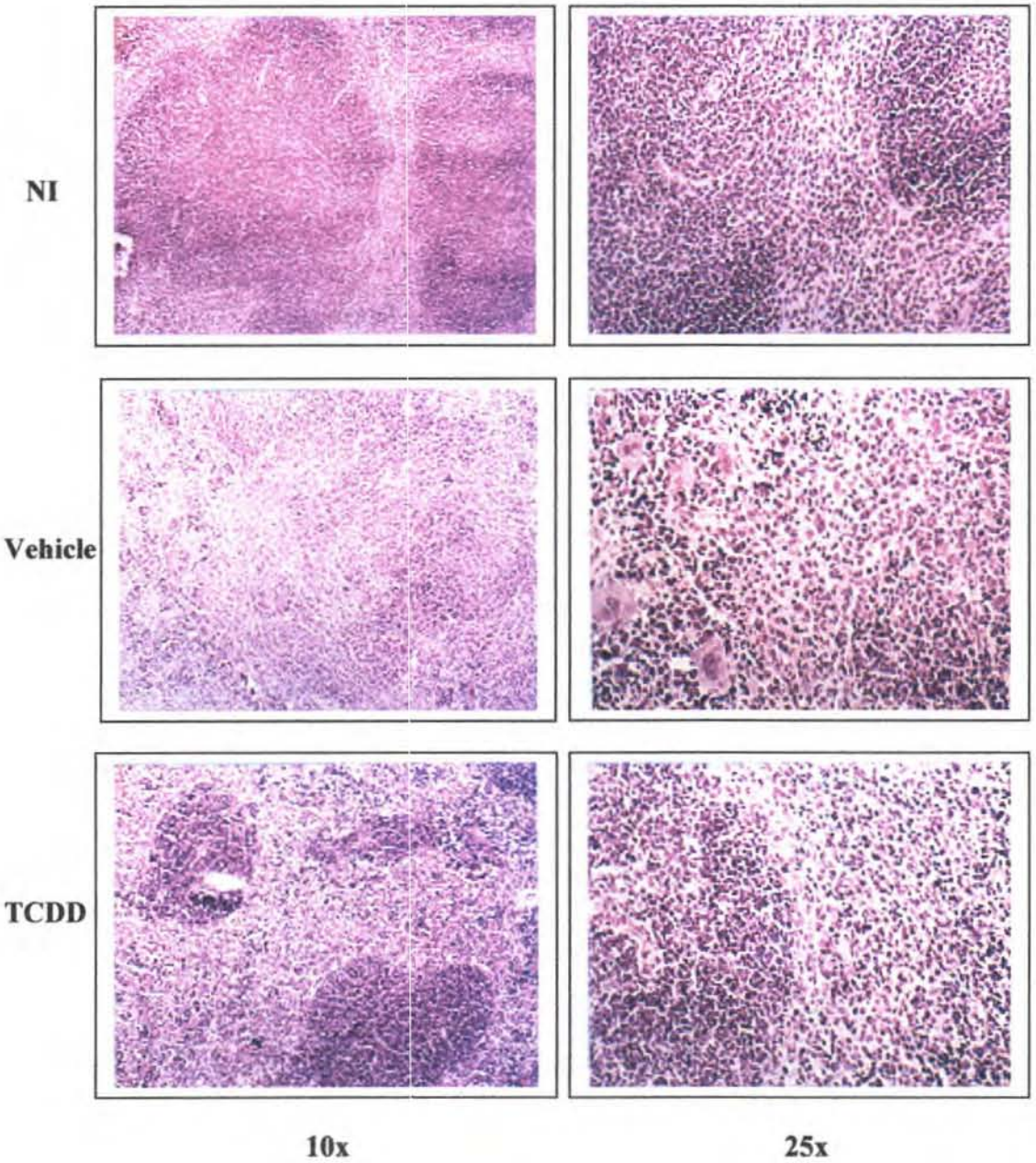


Figure 4-11-1. Histopathology of spleens
Vehicle or TCDD-treated C57Bl/6 mice were injected with allogeneic P815 tumor cells. Nine days after tumor injection, mice were sacrificed. Spleens were snap frozen and stained with H&E, 10x and 25x.

In the expanded red pulp sinusoids, there were abundant neutrophils, numerous large reactive macrophages and mononuclear cells. Occasional megakaryocytes were present in the red pulp regions but they often had condensed basophilic nuclei and reduced amount of smudged condensed eosinophylic cytoplasm. In the white pulp regions, there were distinct periarteriolar lymphoid sheaths and lymphoid nodules. Germinal centers were not observed.

Figure 4-11-2 shows immunohistochemical staining of Mac-1 and CD19 in the spleen on days 5 and 9 after tumor injection. CD19 staining localized in the peripheral white pulp while the majority of Mac-1 staining localized in the red pulp region. The area and number of the cells that were stained for Mac-1 increased on day 9 when compared to day 5 after P815 tumor injection. In TCDD-treated mice, the white pulp diminished in size and the number of cells stained with Mac-1 was further increased when compared to vehicle-treated mice. As expected, double staining for Mac-1 and Gr-1 revealed that cells stained with Mac-1 also stained with Gr-1 as shown in Figure 4-11-3. These results show that physical interaction of Mac-1^+Gr-1^+ cells and T cells is unlikely because Mac-1^+Gr-1^+ cells and T cells did not co-localize within the T cell area of the spleen (the white pulp).

Figure 4-11-4 shows the morphological characteristics of Mac-1^+Gr-1^+ cells in the spleen and blood. In the blood, Mac-1^+Gr-1^+ cells were morphologically identified as neutrophils with the characteristic lobular shaped nuclei, whereas Mac-1^+Gr-1^+ cells from the spleen included not only neutrophils (approximately 50%) but also other cell types that have ring-shaped nuclei. Similar morphological characteristics were observed in the spleen and blood from TCDD-treated mice.

The Mac-1^+Gr-1^+ cells with ring-shaped nuclei in the spleen might represent immature granulocytes as suggested by Biermann. *et al.*, (1999). They reported that murine leukocytes with multilobulated nuclei included monocytes, immature granulocytes or early progeny of hematopoietic stem cells. As shown in Figure 4-6-2, A, the two distinct populations of splenic Mac-1^+Gr-1^+ cells from TCDD-treated mice may represent fully differentiated neutrophils (Gr-1^{hi}) and immature granulocytes (Gr-1^{low}) or other cells which are not fully differentiated.

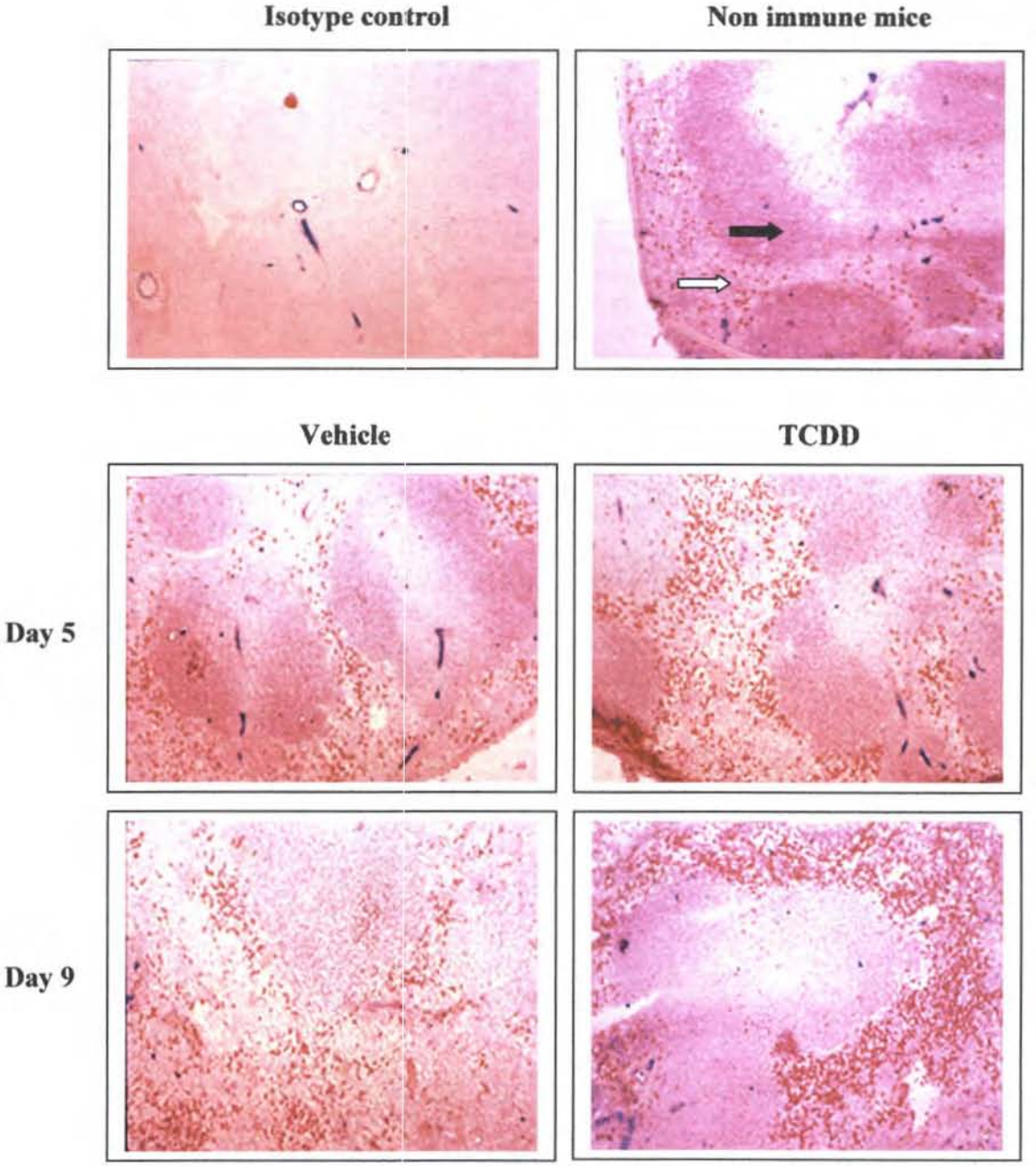


Figure 4-11-2. Immunohistochemical detection of Mac-1 and CD19 stained cells in the spleen

Mice were treated with vehicle or TCDD by gavage one day prior to injection of P815 tumor cells. Mice were sacrificed at indicated days after tumor injection and spleens were snap frozen. Spleen sections were stained with mAbs to Mac-1 and CD19 and visualized by avidin-conjugated alkaline phosphatase or horseradish peroxidase methods as described in Materials and Methods. CD19 staining localized in the peripheral white pulp (➡) and Mac-1 staining localized in the red pulp region (⇨). 10x.

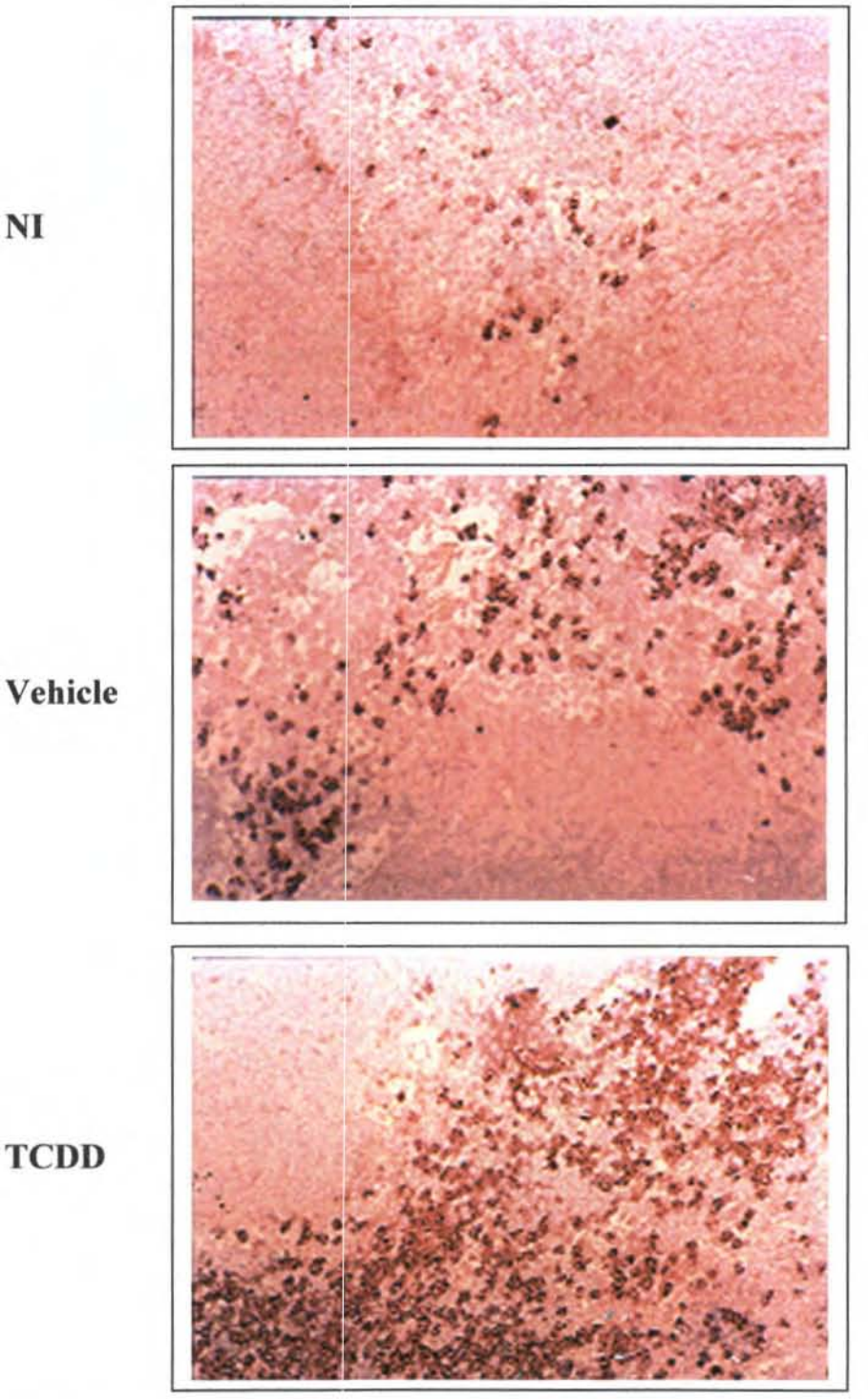


Figure 4-11-3. Immunohistochemical detection of Mac-1 and Gr-1-stained cells in the spleen

Spleen sections were analyzed by immunohistochemical staining for expression of Mac-1 and Gr-1 in vehicle- or TCDD-treated mice nine days after P815 tumor injection. 25x.

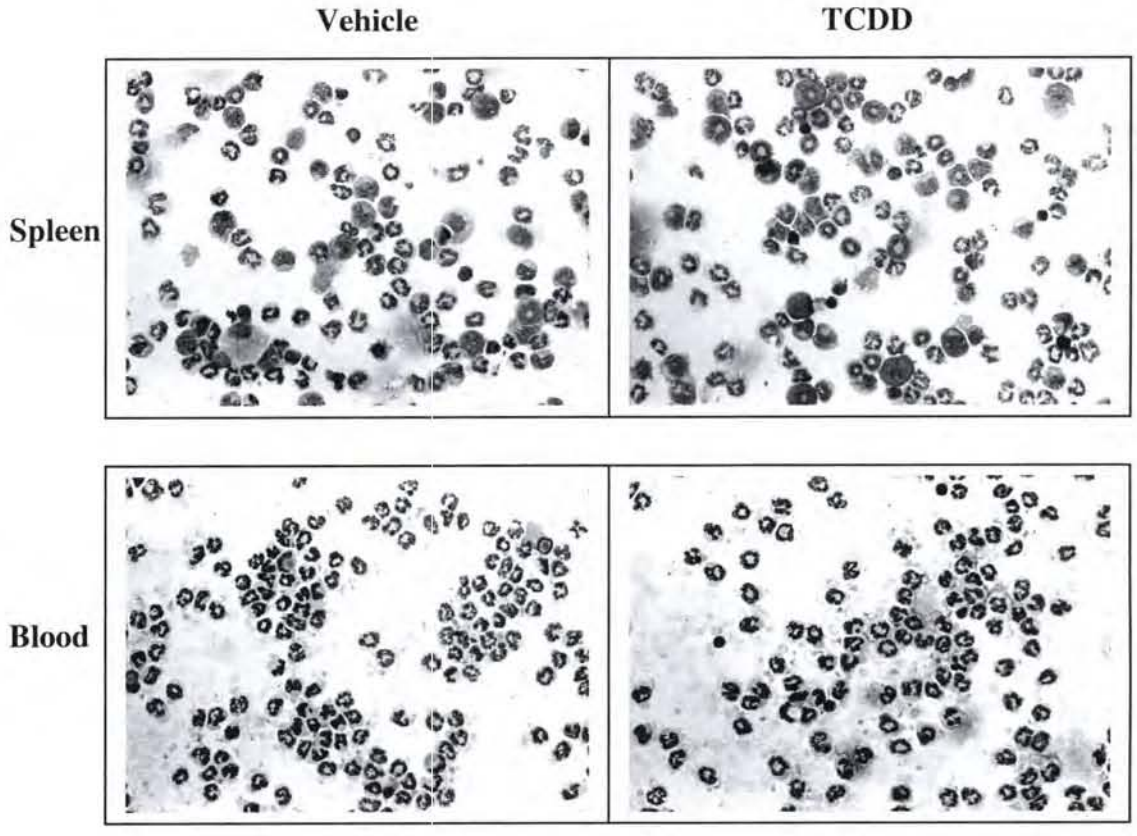


Figure 4-11-4. Morphological identification of Mac-1$^+$Gr-1$^+$ cells from the spleen and blood
Vehicle- or TCDD treated mice were injected with P815 tumor cells. Nine days after tumor injection, Mac-1$^+$Gr-1$^+$ cells from the blood and spleens were enriched, cytospin prepared, and stained with Wright-Giemsa, as described in Materials and Methods. 400x.

4-12. Effect of depleting Mac-1$^+$Gr-1$^+$ cells on the development of CTL activity *in vivo*

In order to determine the inhibitory modulation of Mac-1$^+$Gr-1$^+$ cells on the *in vivo* development of CTL activity, we depleted Mac-1$^+$Gr-1$^+$ cells in TCDD-treated mice using monoclonal RB6-8C5 antibody, which recognizes the Gr1 antigen. *In vivo* treatment of mice with this mAb has been reported to bind and lyse neutrophils,

resulting in neutropenia in the blood and spleen for up to 5 days (Czuprynski *et al.*, 1994). Experiments were conducted to ask the following questions: 1) Are Mac-1^+Gr-1^+ cells necessary in CTL generation? 2) Can an excessive number of Mac-1^+Gr-1^+ cells disrupt normal CTL development?

4-12-1. Comparisons of responses to P815 vs. DNAX-P815 tumor cells

These studies were complicated by the lethality induced by multiple injections of RB6-8C5 antibody in P815-challenged mice, both vehicle and TCDD-treated. To minimize the severe inflammatory response using ascites P815 tumor cells, we characterized the CTL response to DNAX-P815 tumor cells, an *in vitro* P815 tumor cell line.

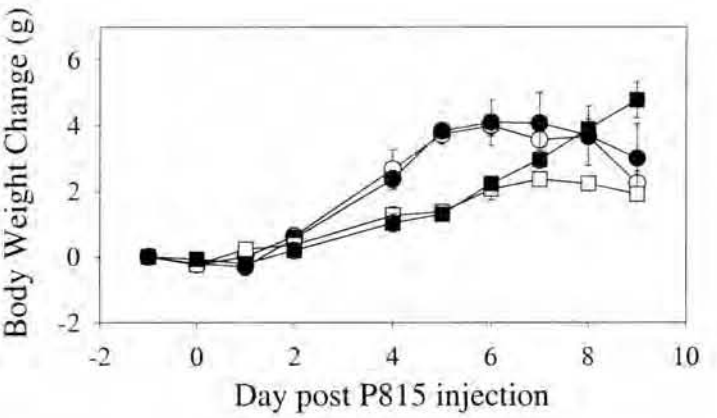
Figure 4-12-1 compares the CTL response in mice injected with cultured DNAX-P815 cells to ascites P815 tumor cells. Figure 4-12-1 A illustrates the changes in body weight. In mice injected with ascites P815 tumor cells, body weight began to increase three days after tumor injection and decrease after the maximal increase on day 7. There was no significant difference in body weight between vehicle and TCDD-treated mice injected with ascites P815 tumor cells at any time tested. Mice injected with cultured DNAX-P815 gained weight more slowly compared to ascites P815-injected mice. In TCDD-treated mice injected with DNAX-P815 tumor cells, body weights continued to increase when compared to vehicle later in the response.

These changes of body weight represent various factors in mice including degree of tumor growth in the peritoneal cavity, clearance of tumor cells by development of CTL_E, and body weight loss induced by TCDD treatment. In vehicle-treated mice, body weights increased along with tumor growth and decreased when mice began to develop CTL activity that coincided with the clearance of either ascites- or DNAX-P815 tumors. In contrast, in TCDD-treated mice, body weight changes were different depending on types of tumors; there was a decrease in body weight after the peak increase in ascites P815, but a continuous increase in DNAX-P815-injected mice. These results should be interpreted with great caution. First, exposure to TCDD has been shown to induce weight loss that is called the wasting syndrome,

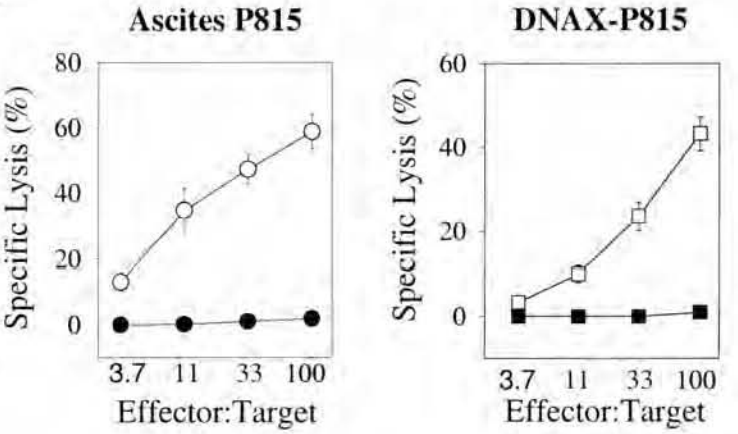
Figure 4-12-1. Comparison of CTL responses in mice injected with ascites P815 cells or DNAX-P815 tumor cell line

Vehicle- or TCDD-treated mice were injected with either ascites P815 cells or cultured DNAX-P815 tumor cells. Mice were sacrificed nine days after tumor injection (A and C). From two independent experiments, mice were sacrificed ten days after tumor injection (B). Body weights were monitored daily (A). CTL activity was measured using a standard ^{51}Cr-release assay (B). In order to identify Mac-1^{+}Gr-1^{+} cells, spleen samples were stained with mAbs against Mac-1 and Gr-1 for flow cytometric analysis (C). Symbols represent groups: vehicle (O)- or TCDD (●)-treated ascites P815-injected mice, vehicle (□)- or TCDD (■)-treated cultured DNAX-P815-injected mice. Data points represent the mean ± SEM of 3-6 mice per group. * represents statistically significant difference ($p<0.05$) when compared to vehicle control group.

(A) Body weight



(B) CTL activity



(C) Mac-1$^+$Gr-1$^+$ Cells in the spleen

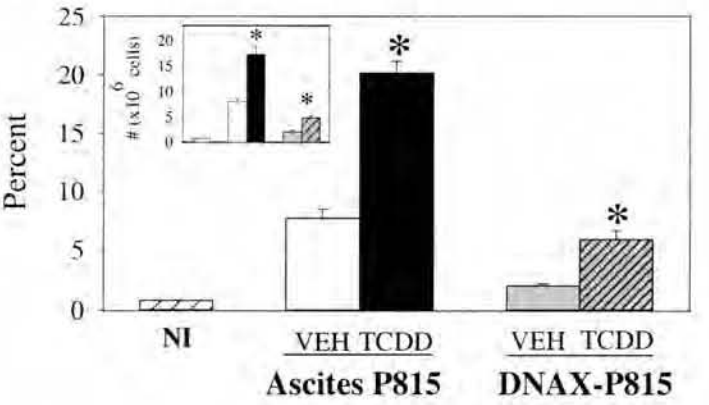


Figure 4-12-1

however, the dose we used was 10-fold lower; secondly, TCDD suppresses CTL development in P815 allografted mice. Therefore, these factors, in addition to the nature of the tumor in growth rate could result in differences in body weight change between P815 and DNAX-P815 tumor cells.

As shown in Figure 4-12-1 B, the CTL response to cultured DNAX-P815 was similar to the CTL response induced by ascites P815. Vehicle-treated mice developed CTL activity, and TCDD treatment suppressed the development of CTL activity.

The number of Mac-1^+Gr-1^+ cells in the spleens of mice injected with DNAX-P815 was compared to ascites P815 tumor-injected mice. As shown in Figure 4-12-1 C, DNAX-P815 cells induced an increase in Mac-1^+Gr-1^+ cells in the spleen, but the magnitude of this increase was considerably less than that induced by ascites P815 tumor cells. However, TCDD treatment still caused a two-fold increase in these cells as compared to vehicle treatment in DNAX-P815-injected mice.

4-12-2. Effect of depletion of Mac-1^+Gr-1^+ cells on normal CTL response in DNAX-P815-allografted mice

In order to determine an appropriate dose of RB6-8C5 antibody to deplete Mac-1^+Gr-1^+ cells in DNAX-P815-allografted mice, a dose-response experiment was conducted using TCDD-treated mice. TCDD-treated mice were used due to the large increase of Mac-1^+Gr-1^+ cells in the spleen and blood. As shown in Figure 4-12-2, RB6-8C5 antibody depleted Mac-1^+Gr-1^+ cells in the spleen and blood in a dose-dependent manner as determined four days after a single injection. A dose of 250 μg RB6-8C5 was used to deplete Mac-1^+Gr-1^+ cells in all subsequent experiments.

In order to examine the effect of depleting Mac-1^+Gr-1^+ cells on the CTL response to DNAX-P815 cells, 250 μg RB6-8C5 antibody were injected at various times after DNAX-P815 cell injection. To control for the effect of RB6-8C5 antibody, a group of DNAX-P815-injected mice received three injections of 250 μg Rat IgG. As shown in Figure 4-12-3, one to three injections of RB6-8C5 antibody had no effect on CTL activity when compared to mice that were injected with Rat IgG. Because

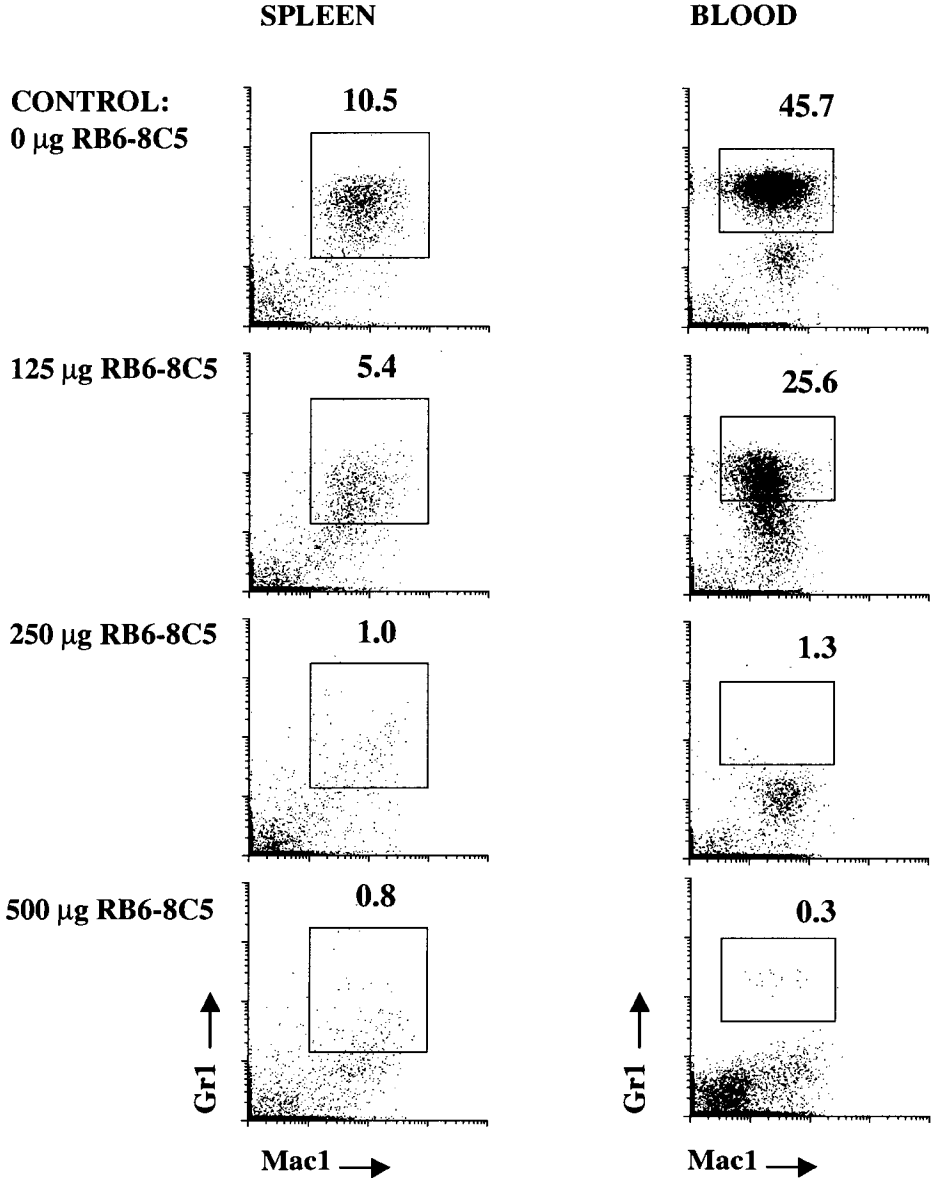


Figure 4-12-2. Dose-response of RB6-8C5 antibody in depleting Mac-1$^+$Gr-1$^+$ cells in the spleen and blood of P815 allografted mice

TCDD-treated mice were injected with DNAX-P815 tumor cells and, four days later, injected ip with a single dose of 125, 250, 500 μg RB6-8C5 antibody. For the control group (0 μg RB6-8C5), mice were injected with PBS. All mice were killed by CO_2 asphyxiation 8 days after tumor injection. Blood and spleen samples were prepared and stained with mAbs against Mac-1 and Gr-1 for flow-cytometric analysis to verify depletion of Mac-1$^+$Gr-1$^+$ cells. Mac-1$^+$Gr-1$^+$ cells are identified in the boxed regions with numbers representing the percentage of Mac-1$^+$Gr-1$^+$ cells in the spleen and blood.

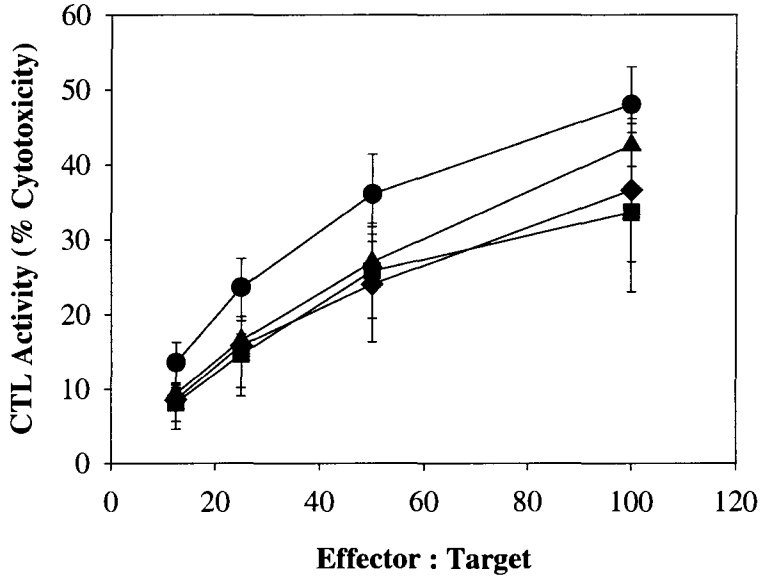


Figure 4-12-3. Effect of Mac-1$^+$Gr-1$^+$ depletion on the development of the CTL response in DNAX-P815-allografted mice

Mice were injected with 250 μg RB6-8C5 on days 1, 4, and 7 after DNAX-P815 injection (■); mice were injected with 250 μg RB6-8C5 on days 4 and 7 (◆); or 250 μg RB6-8C5 on day 7 (▲). For the control group, mice were injected with 250 μg Rat IgG on days 1, 4, and 7 (●). Mice were sacrificed by CO_2 asphyxiation 10 days after tumor injection. The percentage of Mac-1$^+$Gr-1$^+$ cells was <1 % in both the spleen and blood as determined by flow cytometric analysis. Splenic CTL activity was assessed using a standard ^{51}Cr-release assay. Data points represent the mean ± SEM of 4 mice per group.

depleting Mac-1^+Gr-1^+ cells did not alter the CTL activity, these results suggest that Mac-1^+Gr-1^+ cells do not play a role in the development of the CTL response

4-12-3. Effect of depleting Mac-1^+Gr-1^+ cells on CTL development in TCDD-treated mice

We next examined whether the elimination of Mac-1^+Gr-1^+ cells in TCDD-treated mice could restore suppressed CTL activity. Mice were injected with RB6-8C5 antibody on days 4 and 7 after tumor injection, and CTL activity was assessed on day ten, as measured by a ^{51}Cr-release assay. To control for the effect of RB6-8C5 antibody, a group of vehicle and TCDD-treated mice were injected with Rat IgG.

Figure 4-12-4 A illustrates the change in body weight over the course of the response. In mice injected with DNAX-P815 tumor cells, body weight continued to increase in TCDD-treated mice when compared to vehicle-treated mice. In contrast, in mice injected with RB6-8C5 antibody, there was no significant difference in body weight between vehicle and TCDD-treated mice. These results demonstrate that depleting Mac-1^+Gr-1^+ cells protected from TCDD-induced increase in body weight.

Figure 4-12-4 shows the effect of Mac-1^+Gr-1^+ depletion on CTL activity (B) and the allo-antibody-mediated cytotoxic activity (C). Again, in vehicle-treated mice, there was no evident difference in the level of CTL activity in mice treated with RB6-8C5 antibody. Furthermore, RB6-8C5 antibody treatment did not restore suppressed CTL activity response in TCDD-treated mice. Interestingly, when RB6-8C5 antibody was injected in TCDD-treated mice, we observed a small restoration in the allo-antibody response as compared to TCDD-treated mice injected with Rat IgG.

4-13. Ah receptor involvement on Mac-1^+Gr-1^+ cells

The AhR is a ligand-dependent transcription factor that is believed to mediate the toxic effects of TCDD exposure. This is true especially for the immune system as immunotoxic effects of TCDD have been shown to be mediated via an Ah receptor-dependent mechanism. Several pieces of evidence support this statement, such as 1)

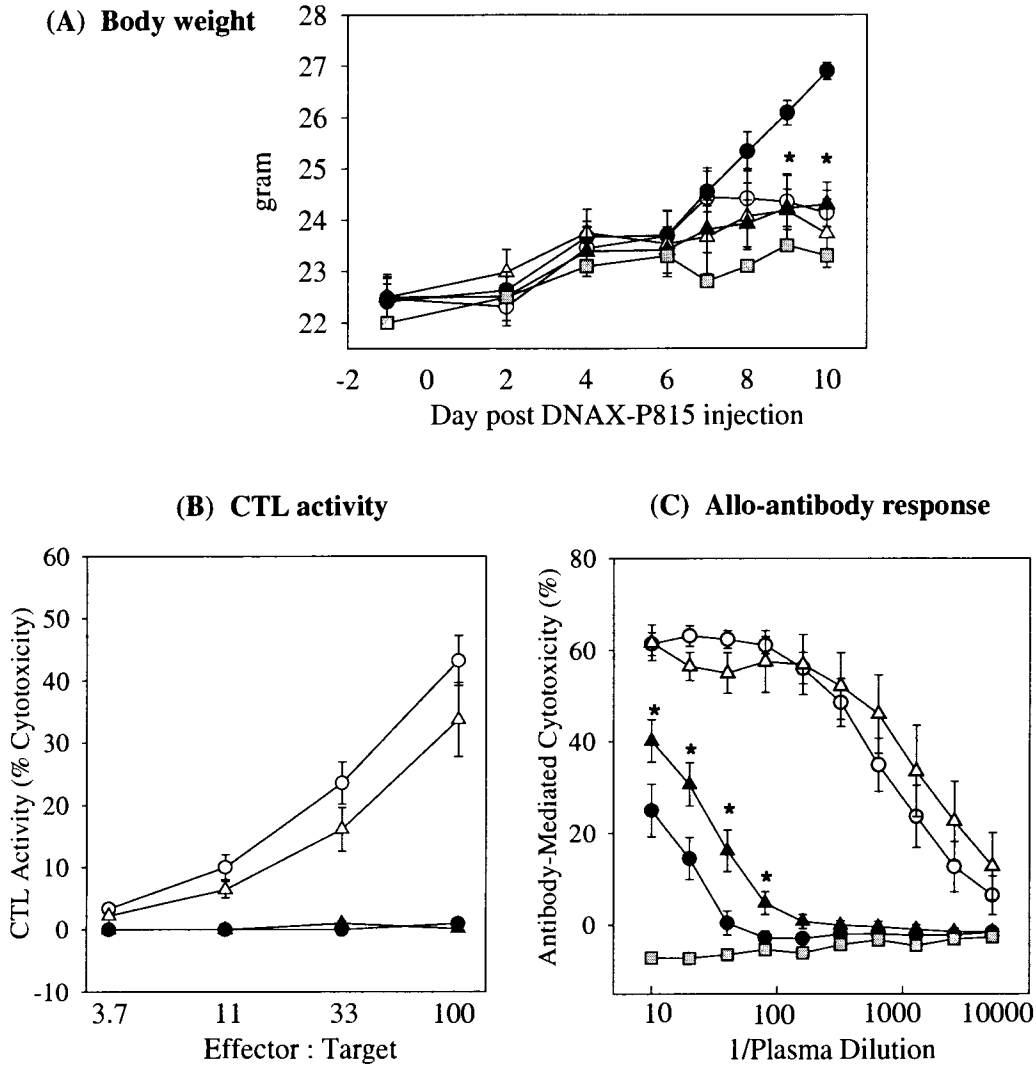


Figure 4-12-4. Effect of Mac-1$^+$Gr-1$^+$ depletion on body weight and CTL development in TCDD-treated mice

Vehicle or TCDD-treated mice were injected with DNAX-P815 cells. On days 4 and 7 after tumor injection, 250 μg RB6-8C5 or Rat IgG were injected ip. Mice were killed by CO_2 asphyxiation at day 10. Blood and spleen samples were prepared for CTL activity and allo-antibody response as described in Materials and Methods and were stained with mAbs against Mac-1 and Gr-1 for flow cytometric analysis to verify efficiency of depletion (< 1% in the spleen and blood). Symbols represent groups: vehicle (O)- or TCDD (●)-treated Rat IgG-injected mice, vehicle (△)- or TCDD (▲)-treated RB6-8C5-injected mice. Non-immune mouse is represented as ■. Data points represent the mean ± SEM of 6-7 mice per group. * represents statistically significant difference (p<0.05) when compared to TCDD-treated Rat IgG-injected mice.

the lower sensitivity for the immune response to SRBC in DBA/2 mice (Ah^{dd}) compared to C57Bl/6 mice (Ah^{bb}; Vecchi *et al.*, 1983) or in congenic C57Bl/6 mice that differ in their Ah locus, Ah^{bb} or Ah^{dd} (Kerkvliet *et al.*, 1990a and b; Silkworth *et al.*, 1993); and 2) the correlation between the immunosuppressive potency of chemicals and their binding affinity for the AhR (Silkworth and Grabstein, 1982; Kerkvliet *et al.*, 1990b).

The recent generation of AhR-deficient ($Ahr^{-/-}$) mice provided the opportunity to assess the role of the AhR on Mac-1^+Gr-1^+ cells in response to allogenic P815 tumor cells (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). We hypothesized that, if the increase in Mac-1^+Gr-1^+ cells was dependent on the presence of the AhR, Mac-1^+Gr-1^+ cells would not increase in $Ahr^{-/-}$ mice.

$Ahr^{-/-}$ and wild type C57Bl/6 mice were treated with vehicle or TCDD one day prior to P815 injection and sacrificed ten days later. Spleen samples were prepared and stained with mAbs against Mac-1, Gr-1, CD8, CD44, and CD62L for flow cytometric analysis. Table 4-13-1 shows spleen cell recoveries and the frequency of Mac-1^+Gr-1^+ cells in the spleens of vehicle- and TCDD-treated $Ahr^{-/-}$ and wild type C57Bl/6 mice.

As expected, in wild type mice, TCDD treatment caused a two-fold increase in splenic Mac-1^+Gr-1^+ cells ten days after P815 cell injection. In contrast, we did not observe any increase in the frequency of these cells in TCDD-treated $Ahr^{-/-}$ mice when compared to vehicle-treated $Ahr^{-/-}$ mice. Moreover, $Ahr^{-/-}$ mice developed CTL_E (Table 4-13-1), as well as CTL activity, and alloantibody responses comparable to those in wild type mice (Data not shown). These results indicate that the absence of the AhR does not affect the ability to generate normal immune response to allogenic P815 tumor cells, and TCDD treatment does not suppress the development of normal CTL_E nor cause an increase in Mac-1^+Gr-1^+ cells in these $Ahr^{-/-}$ mice.

Table 4-13-1. Spleen cell recoveries and Mac-1$^+$Gr-1$^+$ cells from the spleens of vehicle- and TCDD-treated *Ahr*$^{-/-}$ and C57Bl/6 mice ten days after the injection of P815 cells.

	C57Bl/6		*Ahr*$^{-/-}$	
	Vehicle	TCDD	Vehicle	TCDD
Total number of spleen cells[a]	12.9 ± 0.7	5.6 ± 0.9*	13.8 ± 1.2	14.4 ± 0.3
% CTL_E[b]	57.4 ± 2.3	7.7 ± 0.7*	52.4 ± 5.4	57.4 ± 1.3
% Mac-1$^+$Gr-1$^+$	8.6 ± 0.5	17.1 ± 1.9*	9.7 ± 0.6	9.1 ± 0.5

Values represent the mean ± SEM for 3-6 mice per treatment.
* represents statistically significant difference ($p<0.05$) when compared to the corresponding vehicle control.
[a] represents total number of spleen cells recovered from individual animal.
[b] represents the percentage of CD8$^+$ cells expressing the CTL_E phenotype.

CHAPTER 5

DISCUSSION

We have identified and characterized an inflammatory component of the P815 response that is enhanced by TCDD treatment. These studies were prompted by our previous report that Mac-1^+ cells were two to three-fold increased in the spleens of TCDD-treated mice 7-9 days after P815 injection, as compared to vehicle-treated mice (Prell and Kerkvliet, 1997). This increase occurred at the same time that CTL activity failed to increase in P815-injected mice. We speculated that these Mac-1^+ cells represented suppressor cells that might act as an immunoregulatory population by which TCDD suppressed the allo-specific CTL responses. Recently, splenic Mac-1^+Gr-1^+ cells have been reported as an immunoregulatory population by Bronte *et al.* (1998; 1999) demonstrating that Mac-1^+Gr-1^+ cells were induced in the spleen by GM-CSF-producing tumors, and this cell population inhibited antigen-specific CTL function in a direct cell-to-cell contact manner. The goal of these studies was to determine if this inflammatory response contributes to the immune suppressive state in TCDD-treated mice.

Initial studies used antibodies to Gr-1, a marker of granulocyte, to examine if these Mac-1^+ cells were Mac-1^+Gr-1^+ cells observed in the study of Bronte *et al.* Interestingly, the majority of these Mac-1^+ cells in the spleen expressed Gr-1 antigen. Mac-1^+Gr-1^+ cells increased in response to allogeneic P815 tumor cells not only in the spleen but also in the blood. These increases were dramatically enhanced by TCDD, accounting for 25% and 75% cells of total spleen cells and WBC, respectively. This TCDD effect was not observed in Ah receptor-deficient (*Ahr*$^{-/-}$) mice, indicating this enhanced increase in Mac-1^+Gr-1^+ cells is dependent on the presence of AhR.

These initial observations were especially interesting for us. An increase in inflammatory cells is consistent with evidence observed in earlier studies in which

TCDD-induced toxicity was associated with enhanced inflammatory responses (Vos *et al.*, 1973; 1974; Weissberg and Zinkl, 1973; Puhvel and Sakamoto, 1988; Kerkvliet and Oughton, 1993). The enhancement of inflammatory responses by TCDD maybe associated with suppressed immune response. Therefore, we hypothesized that Mac-1^+Gr-1^+ cells reflect the inflammatory response and act as immunomodulatory (immune-inhibitory) cells by which TCDD suppresses the CTL response.

Morphological identification revealed that Mac-1^+Gr-1^+ cells in the blood were neutrophils, whereas cells from the spleen included not only neutrophils but also other cell types having ring-shaped nuclei. These results indicate that TCDD may enhance systemic inflammation in response to P815 tumor cells and also stimulate hematopoiesis in the spleen.

We examined two hematopoietic tissues, the bone marrow and the spleen, in order to determine where these Mac-1^+Gr-1^+ cells originated and if TCDD altered hematopoiesis at the stem cell level. Upon P815 tumor injection, the frequency of Mac-1^+Gr-1^+ cells increased in the bone marrow. Likewise in the spleen, Mac-1^+Gr-1^+ cells consisted of two distinct populations, Gr-1^{hi} and Gr-1^{low}. However, the Gr-1^{low} population was not detected in the blood. In TCDD-treated mice, in the bone marrow, a population expressing Gr-1^{low} was more evident, and the level of Gr-1 expression was reduced in the spleen as well as in the blood. Mac-1^+Gr-1^+ cells in the blood were morphologically identified as neutrophils, whereas Mac-1^+Gr-1^+ cells from the spleen included not only neutrophils but also other cell types having ring-shaped nuclei.

Hestdal *et al.* (1991) found that, in the bone marrow, Gr-1^{high} cells were exclusively neutrophils, while Gr-1^{int} or Gr-1^{low} cells were myelocytes or immature progenitors. Biermann *et al.* (1999) reported that murine leukocytes with ring-shaped nuclei in the bone marrow and peripheral blood included immature granulocytes, monocytes, and the early progeny of hematopoietic stem cells. Considering these reports, our results indicate that Mac-1^+Gr-1^+ cells expressing Gr-1^{low} in TCDD-treated mice may represent myelocytes or immature progenitors and TCDD may

facilitate the production of myelocytes selectively, which resulted in accumulation of immature granulocytes in the bone marrow and spleen.

Megakaryocytes were observed in the spleens of vehicle-treated mice but not in TCDD-treated mice. These results suggest that hematopoiesis occurs in the spleens of mice injected with P815 tumor cells and that Mac-1^+Gr-1^+ cells probably originate from the spleen. Furthermore, it also supports that TCDD selectively stimulates the differentiation of myelocyte lineage cells

In order to provide insight into the function of these Mac-1^+Gr-1^+ cells in immunity, we evaluated their expression of several cell surface molecules important in the generation of antigen-specific immune responses. Although Mac-1^+Gr-1^+ cells from both vehicle- and TCDD-treated mice expressed similar levels of B7-1, B7-2, IA^b and $H2K^b$, expression levels of these molecules were very low. These results suggest that a role of Mac-1^+Gr-1^+ cells as APC is unlikely; rather soluble factors from Mac-1^+Gr-1^+ cells might be more relevant. However, TCDD treatment caused a significant down-regulation in the expression of ICAM-1 and F4/80, as well as very small decreases in the expression of LFA-1 and CD40. Furthermore, one of the most interesting findings from this study was the level of CD62L expression on Mac-1^+Gr-1^+ cells. Although, in the bone marrow, Mac-1^+Gr-1^+ cells constitutively expressed high levels of CD62L in both vehicle- and TCDD-treated mice, CD62L expression was markedly reduced in the spleens of TCDD-treated mice and was completely shed on Mac-1^+Gr-1^+ cells in the blood. Since CD62L is down-regulated upon activation (Kishimoto *et al.,* 1989; Soler-Rodriguez *et al.,* 2000), these results indicate that Mac-1^+Gr-1^+ cells from TCDD-treated mice are functionally different and may be highly activated when compared to cells from vehicle-treated mice.

If Mac-1^+Gr-1^+ cells are activated by TCDD, this might be a pivotal event in TCDD-induced immune suppression. Studies have shown that chronically activated inflammatory cells release oxidative metabolites, leading to tissue damage, and can eventually result in immune hypo-responsiveness (Marcinkiewicz *et al.,* 1999; Catania and Chaudry, 1999). In addition, down-regulation of both CD62L and ICAM-1 may result in an alteration in the ability of Mac-1^+Gr-1^+ cells to migrate to the site of

inflammation by inhibiting initial interaction between these cells and the endothelial cells (von Andrian *et al.,* 1991; Bevilacqua, 1993; Springer, 1994).

Generation of reactive oxygen species is a key feature of activated neutrophils (Bellingan, 1999). However, inappropriate generation of these toxic products also contributes to inflammation-mediated tissue injury in a variety of pathophysiologic conditions (Ward *et al.,* 1988). We measured superoxide anion production by Mac-1^+Gr-1^+ cells as one mechanism by which these cells might suppress *in vitro* CTL development. Interestingly upon stimulation with PMA, generation of superoxide anion was significantly enhanced by TCDD. Splenic Mac-1^+Gr-1^+ cells from TCDD-treated mice produced 5.5-fold higher levels of superoxide, and Mac-1^+Gr-1^+ cells in the blood produced 2.5-fold higher amounts when compared to vehicle-treated mice. These results further indicate that Mac-1^+Gr-1^+ cells from TCDD-treated mice are highly activated and over-production of reactive oxygen species by Mac-1^+Gr-1^+ cells may be associated with TCDD-induced immune suppression.

Next we focused on the function of splenic Mac-1^+Gr-1^+ cells in P815 tumor cell-injected mice. Immunomodulatory functions of these cells were assessed by examining their ability to directly inhibit CTL activity and to interfere with the *in vitro* development of CTL. Mac-1^+Gr-1^+ cells from both vehicle- and TCDD-treated mice did not suppress CTL activity of *in vivo*-activated CTL. However, Mac-1^+Gr-1^+ cells from TCDD-treated mice but not vehicle-treated mice suppressed the generation of allo-reactive CTL in a cell number- and cell contact-dependent manner. These results were consistent with the hypothesis that Mac-1^+Gr-1^+ cells in TCDD-treated mice play a role in suppression of the CTL response.

To directly test the role of the Mac-1^+Gr-1^+ cells *in vivo*, we used an anti Gr-1 specific antibody to deplete these cells during the response to P815 tumor cells.

Interestingly, depletion of Mac-1^+Gr-1^+ cells did not suppress the normal CTL response to P815 tumor cells, suggesting that these cells play no role in the development of the CTL response. Surprisingly depletion of the cells also failed to restore suppressed CTL activity in TCDD-treated mice, indicating that Mac-1^+Gr-1^+ and are not immunomodulatory cells. Immunohistochemistry studies also supported

these unexpected results. We found that Mac-1^+Gr-1^+ cells were localized in the red pulp of the spleen, separated from T cells, suggesting that Mac-1^+Gr-1^+ cells were not in close proximity to affect T cell functions.

However, there was some evidence that depletion of Mac-1^+Gr-1^+ cells protected TCDD-treated mice from P815 tumor-induced increase in body weight associated with tumor ascites. These results suggest that Mac-1^+Gr-1^+ cells have a role in inflammation. The lack of a role for Mac-1^+Gr-1^+ cells in suppression of the CTL response is consistent with a lack of correlation between the magnitude of inflammation and the suppression of the CTL response. During the course of experiments using DNAX-P815 tumor cells, we noticed that the injection of DNAX-P815 tumor cells induced Mac-1^+Gr-1^+ cells in the spleen and blood, but the magnitude of the increase was significantly less when compared to the increase following injection of ascites-P815 tumor cells. Regardless of the magnitude of inflammation however, the CTL response was always suppressed in TCDD-treated mice following the injection of both DNAX-P815 and ascites P815 tumor cells, indicating that Mac-1^+Gr-1^+ cells are not immumomodulatory cells.

It is possible that there were confounding effects from the antibody treatment that prevented the protective effect of depleting Mac-1^+Gr-1^+ in TCDD-treated mice. Previous experiments with a anti-neutrophil monoclonal antibody (1F12), which causes neutropenia in the rat, showed that the majority of neutrophils accumulated in the liver after treatment (Bautista *et al.,* 1994). These neutrophils were functionally inactivated and were phagocytosed by Kupffer cells, which resulted in the priming of Kupffer cells, as demonstrated by enhanced releases of superoxide (Bautista *et al.,* 1994) and TNF (Hewett *et al.,* 1993). Thus in our study, the beneficial effect of eliminating Mac-1^+Gr-1^+ cells by RB6-8C5 might be masked by activating Kupffer cells that then produced significant amounts of proinflammatory cytokines and reactive oxygen metabolites. Although this explanation does not explain why depletion of Mac-1^+Gr-1^+ cells did not alter the normal CTL response, it is possible that TCDD would also enhance the Kupffer cell response.

We also examined other systemic inflammatory components that might act systemically and could contribute to modulate immune response. Because of the essential role of TNF in physiology (Dinarello *et al.,* 1986), not only in stimulating immune responses but also in amplifying inflammatory responses (Baumann and Gauldie, 1994; Joseph *et al.,* 1998; Hart *et al.,* 1998), we hypothesized that TCDD exposure in the presence of inflammatory P815 tumor cells resulted in enhanced TNF production that would ultimately lead to the over-production of SAA. An increase in inflammatory cells could result from the increase in production of TNF and SAA, or alternatively, Mac-1^+Gr-1^+ cells may produce TNF which results in the over-production of SAA.

Disappointedly, we were unable to detect TNF in the plasma and the peritoneal supernatant in both vehicle- and TCDD-treated mice following the injection of P815 tumor cells. Interestingly, an increase in plasma levels of SAA appeared to be associated with an increase in tumor burden in P815 tumor-allografted mice. However, SAA levels were not different in TCDD-treated mice when compared to vehicle-treated mice. In addition, we tested two classical inflammatory stimuli, LPS and SRBC, in order to determine if TCDD enhanced the production of TNF and SAA. As previously, TCDD treatment did not enhance LPS-induced TNF or LPS-induced SAA production and TNF or SAA was not detected in either vehicle or TCDD-treated mice upon SRBC injection. These results suggest that alteration in the production of TNF or SAA is not involved in the increase in inflammatory cells in TCDD-treated mice. TCDD itself at the dose used (15 μg/kg) does not act as a proinflammatory factor.

We also assessed the development of CTL activity in response to P815 tumor cells using TNF-R KO mice. Interestingly, TNF-R KO mice generated a CTL response to P815 tumor cells comparable to wild-type mice. Moreover, TCDD did not suppress CTL activity or the development of CTL_E in KO mice to the same degree as for C57Bl/6 wild type mice. These data indicate that TNF is clearly not required for the development of allogeneic CTL responses, moreover that the absence of TNF

activity might be beneficial, in part, in preventing TCDD-induced suppression in CTL activity.

Regarding TNF, despite a great deal of effort in the SRBC model, we could not find any association between attenuation of the recruitment of inflammatory cells and restoration of suppressed antibody responses in TCDD-treated mice. Blocking TNF activity dramatically reduced TCDD-induced increased recruitment of inflammatory cells in response to SRBC, but it did not restore the suppressed antibody response in TCDD-treated mice (Moos and Kerkvliet, 1995). These reports also indicate that enhanced inflammatory cells were not the mechanism by which TCDD suppresses the antibody response.

Additionally, we obtained an unexpected, but very exciting finding from the bone marrow studies. TCDD treatment caused an alteration in the population of cells expressing CD19, a B cell-specific transmembrane protein and a B cell differentiation antigen, that is expressed from the pro-B cell through the mature B cell stages (Krop *et al.,* 1996). In NI mice, $CD19^+$ cells comprised approximately 50% of the $CD45^+$ leukocytes with two distinct subpopulations of $CD19^+$ cells, $CD19^+/CD45^{low}$ and $CD19^+/CD45^{hi}$. In response to P815 tumor cells, there was a reduction in $CD19^+$ cells in vehicle-treated mice (16.7%) and even fewer in TCDD-treated mice (7.5%). Both $CD19^+$ subpopulations were evident in vehicle-treated mice, but only one, the $CD19^+/CD45^{hi}$ subpopulation, was present in TCDD-treated mice. These results suggest that TCDD might perturb the normal development of B cells that could result in functional immune alterations.

CD19 has been shown to be essential for murine B cell development and for T cell-dependent B cell immune responses (Krop *et al.,* 1996). Other studies suggested that small changes in CD19 expression on pre-B cells as they progress through different stages of maturation may have an impact on their functional responses (Sato *et al.,* 1996; Martensson *et al.,* 1997). In contrast, studies with CD19-deficient mice have suggested that this molecule may not be required for normal generation and maturation of B cells in the bone marrow (Rickert *et al.,* 1995; Sato *et al.,* 1996).

Despite conflicting information, the alteration of CD19 expressing cell populations is interesting and deserving of additional study. A number of studies have suggested several possible mechanisms by which TCDD may suppress humoral immunity, such as perturbed B cell activation and differentiation in the periphery (Tucker *et al.,* 1986; Morris and Holsapple, 1991), or a toxic effect on immature B cells in the bone marrow (Chastain and Pazdernik, 1985). Furthermore, Masten and Shiverick (1995) demonstrated that TCDD reduced CD19 mRNA and that the AhR complex bound to the same DNA binding site as B cell lineage-specific activator protein (BSAP), the transcription factor which mediates CD19 gene transcription. Because CD19 signaling is important for B cell development, they hypothesized that TCDD-mediated down-regulation of CD19 expression may lead to suppressed antibody production. These results, in addition to alteration in $CD19^+$ expression in bone marrow cells by TCDD, might explain, at least partly, the mechanisms by which TCDD suppresses immune responses.

In summary, TCDD was shown to enhance the increase in inflammatory cells in response to allogeneic P815 tumor cells and SRBC. Inflammatory cells induced in response to P815 tumor cells expressed Mac-1 as well as Gr-1, and increased in the spleen, blood as well as bone marrow. Mac-1^+Gr-1^+ cells were identified as neutrophils and these cells from TCDD-treated mice included Gr-1^{low} cells, indicating that TCDD enhances the production of immature granulocytes in the spleen and bone marrow. TCDD treatment caused a down-regulation of expression of a number of surface molecules, suggesting that these cells have altered functions. The complete shedding of CD62L and the significantly enhanced oxidative burst indicated that Mac-1^+Gr-1^+ cells from TCDD-treated mice were highly activated. Furthermore, Mac-1^+Gr-1^+ cells from TCDD-treated mice suppressed the generation of allo-reactive CTL in a cell number- and cell contact-dependent manner, suggesting these cells are immunomodulatory cells. However, depletion of Mac-1^+Gr-1^+ cells did not restore the suppressed CTL response in TCDD-treated mice. Furthermore, immunohistochemical staining of Mac-1 and Gr-1 showed that Mac-1^+Gr-1^+ cells were located in the red pulp, separated from T cells in the spleen, suggesting that interactions between

Mac-1^+Gr-1^+ cells and T cells are unlikely. We conclude from these studies that Mac-1^+Gr-1^+ cells are not an immunomodulatory population in P815-allografted mice. In addition, we found no evidence for the involvement of other systemic inflammatory components in TCDD-induced immune suppression.

BIBLIOGRAPHY

Abraham, K., Krowke, R., and Neubert, D. (1988). Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo- p-dioxin. 1. Dose-dependent tissue distribution and induction of hepatic ethoxyresorufin O-deethylase in rats following a single injection. *Arch.Toxicol.* **62**, 359-368.

Ackermann, M. F., Gasiewicz, T. A., Lamm, K. R., Germolec, D. R., and Luster, M. I. (1989a). Selective inhibition of polymorphonuclear neutrophil activity by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol.Appl.Pharmacol.* **101**, 470-480.

Ackermann, M. F., Lamm, K. R., Wiegand, G. W., and Luster, M. I. (1989b). Antitumor activity of murine neutrophils demonstrated by cytometric analysis. *Cancer Res.* **49**, 528-532.

Aldo-Benson, M. A., and Benson, M. D. (1982). SAA suppression of immune response in vitro: evidence for an effect on T cell-macrophage interaction. *J.Immunol.* **128**, 2390-2392.

Ault, K. A., and Springer, T. A. (1981). Cross-reaction of a rat-anti-mouse phagocyte-specific monoclonal antibody (anti-Mac-1) with human monocytes and natural killer cells. *J.Immunol.* **126**, 359-364.

Ayala, A., Perrin, M. M., and Chaudry, I. H. (1990). Defective macrophage antigen presentation following haemorrhage is associated with the loss of MHC class II (Ia) antigens. *Immunology* **70**, 33-39.

Baumann, H., and Gauldie, J. (1994). The acute phase response. *Immunol.Today* **15**, 74-80.

Bautista, A. P., Spolarics, Z., Jaeschke, H., Smith, C. W., and Spitzer, J. J. (1994). Antineutrophil monoclonal antibody (1F12) alters superoxide anion release by neutrophils and Kupffer cells. *J.Leukoc.Biol.* **55**, 328-335.

Bazzoni, F., and Beutler, B. (1996). The tumor necrosis factor ligand and receptor families. *N.Engl.J.Med.* **334**, 1717-1725.

Bellingan, G. (1999). Inflammatory cell activation in sepsis. *Br.Med.Bull.* **55**, 12-29.

Benson, M. D., and Aldo-Benson, M. (1979). Effect of purified protein SAA on immune response in vitro: mechanisms of suppression. *J.Immunol.* **122**, 2077-2082.

Bevilacqua, M. P. (1993). Endothelial-leukocyte adhesion molecules. *Annu.Rev.Immunol.* **11**, 767-804.

Biermann, H., Pietz, B., Dreier, R., Schmid, K. W., Sorg, C., and Sunderkotter, C. (1999). Murine leukocytes with ring-shaped nuclei include granulocytes, monocytes, and their precursors. *J.Leukoc.Biol.* **65**, 217-231.

Blankenship, A., and Matsumura, F. (1997). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-induced activation of a protein tyrosine kinase, pp60src, in murine hepatic cytosol using a cell-free system. *Mol.Pharmacol.* **52**, 667-675.

Blaylock, B. L., Holladay, S. D., Comment, C. E., Heindel, J. J., and Luster, M. I. (1992). Exposure to tetrachlorodibenzo-p-dioxin (TCDD) alters fetal thymocyte maturation. *Toxicol.Appl.Pharmacol.* **112**, 207-213.

Bronte, V., Wang, M., Overwijk, W. W., Surman, D. R., Pericle, F., Rosenberg, S. A., and Restifo, N. P. (1998). Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. *J.Immunol.* **161**, 5313-5320.

Bronte, V., Chappell, D. B., Apolloni, E., Cabrelle, A., Wang, M., Hwu, P., and Restifo, N. P. (1999). Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. *J.Immunol.* **162**, 5728-5737.

Burleson, G. R., Lebrec, H., Yang, Y. G., Ibanes, J. D., Pennington, K. N., and Birnbaum, L. S. (1996). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on influenza virus host resistance in mice. *Fundam.Appl.Toxicol.* **29**, 40-47.

Burton, J. L., Kehrli, M. E., Jr., Kapil, S., and Horst, R. L. (1995). Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. *J.Leukoc.Biol.* **57**, 317-325.

Cassatella, M. A. (1995). The production of cytokines by polymorphonuclear neutrophils. *Immunol.Today* **16**, 21-26.

Catania, R. A., and Chaudry, I. H. (1999). Immunological consequences of trauma and shock. *Ann.Acad.Med.Singapore* **28**, 120-132.

Chastain, J. E., Jr., and Pazdernik, T. L. (1985). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)-induced immunotoxicity. *Int.J.Immunopharmacol.* **7**, 849-856.

Clark, D. A., Gauldie, J., Szewczuk, M. R., and Sweeney, G. (1981). Enhanced suppressor cell activity as a mechanism of immunosuppression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Proc.Soc.Exp.Biol.Med.* **168**, 290-299.

Clark, D. A., Sweeney, G., Safe, S., Hancock, E., Kilburn, D. G., and Gauldie, J. (1983). Cellular and genetic basis for suppression of cytotoxic T cell generation by haloaromatic hydrocarbons. *Immunopharmacology* **6**, 143-153.

Clark, G. C., Taylor, M. J., Tritscher, A. M., and Lucier, G. W. (1991a). Tumor necrosis factor involvement in 2,3,7,8-tetrachlorodibenzo-p- dioxin-mediated endotoxin hypersensitivity in C57BL/6J mice congenic at the Ah locus. *Toxicol.Appl.Pharmacol.* **111**, 422-431.

Clark, G. C., Blank, J. A., Germolec, D. R., and Luster, M. I. (1991b). 2,3,7,8-Tetrachlorodibenzo-p-dioxin stimulation of tyrosine phosphorylation in B lymphocytes: potential role in immunosuppression. *Mol.Pharmacol.* **39**, 495-501.

Clark, R. A., and Klebanoff, S. J. (1975). Neutrophil-mediated tumor cell cytotoxicity: role of the peroxidase system. *J.Exp.Med.* **141**, 1442-1447.

Coligan, J.E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober. W. (1996). Isolation and functional analysis of neutrophils. *In Current protocols in immunology* p. 7.23.1-7.23.17. John Wiley and Sons, New York.

Cox, G. (1995). Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. *J.Immunol.* **154**, 4719-4725.

Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K., and Mescher, M. F. (1999). Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *J.Immunol.* **162**, 3256-3262.

Czuprynski, C. J., Brown, J. F., Maroushek, N., Wagner, R. D., and Steinberg, H. (1994). Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection. *J.Immunol.* **152**, 1836-1846.

Daffern, P. J., Jagels, M. A., and Hugli, T. E. (1999). Multiple epithelial cell-derived factors enhance neutrophil survival. Regulation by glucocorticoids and tumor necrosis factor-alpha. *Am.J.Respir.Cell Mol.Biol.* **21**, 259-267.

Davis, D., and Safe, S. (1991). Halogenated aryl hydrocarbon-induced suppression of the in vitro plaque- forming cell response to sheep red blood cells is not dependent on the Ah receptor. *Immunopharmacology* **21**, 183-190.

De Krey, G. K., Baecher-Steppan, L., Deyo, J. A., Smith, B., and Kerkvliet, N. I. (1993). Polychlorinated biphenyl-induced immune suppression: castration, but not adrenalectomy or RU 38486 treatment, partially restores the suppressed cytotoxic T lymphocyte response to alloantigen. *J.Pharmacol.Exp.Ther.* **267**, 308-315.

De Krey, G. K., and Kerkvliet, N. I. (1995). Suppression of cytotoxic T lymphocyte activity by 2,3,7,8- tetrachlorodibenzo-p-dioxin occurs in vivo, but not in vitro, and is independent of corticosterone elevation. *Toxicology* **97**, 105-112.

De Vito, M. J., and Birnbaum, L. S. (1994). *In dioxin and health* (A.Schecter, Ed.), pp. 139-162. Plenum Press, NewYork.

Diaz-Gonzalez, F., Gonzalez-Alvaro, I., Campanero, M. R., Mollinedo, F., del Pozo, M. A., Munoz, C., Pivel, J. P., and Sanchez-Madrid, F. (1995). Prevention of in vitro neutrophil-endothelial attachment through shedding of L-selectin by nonsteroidal antiinflammatory drugs. *J.Clin.Invest* **95**, 1756-1765.

Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., Jr., and O'Connor, J. V. (1986). Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J.Exp.Med.* **163**, 1433-1450.

Dooley, R. K., and Holsapple, M. P. (1988). Elucidation of cellular targets responsible for tetrachlorodibenzo-p- dioxin (TCDD)-induced suppression of antibody responses: I. The role of the B lymphocyte. *Immunopharmacology* **16**, 167-180.

Enan, E., and Matsumura, F. (1995). Regulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) of the DNA binding activity of transcriptional factors via nuclear protein phosphorylation in guinea pig adipose tissue. *Biochem.Pharmacol.* **50**, 1199-1206.

Erickson, S. L., de Sauvage, F. J., Kikly, K., Carver-Moore, K., Pitts-Meek, S., Gillett, N., Sheehan, K. C., Schreiber, R. D., Goeddel, D. V., and Moore, M. W. (1994). Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* **372**, 560-563.

Ertel, W., Morrison, M. H., Ayala, A., and Chaudry, I. H. (1991). Insights into the mechanisms of defective antigen presentation after hemorrhage. *Surgery* **110**, 440-445.

Ertel, W., Morrison, M. H., Ayala, A., and Chaudry, I. H. (1993). Modulation of macrophage membrane phospholipids by n-3 polyunsaturated fatty acids increases interleukin 1 release and prevents suppression of cellular immunity following hemorrhagic shock. *Arch.Surg.* **128**, 15-20.

Faith, R. E., Luster, M. I., and Moore, J. A. (1978). Chemical separation of helper cell function and delayed hypersensitivity responses. *Cell Immunol.* **40**, 275-284.

Fan, F., Wierda, D., and Rozman, K. K. (1996). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on humoral and cell- mediated immunity in Sprague-Dawley rats. *Toxicology* **106**, 221-228.

Fearon, D. T., and Austen, K. F. (1980). Current concepts in immunology: the alternative pathway of complement-- a system for host resistance to microbial infection. *N.Engl.J.Med.* **303**, 259-263.

Fearon, D. T., and Locksley, R. M. (1996). The instructive role of innate immunity in the acquired immune response. *Science* **272**, 50-53.

Fernandez-Salguero, P., Pineau, T., Hilbert, D. M., McPhail, T., Lee, S. S., Kimura, S., Nebert, D. W., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1995). Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor [see comments]. *Science* **268**, 722-726.

Fernandez-Salguero, P. M., Hilbert, D. M., Rudikoff, S., Ward, J. M., and Gonzalez, F. J. (1996). Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced toxicity. *Toxicol.Appl.Pharmacol.* **140**, 173-179.

Funseth, E., and Ilback, N. G. (1992). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on blood and spleen natural killer (NK) cell activity in the mouse. *Toxicol.Lett.* **60**, 247-256.

Gaido, K. W., Maness, S. C., Leonard, L. S., and Greenlee, W. F. (1992). 2,3,7,8-Tetrachlorodibenzo-p-dioxin-dependent regulation of transforming growth factors-alpha and -beta 2 expression in a human keratinocyte cell line involves both transcriptional and post- transcriptional control. *J.Biol.Chem.* **267**, 24591-24595.

Gasiewicz, T. A., Geiger, L. E., Rucci, G., and Neal, R. A. (1983). Distribution, excretion, and metabolism of 2,3,7,8-tetrachlorodibenzo-p- dioxin in C57BL/6J, DBA/2J, and B6D2F1/J mice. *Drug Metab Dispos.* **11**, 397-403.

Gasiewicz, T. A., Rucci, G., Henry, E. C., and Baggs, R. B. (1986). Changes in hamster hepatic cytochrome P-450, ethoxycoumarin O- deethylase, and reduced NAD(P): menadione oxidoreductase following treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. Partial dissociation of temporal and dose-response relationships from elicited toxicity. *Biochem.Pharmacol.* **35**, 2737-2742.

Gehrs, B. C., Riddle, M. M., Williams, W. C., and Smialowicz, R. J. (1997). Alterations in the developing immune system of the F344 rat after perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin: II. Effects on the pup and the adult [published erratum appears in Toxicology 1998 Sep 1;130(1):71]. *Toxicology* **122**, 229-240.

Gehrs, B. C., and Smialowicz, R. J. (1999). Persistent suppression of delayed-type hypersensitivity in adult F344 rats after perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology* **134**, 79-88.

Gonzalez, F. J., and Fernandez-Salguero, P. (1998). The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metab Dispos.* **26**, 1194-1198.

Greenlee, W. F., Dold, K. M., Irons, R. D., and Osborne, R. (1985). Evidence for direct action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on thymic epithelium. *Toxicol.Appl.Pharmacol.* **79**, 112-120.

Greig, J. B., Jones, G., Butler, W. H., and Barnes, J. M. (1973). Toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Food Cosmet.Toxicol.* **11**, 585-595.

Gupta, B. N., Vos, J. G., Moore, J. A., Zinkl, J. G., and Bullock, B. C. (1973). Pathologic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals. *Environ.Health Perspect.* **5**, 125-140.

Hanson, C. D., and Smialowicz, R. J. (1994). Evaluation of the effect of low-level 2,3,7,8-tetrachlorodibenzo-p- dioxin exposure on cell mediated immunity. *Toxicology* **88**, 213-224.

Harper, N., Steinberg, M., Thomsen, J., and Safe, S. (1995). Halogenated aromatic hydrocarbon-induced suppression of the plaque- forming cell response in B6C3F1 splenocytes cultured with allogenic mouse serum: Ah receptor structure activity relationships. *Toxicology* **99**, 199-206.

Hart, P. H., Grimbaldeston, M. A., Swift, G. J., Sedgwick, J. D., Korner, H., and Finlay-Jones, J. J. (1998). TNF modulates susceptibility to UVB-induced systemic immunomodulation in mice by effects on dermal mast cell prevalence. *Eur.J.Immunol.* **28**, 2893-2901.

Hauschildt, S., and Kleine, B. (1995). Bacterial stimulators of macrophages. *Int.Rev.Cytol.* **161**, 263-331.

Hestdal, K., Ruscetti, F. W., Ihle, J. N., Jacobsen, S. E., Dubois, C. M., Kopp, W. C., Longo, D. L., and Keller, J. R. (1991). Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. *J.Immunol.* **147**, 22-28.

Hewett, J. A., Jean, P. A., Kunkel, S. L., and Roth, R. A. (1993). Relationship between tumor necrosis factor-alpha and neutrophils in endotoxin-induced liver injury. *Am.J.Physiol* **265**, G1011-G1015.

Holmskov, U., Malhotra, R., Sim, R. B., and Jensenius, J. C. (1994). Collectins: collagenous C-type lectins of the innate immune defense system. *Immunol.Today* **15**, 67-74.

Holsapple, M. P., McCay, J. A., and Barnes, D. W. (1986). Immunosuppression without liver induction by subchronic exposure to 2,7- dichlorodibenzo-p-dioxin in adult female B6C3F1 mice. *Toxicol.Appl.Pharmacol.* **83**, 445-455.

House, R. V., Lauer, L. D., Murray, M. J., Thomas, P. T., Ehrlich, J. P., Burleson, G. R., and Dean, J. H. (1990). Examination of immune parameters and host resistance mechanisms in B6C3F1 mice following adult exposure to 2,3,7,8-tetrachlorodibenzo-p- dioxin. *J.Toxicol.Environ.Health* **31**, 203-215.

Huff, J., Lucier, G., and Tritscher, A. (1994). Carcinogenicity of TCDD: experimental, mechanistic, and epidemiologic evidence. *Annu.Rev.Pharmacol.Toxicol.* **34**, 343-372.

Inoue, T., and Sendo, F. (1983). In vitro induction of cytotoxic polymorphonuclear leukocytes by supernatant from a concanavalin A-stimulated spleen cell culture. *J.Immunol.* **131**, 2508-2514.

Jablonska, E., Jablonski, J., and Holownia, A. (1999). Role of neutrophils in release of some cytokines and their soluble receptors. *Immunol.Lett.* **70**, 191-197.

Jacob, C. O. (1992). Tumor necrosis factor alpha in autoimmunity: pretty girl or old witch? *Immunol.Today* **13**, 122-125.

Jaovisidha, P., Peeples, M. E., Brees, A. A., Carpenter, L. R., and Moy, J. N. (1999). Respiratory syncytial virus stimulates neutrophil degranulation and chemokine release. *J.Immunol.* **163**, 2816-2820.

Jones, G., and Butler, W. H. (1974). A morphological study of the liver lesion induced by 2,3,7,8- tetrachlorodibenzo-p-dioxin in rats. *J.Pathol.* **112**, 93-97.

Jones, G., and Greig, J. B. (1975). Pathological changes in the liver of mice given 2,3,7,8- tetrachlorodibenzo-p-dioxin. *Experientia* **31**, 1315-1317.

Joseph, S. B., Miner, K. T., and Croft, M. (1998). Augmentation of naive, Th1 and Th2 effector CD4 responses by IL-6, IL-1 and TNF. *Eur.J.Immunol.* **28**, 277-289.

Kaminski, N. E., and Holsapple, M. P. (1987). Inhibition of macrophage accessory cell function in casein-treated B6C3F1 mice. *J.Immunol.* **139**, 1804-1810.

Kaminski, N. E., and Holsapple, M. P. (1992). A functional characterization of macrophage alterations in casein- treated B6C3F1 mice. *Immunopharmacology* **24**, 229-240.

Kantor, A. B., Stall, A. M., Adams, S., Herzenberg, L. A., and Herzenberg, L. A. (1992). Differential development of progenitor activity for three B-cell lineages. *Proc.Natl.Acad.Sci.U.S.A* **89**, 3320-3324.

Katz, L. B., Theobald, H. M., Bookstaff, R. C., and Peterson, R. E. (1984). Characterization of the enhanced paw edema response to carrageenan and dextran in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated rats. *J.Pharmacol.Exp.Ther.* **230**, 670-677.

Kelly, C. J., Gallagher, H., Wolf, B. A., and Daly, J. M. (1994). Alterations in macrophage signal transduction pathways mediate post- traumatic changes in macrophage function. *J.Surg.Res.* **57**, 221-226.

Kerkvliet, N. I., Brauner, J. A., and Matlock, J. P. (1985). Humoral immunotoxicity of polychlorinated diphenyl ethers, phenoxyphenols, dioxins and furans present as contaminants of technical grade pentachlorophenol. *Toxicology* **36**, 307-324.

Kerkvliet, N. I., Steppan, L. B., Brauner, J. A., Deyo, J. A., Henderson, M. C., Tomar, R. S., and Buhler, D. R. (1990a). Influence of the Ah locus on the humoral immunotoxicity of 2,3,7,8- tetrachlorodibenzo-p-dioxin: evidence for Ah-receptor-dependent and Ah- receptor-independent mechanisms of immunosuppression. *Toxicol.Appl.Pharmacol.* **105**, 26-36.

Kerkvliet, N. I., Baecher-Steppan, L., Smith, B. B., Youngberg, J. A., Henderson, M. C., and Buhler, D. R. (1990b). Role of the Ah locus in suppression of cytotoxic T lymphocyte activity by halogenated aromatic hydrocarbons (PCBs and TCDD): structure- activity relationships and effects in C57Bl/6 mice congenic at the Ah locus. *Fundam.Appl.Toxicol.* **14**, 532-541.

Kerkvliet, N. I., and Brauner, J. A. (1990). Flow cytometric analysis of lymphocyte subpopulations in the spleen and thymus of mice exposed to an acute immunosuppressive dose of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD). *Environ.Res.* **52**, 146-154.

Kerkvliet, N. I., and Oughton, J. A. (1993). Acute inflammatory response to sheep red blood cell challenge in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD): phenotypic and functional analysis of peritoneal exudate cells. *Toxicol.Appl.Pharmacol.* **119**, 248-257.

Kerkvliet, N. I., Baecher-Steppan, L., Shepherd, D. M., Oughton, J. A., Vorderstrasse, B. A., and DeKrey, G. K. (1996). Inhibition of TC-1 cytokine production, effector cytotoxic T lymphocyte development and alloantibody production by 2,3,7,8-tetrachlorodibenzo-p- dioxin. *J.Immunol.* **157**, 2310-2319.

Kishimoto, T. K., Jutila, M. A., Berg, E. L., and Butcher, E. C. (1989). Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. *Science* **245**, 1238-1241.

Kondo, S., Wang, B., Fujisawa, H., Shivji, G. M., Echtenacher, B., Mak, T. W., and Sauder, D. N. (1995). Effect of gene-targeted mutation in TNF receptor (p55) on contact hypersensitivity and ultraviolet B-induced immunosuppression. *J.Immunol.* **155**, 3801-3805.

Korte, M., Stahlmann, R., Kubicka-Muranyi, M., Gleichmann, E., and Neubert, D. (1991). Polyhalogenated dibenzo-p-dioxins and dibenzofurans and the immune system. 3. No immunosuppressive effect of 2,3,7,8-TCDD in the popliteal lymph node assay (PLNA) in rats. *Arch.Toxicol.* **65**, 656-660.

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546-549.

Krop, I., Shaffer, A. L., Fearon, D. T., and Schlissel, M. S. (1996). The signaling activity of murine CD19 is regulated during cell development. *J.Immunol.* **157**, 48-56.

Kurimoto, I., and Streilein, J. W. (1992). Deleterious effects of cis-urocanic acid and UVB radiation on Langerhans cells and on induction of contact hypersensitivity are mediated by tumor necrosis factor-alpha. *J.Invest Dermatol.* **99**, 69S-70S.

Kurt-Jones, E. A., Virgin, H. W., and Unanue, E. R. (1986). In vivo and in vitro expression of macrophage membrane interleukin 1 in response to soluble and particulate stimuli. *J.Immunol.* **137**, 10-14.

Lahvis, G. P., and Bradfield, C. A. (1998). Ahr null alleles: distinctive or different? *Biochem.Pharmacol.* **56**, 781-787.

Lai, Z. W., Pineau, T., and Esser, C. (1996). Identification of dioxin-responsive elements (DREs) in the 5' regions of putative dioxin-inducible genes. *Chem.Biol.Interact.* **100**, 97-112.

Lakshmanan, M. R., Campbell, B. S., Chirtel, S. J., Ekarohita, N., and Ezekiel, M. (1986). Studies on the mechanism of absorption and distribution of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. *J.Pharmacol.Exp.Ther.* **239**, 673-677.

Lampson, G. P., Tytell, A. A., Field, A. K., Nemes, M. M., and Hilleman, M. R. (1967). Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of Penicillium funiculosum. *Proc.Natl.Acad.Sci.U.S.A* **58**, 782-789.

Lang, D. S., Becker, S., Devlin, R. B., and Koren, H. S. (1998). Cell-specific differences in the susceptibility of potential cellular targets of human origin derived from blood and lung following treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Cell Biol.Toxicol.* **14**, 23-38.

Lawrence, B. P., Leid, M., and Kerkvliet, N. I. (1996). Distribution and behavior of the Ah receptor in murine T lymphocytes. *Toxicol.Appl.Pharmacol.* **138**, 275-284.

Lichtenstein, A. (1986). Spontaneous tumor cytolysis mediated by inflammatory neutrophils: dependence upon divalent cations and reduced oxygen intermediates. *Blood* **67**, 657-665.

Lichtenstein, A. K., Kahle, J., Berek, J., and Zighelboim, J. (1984a). Successful immunotherapy with intraperitoneal Corynebacterium parvum in a murine ovarian cancer model is associated with the recruitment of tumor-lytic neutrophils into the peritoneal cavity. *J.Immunol.* **133**, 519-526.

Lichtenstein, A. K., Berek, J., Kahle, J., and Zighelboim, J. (1984b). Role of inflammatory neutrophils in antitumor effects induced by intraperitoneal administration of Corynebacterium parvum in mice. *Cancer Res.* **44**, 5118-5123.

Lillard, J. W., Jr., Boyaka, P. N., Chertov, O., Oppenheim, J. J., and McGhee, J. R. (1999). Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc.Natl.Acad.Sci.U.S.A* **96**, 651-656.

Liu, M. A., Friedman, A., Oliff, A. I., Tai, J., Martinez, D., Deck, R. R., Shieh, J. T., Jenkins, T. D., Donnelly, J. J., and Hawe, L. A. (1992). A vaccine carrier derived

from Neisseria meningitidis with mitogenic activity for lymphocytes. *Proc.Natl.Acad.Sci.U.S.A* **89**, 4633-4637.

Luebke, R. W., Copeland, C. B., Diliberto, J. J., Akubue, P. I., Andrews, D. L., Riddle, M. M., Williams, W. C., and Birnbaum, L. S. (1994). Assessment of host resistance to Trichinella spiralis in mice following preinfection exposure to 2,3,7,8-TCDD. *Toxicol.Appl.Pharmacol.* **125**, 7-16.

Luebke, R. W., Copeland, C. B., and Andrews, D. L. (1995). Host resistance to Trichinella spiralis infection in rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). *Fundam.Appl.Toxicol.* **24**, 285-289.

Lundberg, K., Gronvik, K. O., and Dencker, L. (1991). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) induced suppression of the local immune response. *Int.J.Immunopharmacol.* **13**, 357-368.

Luster, M. I., Boorman, G. A., Dean, J. H., Harris, M. W., Luebke, R. W., Padarathsingh, M. L., and Moore, J. A. (1980). Examination of bone marrow, immunologic parameters and host susceptibility following pre- and postnatal exposure to 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD). *Int.J.Immunopharmacol.* **2**, 301-310.

Luster, M. I., Hong, L. H., Boorman, G. A., Clark, G., Hayes, H. T., Greenlee, W. F., Dold, K., and Tucker, A. N. (1985). Acute myelotoxic responses in mice exposed to 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD). *Toxicol.Appl.Pharmacol.* **81**, 156-165.

Mantovani, A., Vecchi, A., Luini, W., Sironi, M., Candiani, G. P., Spreafico, F., and Garattini, S. (1980). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on macrophage and natural killer cell-mediated cytotoxicity in mice. *Biomedicine.* **32**, 200-204.

Marcinkiewicz, J., Nowak, B., Grabowska, A., Bobek, M., Petrovska, L., and Chain, B. (1999). Regulation of murine dendritic cell functions in vitro by taurine chloramine, a major product of the neutrophil myeloperoxidase-halide system. *Immunology* **98**, 371-378.

Marinovich, M., Sirtori, C. R., Galli, C. L., and Paoletti, R. (1983). The binding of 2,3,7,8-tetrachlorodibenzodioxin to plasma lipoproteins may delay toxicity in experimental hyperlipidemia. *Chem.Biol.Interact.* **45**, 393-399.

Martensson, I. L., Melchers, F., and Winkler, T. H. (1997). A transgenic marker for mouse B lymphoid precursors. *J.Exp.Med.* **185**, 653-661.

Masten, S. A., and Shiverick, K. T. (1995). The Ah receptor recognizes DNA binding sites for the B cell transcription factor, BSAP: a possible mechanism for dioxin-mediated alteration of CD19 gene expression in human B lymphocytes. *Biochem.Biophys.Res.Commun.* **212**, 27-34.

Masuda, Y. (1994). The Yusho rice oil poisoning incident. *In Dioxins and health* (A. Schecter, Ed.), pp. 633-659. Plenum, NewYork.

Medzhitov, R., and Janeway, C. A., Jr. (1997). Innate immunity: impact on the adaptive immune response. *Curr.Opin.Immunol.* **9**, 4-9.

Miller, M. W., Shearer, G. M., and McAdam, K. P. (1980). Suppression of T cell-mediated cytotoxic responses by spleen cells from mice rendered pre-amyloid by a single injection of complete Freund's adjuvant and Mycobacterium butyricum. *J.Immunol.* **125**, 2559-2564.

Moodycliffe, A. M., Kimber, I., and Norval, M. (1994). Role of tumour necrosis factor-alpha in ultraviolet B light-induced dendritic cell migration and suppression of contact hypersensitivity. *Immunology* **81**, 79-84.

Moos, A. B., Baecher-Steppan, L., and Kerkvliet, N. I. (1994). Acute inflammatory response to sheep red blood cells in mice treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin: the role of proinflammatory cytokines, IL-1 and TNF. *Toxicol.Appl.Pharmacol.* **127**, 331-335.

Moos, A. B., and Kerkvliet, N. I. (1995). Inhibition of tumor necrosis factor activity fails to restore 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD)-induced suppression of the antibody response to sheep red blood cells. *Toxicol.Lett.* **81**, 175-181.

Moos, A. B., Oughton, J. A., and Kerkvliet, N. I. (1997). The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on tumor necrosis factor (TNF) production by peritoneal cells. *Toxicol.Lett.* **90**, 145-153.

Morikawa, K., Kamegaya, S., Yamazaki, M., and Mizuno, D. (1985). Hydrogen peroxide as a tumoricidal mediator of murine polymorphonuclear leukocytes

induced by a linear beta-1,3-D-glucan and some other immunomodulators. *Cancer Res.* **45**, 3482-3486.

Morris, D. L., and Holsapple, M. P. (1991). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on humoral immunity: II. B cell activation. *Immunopharmacology* **21**, 171-181.

Morris, D. L., Jeong, H. G., Jordan, S. D., Kaminski, N. E., and Holsapple, M. P. (1998). Characterization of the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in B6C3F1 and DBA/2 mice following single and repeated exposures. *Arch.Toxicol.* **72**, 157-168.

Moshage, H. (1997). Cytokines and the hepatic acute phase response. *J.Pathol.* **181**, 257-266.

Murphy, P. M. (1994). The molecular biology of leukocyte chemoattractant receptors. *Annu.Rev.Immunol.* **12**, 593-633.

Nakagawa, M., Bondy, G. P., Waisman, D., Minshall, D., Hogg, J. C., and van Eeden, S. F. (1999). The effect of glucocorticoids on the expression of L-selectin on polymorphonuclear leukocyte. *Blood* **93**, 2730-2737.

Nebert, D. W., Robinson, J. R., Niwa, A., Kumaki, K., and Poland, A. P. (1975). Genetic expression of aryl hydrocarbon hydroxylase activity in the mouse. *J.Cell Physiol* **85**, 393-414.

Olson, J. R., Gasiewicz, T. A., and Neal, R. A. (1980). Tissue distribution, excretion, and metabolism of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) in the Golden Syrian hamster. *Toxicol.Appl.Pharmacol.* **56**, 78-85.

Oughton, J. A., and Kerkvliet, N. I. (1999). Novel phenotype associated with in vivo activated CTL precursors. *Clin.Immunol.* **90**, 323-333.

Panja, A., Goldberg, S., Eckmann, L., Krishen, P., and Mayer, L. (1998). The regulation and functional consequence of proinflammatory cytokine binding on human intestinal epithelial cells. *J.Immunol.* **161**, 3675-3684.

Pape, K. A., Khoruts, A., Mondino, A., and Jenkins, M. K. (1997). Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells. *J.Immunol.* **159**, 591-598.

Petty, H. R., and Todd, R. F., III (1996). Integrins as promiscuous signal transduction devices. *Immunol.Today* **17**, 209-212.

Pfeffer, K., Matsuyama, T., Kundig, T. M., Wakeham, A., Kishihara, K., Shahinian, A., Wiegmann, K., Ohashi, P. S., Kronke, M., and Mak, T. W. (1993). Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. *Cell* **73**, 457-467.

Piper, W. N., Rose, J. Q., and Gehring, P. J. (1973). Excretion and tissue distribution of 2,3,7,8-tetrachlorodibenzo-p- dioxin in the rat. *Environ.Health Perspect.* **5**, 241-244.

Pohjanvirta, R., Kulju, T., Morselt, A. F., Tuominen, R., Juvonen, R., Rozman, K., Mannisto, P., Collan, Y., Sainio, E. L., and Tuomisto, J. (1989). Target tissue morphology and serum biochemistry following 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) exposure in a TCDD-susceptible and a TCDD-resistant rat strain. *Fundam.Appl.Toxicol.* **12** , 698-712.

Pohjanvirta, R., and Tuomisto, J. (1994). Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in laboratory animals: effects, mechanisms, and animal models. *Pharmacol.Rev.* **46**, 483-549.

Prell, R. A., and Kerkvliet, N. I. (1997). Involvement of altered B7 expression in dioxin immunotoxicity: B7 transfection restores the CTL but not the autoantibody response to the P815 mastocytoma. *J.Immunol.* **158**, 2695-2703.

Puga, A., Nebert, D. W., and Carrier, F. (1992). Dioxin induces expression of c-fos and c-jun proto-oncogenes and a large increase in transcription factor AP-1. *DNA Cell Biol.* **11** , 269-281.

Puhvel, S. M., and Sakamoto, M. (1988). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on murine skin. *J.Invest Dermatol.* **90**, 354-358.

Rickert, R. C., Rajewsky, K., and Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature* **376**, 352-355.

Rivas, J. M., and Ullrich, S. E. (1994). The role of IL-4, IL-10, and TNF-alpha in the immune suppression induced by ultraviolet radiation. *J.Leukoc.Biol.* **56**, 769-775.

Rosenthal, G. J., Lebetkin, E., Thigpen, J. E., Wilson, R., Tucker, A. N., and Luster, M. I. (1989). Characteristics of 2,3,7,8-tetrachlorodibenzo-p-dioxin induced endotoxin hypersensitivity: association with hepatotoxicity. *Toxicology* **56**, 239-251.

Rothe, J., Mackay, F., Bluethmann, H., Zinkernagel, R., and Lesslauer, W. (1994). Phenotypic analysis of TNFR1-deficient mice and characterization of TNFR1-deficient fibroblasts in vitro. *Circ.Shock* **44**, 51-56.

Rouzer, C. A., and Cerami, A. (1980). Hypertriglyceridemia associated with Trypanosoma brucei brucei infection in rabbits: role of defective triglyceride removal. *Mol.Biochem.Parasitol.* **2**, 31-38.

Safe, S. (1990). Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *Crit Rev.Toxicol.* **21**, 51-88.

Sato, S., Ono, N., Steeber, D. A., Pisetsky, D. S., and Tedder, T. F. (1996). CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. *J.Immunol.* **157**, 4371-4378.

Schmidt, J. V., Su, G. H., Reddy, J. K., Simon, M. C., and Bradfield, C. A. (1996). Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc.Natl.Acad.Sci.U.S.A* **93**, 6731-6736.

Schmidt, J. V., and Bradfield, C. A. (1996). Ah receptor signaling pathways. *Annu.Rev.Cell Dev.Biol.* **12**, 55-89.

Sen, G. C., and Lengyel, P. (1992). The interferon system. A bird's eye view of its biochemistry. *J.Biol.Chem.* **267**, 5017-5020.

Shen, E. S., Gutman, S. I., and Olson, J. R. (1991). Comparison of 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated hepatotoxicity in C57BL/6J and DBA/2J mice. *J.Toxicol.Environ.Health* **32**, 367-381.

Shimizu, Y., Newman, W., Tanaka, Y., and Shaw, S. (1992). Lymphocyte interactions with endothelial cells. *Immunol.Today* **13**, 106-112.

Silkworth, J. B., and Grabstein, E. M. (1982). Polychlorinated biphenyl immunotoxicity: dependence on isomer planarity and the Ah gene complex. *Toxicol.Appl.Pharmacol.* **65**, 109-115.

Silkworth, J. B., Cutler, D. S., O'Keefe, P. W., and Lipinskas, T. (1993). Potentiation and antagonism of 2,3,7,8-tetrachlorodibenzo-p-dioxin effects in a complex environmental mixture. *Toxicol.Appl.Pharmacol.* **119**, 236-247.

Smialowicz, R. J., Riddle, M. M., Williams, W. C., and Diliberto, J. J. (1994). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on humoral immunity and lymphocyte subpopulations: differences between mice and rats. *Toxicol.Appl.Pharmacol.* **124**, 248-256.

Smialowicz, R. J., Williams, W. C., and Riddle, M. M. (1996). Comparison of the T cell-independent antibody response of mice and rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Fundam.Appl.Toxicol.* **32**, 293-297.

Snick, v. J. (1990). Interleukin-6: an overview. *Annu.Rev.Immunol.* **8**, 253-278.

Soler-Rodriguez, A. M., Zhang, H., Lichenstein, H. S., Qureshi, N., Niesel, D. W., Crowe, S. E., Peterson, J. W., and Klimpel, G. R. (2000). Neutrophil activation by bacterial lipoprotein versus lipopolysaccharide: differential requirements for serum and CD14. *J.Immunol.* **164**, 2674-2683.

Springer, T., Galfre, G., Secher, D. S., and Milstein, C. (1979). Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur.J.Immunol.* **9**, 301-306.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* **76**, 301-314.

Stahl, P. D. (1992). The mannose receptor and other macrophage lectins. *Curr.Opin.Immunol.* **4**, 49-52.

Steel, D. M., and Whitehead, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol.Today* **15**, 81-88.

Steppan, L. B., and Kerkvliet, N. I. (1991). Influence of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the production of inflammatory cytokine mRNA by C57Bl/6 macrophages. *Toxicologist* **11**, 35 (abstract).

Sutter, T. R., Guzman, K., Dold, K. M., and Greenlee, W. F. (1991). Targets for dioxin: genes for plasminogen activator inhibitor-2 and interleukin-1 beta. *Science* **254**, 415-418.

Taylor, M. J., Lucier, G. W., Mahler, J. F., Thompson, M., Lockhart, A. C., and Clark, G. C. (1992). Inhibition of acute TCDD toxicity by treatment with anti-tumor necrosis factor antibody or dexamethasone. *Toxicol.Appl.Pharmacol.* **117**, 126-132.

Theobald, H. M., Moore, R. W., Katz, L. B., Pieper, R. O., and Peterson, R. E. (1983). Enhancement of carrageenan and dextran-induced edemas by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *J.Pharmacol.Exp.Ther.* **225**, 576-583.

Theobald, H. M., and Peterson R.E. (1994). Developmental and reproductive toxicity of dioxins and other Ah receptor agonists. *In Dioxins and health* (A. Schecter, Ed.), pp. 309-346. Plenum, NewYork.

Thigpen, J. E., Faith, R. E., McConnell, E. E., and Moore, J. A. (1975). Increased susceptibility to bacterial infection as a sequela of exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Infect.Immun.* **12**, 1319-1324.

Thomas, P. T., and Hinsdill, R. D. (1979). The effect of perinatal exposure to tetrachlorodibenzo-p-dioxin on the immune response of young mice. *Drug Chem.Toxicol.* **2**, 77-98.

Thurmond, T. S., Silverstone, A. E., Baggs, R. B., Quimby, F. W., Staples, J. E., and Gasiewicz, T. A. (1999). A chimeric aryl hydrocarbon receptor knockout mouse

model indicates that aryl hydrocarbon receptor activation in hematopoietic cells contributes to the hepatic lesions induced by 2,3,7, 8- tetrachlorodibenzo-p-dioxin. *Toxicol.Appl.Pharmacol.* **158**, 33-40.

Tracey, K. J., Lowry, S. F., Beutler, B., Cerami, A., Albert, J. D., and Shires, G. T. (1986). Cachectin/tumor necrosis factor mediates changes of skeletal muscle plasma membrane potential. *J.Exp.Med.* **164**, 1368-1373.

Tucker, A. N., Vore, S. J., and Luster, M. I. (1986). Suppression of B cell differentiation by 2,3,7,8-tetrachlorodibenzo-p- dioxin. *Mol.Pharmacol.* **29**, 372-377.

Ulevitch, R. J., and Tobias, P. S. (1995). Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu.Rev.Immunol.* **13**, 437-457.

van Eeden, S. F., Bicknell, S., Walker, B. A., and Hogg, J. C. (1997). Polymorphonuclear leukocytes L-selectin expression decreases as they age in circulation. *Am.J.Physiol* **272**, H401-H408.

Vassalli, P. (1992). The pathophysiology of tumor necrosis factors. *Annu.Rev.Immunol.* **10**, 411-452.

Vecchi, A., Mantovani, A., Sironi, M., Luini, W., Cairo, M., and Garattini, S. (1980). Effect of acute exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin on humoral antibody production in mice. *Chem.Biol.Interact.* **30**, 337-342.

Vecchi, A., Sironi, M., Canegrati, M. A., Recchia, M., and Garattini, S. (1983). Immunosuppressive effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in strains of mice with different susceptibility to induction of aryl hydrocarbon hydroxylase. *Toxicol.Appl.Pharmacol.* **68**, 434-441.

von Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E., and Butcher, E. C. (1991). Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. *Proc.Natl.Acad.Sci.U.S.A* **88**, 7538-7542.

Vos, J. G., Moore, J. A., and Zinkl, J. G. (1973). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the immune system of laboratory animals. *Environ.Health Perspect.* **5**, 149-162.

Vos, J. G., Moore, J. A., and Zinkl, J. G. (1974). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in C57B1/6 mice. *Toxicol.Appl.Pharmacol.* **29**, 229-241.

Vos, J. G., and Moore, J. A. (1974). Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Int.Arch.Allergy Appl.Immunol.* **47**, 777-794.

Vos, J. G., Kreeftenberg, J. G., Engel, H. W., Minderhoud, A., and Van Noorle, J. L. (1978). Studies on 2,3,7,8-tetrachlorodibenzo-p-dioxin induced immune suppression and decreased resistance to infection: endotoxin hypersensitivity, serum zinc concentrations and effect of thymosin treatment. *Toxicology* **9**, 75-86.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., and Shortman, K. (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J.Exp.Med.* **176**, 47-58.

Walker, C., Kristensen, F., Bettens, F., and deWeck, A. L. (1983). Lymphokine regulation of activated (G1) lymphocytes. I. Prostaglandin E2-induced inhibition of interleukin 2 production. *J.Immunol.* **130**, 1770-1773.

Wang, B., Kondo, S., Shivji, G. M., Fujisawa, H., Mak, T. W., and Sauder, D. N. (1996). Tumour necrosis factor receptor II (p75) signalling is required for the migration of Langerhans' cells. *Immunology* **88**, 284-288.

Ward, P. A., Warren, J. S., and Johnson, K. J. (1988). Oxygen radicals, inflammation, and tissue injury. *Free Radic.Biol.Med.* **5**, 403-408.

Watson, G. A., and Lopez, D. M. (1995). Aberrant antigen presentation by macrophages from tumor-bearing mice is involved in the down-regulation of their T cell responses. *J.Immunol.* **155**, 3124-3134.

Webster, T., and Commoner, B. (1994). Overview, the dioxin debate. *In Dioxins and Health* (A. Schecter, Ed.), pp. 1-50. Plenum, NewYork.

Weissberg, J. B., and Zinkl, J. G. (1973). Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin upon hemostasis and hematologic function in the rat. *Environ.Health Perspect.* **5**, 119-123.

White, K. L., Jr., Lysy, H. H., McCay, J. A., and Anderson, A. C. (1986). Modulation of serum complement levels following exposure to polychlorinated dibenzo-p-dioxins. *Toxicol.Appl.Pharmacol.* **84**, 209-219.

Wilson, C. L., and Safe, S. (1998). Mechanisms of ligand-induced aryl hydrocarbon receptor-mediated biochemical and toxic responses. *Toxicol.Pathol.* **26**, 657-671.

Yang, Y. G., Lebrec, H., and Burleson, G. R. (1994). Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on pulmonary influenza virus titer and natural killer (NK) activity in rats. *Fundam.Appl.Toxicol.* **23**, 125-131.

Yin, H., Li, Y., and Sutter, T. R. (1994). Dioxin-enhanced expression of interleukin-1 beta in human epidermal keratinocytes: potential role in the modulation of immune and inflammatory responses. *Exp.Clin.Immunogenet.* **11**, 128-135.

Zu, Y. L., Qi, J., Gilchrist, A., Fernandez, G. A., Vazquez-Abad, D., Kreutzer, D. L., Huang, C. K., and Sha'afi, R. I. (1998). p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. *J.Immunol.* **160**, 1982-1989.

APPENDIX

TCDD-induced immunotoxicity; review of literature

1) Mice

Immunity	Strain (sex)	Dose (μg/kg)	Route	Antigen	Measurement	Responses	Reference
Humoral immunity	B6C3F1 (F)	4.2-42.0, single 0.3-3.0, everyday 14x	Oral	SRBC	PFC	↓ (single<repeated) dose-dependent	Morris *et al.*, 1998
	B6C3F1 (F)	1.0-30.0, single, 7 days prior to antigen	ip	TNP-LPS	PFC	↓ at dose of 10 & 30	Smialowicz *et al.*, 1996
	B6C3F1 (F)	0.58-7.0, single	ip	TNP-LPS	PFC	↓(dose-dependent)	Harper *et al.*, 1995
	C57Bl/6J (F)	0.24-7.2, single	ip	SRBC	PFC	No Δ	Hanson and Smialowicz, 1994
		0.01-3.0, weekly 4x	ip	SRBC	PFC	↓	
	C57Bl/6 (M)	50.0, single	ip	OVA+CFA	ELISPOT/ Anti-OVA Ab in serum	↓	Lundberg *et al.*,1991
	C57Bl/6J (F & M)	1.0-20.0, single	Oral	SRBC TNP-LPS	HAIR	↓ ↓	Kerkvliet *et al.*, 1990a
	B6C3F1 (F)	0.1-10.0, single	ip	Viral hemagglutinin	PFC	↓	House *et al.*, 1990
	B6C3F1 (F)	1.0, everyday 14x	Oral	SRBC DNP-Ficoll	IgM AFC	↓ ↓	Holsapple *et al.*, 1986
		1.0 or 2.0, single	Oral	SRBC	IgM AFC	↓	
	C57Bl/6, DBA2, C3H/HCN,B6C3F1, B6D2F1	1.2-30.0, single or weekly 5x or 8x	ip	SRBC	PFC	↓ (different susceptibility depending of AhR locus)	Vecchi *et al.*, 1983

	C57Bl/6J (M)	0.4-40.0, weekly 4x	ip	SRBC	PFC	↓	Clark *et al.*, 1981
	C57Bl/6J (M) & B6D2F1 (M)	1.0-30.0, single	ip	SRBC	PFC	↓ (dose-dependent)	Vecchi *et al.*, 1980
				TypeIII *Pneumococcal polisaccharide*	PFC	↓(dose-dependent)	
	Swiss webster (F)	1-20 ppb TCDD-containing chow (from 4wks before gestation until 3wks after giving birth)	pre/postnatal	SRBC	PFC	↓	Thomas and Hinsdill, 1979
Cell-mediated immunity	C57Bl/6J (F)	0.24-7.2, single	ip	P815	CTL	No Δ	Hanson and Smialowicz, 1994
		0.01-3.0, weekly 4x	ip	P815	CTL	No Δ	
	C57Bl/6 (M)	50.0, single	ip	OVA+CFA	DTH	↓	Lundberg *et at.*,1991
	C57Bl/6J (M) Ah^{bb}/Ab^{d}_{d}	10.0 or 20.0, single	Oral	P815	CTL	↓ (Ah locus dependent)	Kerkvliet *et al.*,1990b
	C57Bl/6, DBA2, C3H/HCN,B6C3F1, B6D2F1	1.2-30.0, single or weekly 5x or 8x	ip	Splenocytes from C57Bl/6 or DBA2 mice	GVH, popliteal lymph node weight gain assay	↓ (different susceptibility depending on AhR locus)	Vecchi *et al.*, 1983
	C57Bl/6J (M)	0.4-40.0, weekly 4x	ip	Oxazalone	DTH	↓ at 40 μg/kg dose	Clark *et al.*, 1981
				P815	CTL	↓	

	C57Bl/6J (M) & B6D2F1 (M)	1.0-30.0, single	ip	Splenocytes from C57Bl/6 mice	GVH, popliteal lymph node weight gain assay	No Δ	Vecchi *et al.*, 1980
	Swiss webster (F)	1-20 ppb TCDD-containing chow (4wks before gestation to 3wks after giving birth)	Pre & postnatal	2,4-Dinitro, 1-fluorobenzene (DNFB)	Contact hypersensitivity	↓	Thomas and Hinsdill, 1979
	C57Bl/6 Sh	1.0 or 5.0 at GD11 or 18 postnatal d4,11 & 18 5.0 at postnatal d0, 7 & 14	Pre & postnatal or Postnatal	C57bl/6 splenocytes	GVH (popliteal lymph node)	↓	Vos and Moore, 1974
				Skin graft	Rejection time	↓	
	C57Bl/6	0.2-25.0, weekly 4x		Splenocytes	GVH (popliteal lymph node assay)	↓	Vos *et al.*, 1973
Host resistance	B6C3F1 (F)	0.001-6.0, single	Oral	Influenza virus	Virus titer, Survival	No Δ ↓	Burleson *et al.*, 1996
	B6C3F1 (F)	0.1-30.0, single	ip	*Trichinella spiralis*	Elimination day	↑	Luebke *et al.*, 1994
	B6C3F1 (F)	0.1-10.0, single	ip	Influenza virus	Mortality	↑	House *et al.*, 1990
				Listeria monocytogenes	Mortality	No Δ	
	B6C3F1 (M & F)	1.0-15.0, 4x at GD14, & postnatal d1, 7 &14	Pre & postnatal	PYB6 tumor cell/ *Listeria monocytogenes*	Mortality	↑	Luster *et al.*, 1980
	Swiss webster (F)	1.0-20.0 ppb TCDD-containing chow (4wks before gestation to 3wks after giving birth)	Pre & postnatal	EndotoXin	Sensitivity	↑	Thomas & Hinsdill, 1979

				Listeria monocytogenes	Mortality	No Δ	
				Survival of offsprings	Mortality	↑	
	Swiss mice (M)	50.0, weekly 4x	Oral	*Listeria monocytogenes* iv	mortality	No Δ	Vos *et al.*, 1978
		1.5-50.0, weekly 4x		Endotoxin	Sensitivity	↑	
	C57Bl/6J fh (M)	0.5-20.0, weekly 4x	Oral	*Salmonella bern*	Mortality	↑	Thigpen *et al.*, 1975
				Herpesvurus suis	Mortality	No Δ	
Innate immunity	A/J (M)	5.0 & 3x of 1.42 weekly	ip	Non-specific	NK cell activity	↑	Funseth and Ilback, 1992
					T cell proliferation	No Δ	
	B6C3F1 (F)	0.1-10.0, single	ip	Non-specific	NK cell & Mϕ activity	No Δ	House *et al.*, 1990
	B6C3F1 (F)	0.01-2.0, repeat 14x	Oral	Non-specific	Complement hemolytic activity	↓ (dose-dependent)	White *et al.*, 1986
	C57Bl/6J (M)	1.0-30.0, single	ip	Non-specific	NK cell activity	No Δ	Mantovani *et al.*, 1980
					Peritoneal Mϕ activity	No Δ	
	B6C3F1 (M & F)	1.0-15.0, 4x at GD14 & postnatal d1, 7, &14	pre/postnatal	Non-specific	Peritoneal Mϕ activity	No Δ	Luster *et al.*, 1980
	Swiss mice	50.0, weekly 4x	oral	Non-specific	Mϕ activity	No Δ	Vos *et al.*, 1978

2) Rat

Immunity	Strain (sex)	Dose (μg/kg)	Route	Antigen	Measurement	Responses	Reference
Humoral immunity	F344 (F)	3.0, single at GD14	Prenatal	SRBC	PFC	No Δ	Gehrs *et al.*, 1997
	F344 (F)	1.0-30.0, single	ip	TNP-LPS	PFC	↓	Smialowicz *et al.*, 1996
	SD (M)	10.0-40.0, single	Oral	SRBC	Anti-SRBC IgM	No Δ	Fan *et al.*, 1996
					Anti-SRBC IgG	↑	
	F344 (F)	0.1-30.0, single	ip	SRBC iv	PFC	↑	Smialowicz, 1994
	F344 (F)	5.0, single at GD18 or post natal at d0, 7&14 or pre/post natal at GD18 & d0, 7 & 14	Prenatal, Postnatal Or Pre & post natal exposure	Bovine gamma globulin-CFA, 2x ip	Antibody titer in the serum	No Δ	Faith *et al.*, 1978
				Con A & PHA	Proliferation	↓	
Cell-mediated immunity	F344 (M & F)	3.0, single at GD14	Prenatal	BSA or KLH	DTH	↓ in 19 month old male offsprings ↓ in 4 month old female offsprings	Gehrs and Smialowicz, 1999
	F344 (F)	3.0, single at GD14	Prenatal	BSA	DTH at 14-17 wks old offsprings	↓	Gehrs *et al.*, 1997
		1.0 single at GD14	Prenatal	BSA	DTH at 5 month old offsprings	↓	
	SD (M)	10.0, every other days 3x	Oral	KLH-CFA sc, 2x	DTH (tibiotarsal joint weight)	↑	Fan *et al.*, 1996

		0.00006-0.6, single		HRBC Foot pad sc	Popliteal lymph node assay	No Δ	Korte *et al.*, 1991
	F344 (F)	5.0, single at GD18 or post natal at d0, 7 &14 or pre & post natal at GD18 & d0, 7 & 14	Prenatal, Postnatal or Pre & post natal exposure	Oxazolone	DTH at age 41,68 & 145 days old offsprings (Lefford method)	↓ in both postnatal exposed and pre & postnatal exposed group	Faith *et al.*, 1978
	F344 (F)	1.0 or 5.0 at GD11, 18 & postnatal d4,11 &18 or 5.0 at postnatal d0, 7 &14	Pre & postnatal or Postnatal	F344-BNF1	GVH (popliteal lymph node)	↓ in postnatal, but not in pre & postnatal exposed group	Vos and Moore, 1974
				Skin graft	Rejection time	↑	
	Albino	0.2-5.0, weekly 6x		*Mycobacterium tuberculosis*	DTH (skin test)	No Δ	Vos *et al.*, 1973
Host resistance	F344 (F)	1.0-30.0, single	ip	*Trichinella spiralis*	Elimination	No Δ	Luebke *et al.*, 1995
	F344 (F)	3.0-30.0, single	Oral	Influenza virus	Virus titer		Yang *et al.*, 1994
Innate	F344 (F)	3.0-30.0, single	Oral	Non-specific	NK activity	No Δ	Yang *et al.*, 1994
				Influenza virus	NK activity	↓ at 48hrs post-infection	

Symbols represent that an increase (↑), suppression (↓), and no change (no Δ). GD, gestation days.