AN ABSTRACT OF THE DISSERTATION OF

Erica A. Dearstyne for the degree of Doctor of Philosophy in Toxicology presented on June 2, 2000. Title: T Lymphocyte Death as a Potential Mechanism of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Induced Immune Suppression.

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Abstract approved: _____

Nancy I. Kerkvliet

The immune system is a sensitive target for the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin). T lymphocyte-dependent responses are particularly sensitive to the suppressive effects of TCDD. Exposure to TCDD causes a reduction in T cell numbers and function by unknown mechanism(s). We tested the hypothesis that induction of cell death is a mechanism by which TCDD causes a decrease in T cell numbers following stimulation. We used annexin v, a protein that binds to phosphatidylserine (PS), and 7-aminoactinomycin D (7-AAD) to discriminate between viable (annexin-/7-AAD-), apoptotic (annexin+/7-AAD-) and dead (annexin+/7-AAD+) cells. We tested our hypothesis in three different in vivo models in which TCDD has been shown to affect T cells. In mice treated with anti-CD3, TCDD enhanced the depletion of CD4+, and to a lesser extent, CD8+ T cells. This decrease correlated with an increase in the percentage of T cells that were annexin+/7-AAD+. In addition, Fas/FasL interaction appeared to play a role in the TCDD-enhanced decrease of T cells in this model. In contrast, in mice injected with P815 tumor cells, TCDD treatment significantly decreased the percentage of apoptotic CD4+ and CD8+ T cells. However, in this model, the data represent a measure of cell death in the entire population of T

cells and not exclusively the antigen-specific cells. In order to determine the effects of TCDD on cell death of activated antigen-specific CD4+ T cells, we used the DO11.10 adoptive transfer model. Exposure to TCDD caused a dose-dependent decrease in activated OVA-specific CD4+ T cells, but had no effect on the initial expansion of these cells. In addition, TCDD caused an increase in the percent of activated CD4+ cells staining positive for annexin. Furthermore, injection of an agonistic anti-OX40 antibody, a CD4+ T cell survival signal, reversed the deletion of OVA-specific CD4+ T cells in TCDD treated mice. Taken together, these data support our hypothesis that TCDD causes a decrease in T cells in part by increased cell death.

T Lymphocyte Death as a Potential Mechanism of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)-Induced Immune Suppression

by

Erica A. Dearstyne

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented on June 2, 2000

Commencement June, 2001

Doctor of Philosophy dissertation of Erica A. Dearstyne presented on June 2, 2000

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Chair of Department of Environmental and Molecular Toxicology

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Dean of Graduate School

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Erica A. Dearstyne, Author

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the many people who have contributed to the completion of this dissertation. First and foremost, I would like to thank Dr. Nancy Kerkvliet. She has been a terrific mentor over the past five years and has taught me a great deal about research, science, writing, and life in general. Nancy has been patient, critical, supportive, thoughtful, and positive over the years, and is not only a mentor but also a friend. Thank you so much for all you have taught me and for your confidence in me. I have had a great time working in the lab, and although at times research was frustrating, I know that I will look very fondly upon the years that I spent here.

This dissertation would have never been completed without help from present and former members of the Kerkvliet lab. A special thanks goes to Linda Steppan and Julie Oughton for all their time and patience with me over the years. In addition, many thanks go to Dr. Paige Lawrence for her guidance and for being a friend, both personally and professionally. I would also like to thank Drs. Rod Prell and Dave Shepherd for their insightful (however, not always appreciated) comments about my research and for help with numerous experiments. Finally, to the rest of lab members, Beth, Jin-Young, and Carl, thanks for all the helpful discussions over the years, for making this a great place to work, and for your friendship.

I would also like to thank my family for all their support, both emotional and financial over the years. And yes, I may actually enter the "real world" someday! Finally, a special thanks goes to Dr. Roger Higdon, who has definitely made the last three years very special and wonderful.

CONTRIBUTION OF AUTHORS

The various coauthors of the chapters are listed in alphabetical order together with their major contributions to the study.

Kerkvliet, Nancy I.	Critical review of the experimental design, data, and manuscripts; financial support.
Shepherd, David M.	Technical support, critical review of data and manuscript

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Chapter 1

Introduction

1.1 Statement of Research Purpose and Research Objectives

The immune system is very sensitive to the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). TCDD causes suppression of both cell-mediated and humoral immune responses in mice. In addition, T lymphocyte-dependent responses tend to be suppressed at lower doses of TCDD than T-independent responses (reviewed in Kerkvliet and Burleson, 1994). Therefore, T lymphocytes represent a potential target for the suppressive effects of TCDD. Exposure to TCDD causes a decrease in the percent and number of peripheral T lymphocytes in a number of immune models (Lundberg et al., 1991; Lundberg et al., 1992; Prell et al., 1995; Kerkvliet et al., 1996). T cell cytokine production and proliferation are also suppressed after exposure to TCDD (Lundberg et al., 1992; Kerkvliet et al., 1996). The mechanism(s) by which TCDD affects T cells has not been fully elucidated.

The studies presented in this thesis address the hypothesis that exposure to TCDD causes a decrease in T cells by inducing or augmenting cell death. Since functional T cells are important in the development of many immune responses, the induction of cell death in T cells could result in suppression of the overall immune response in TCDD-treated mice. The objectives of the research are: 1) to determine whether exposure to TCDD increases cell death of T cells, 2) to determine which signaling pathways may be involved in TCDD-induced cell death, and 3) to

determine if blocking cell death pathways reverses the TCDD-induced deletion of the T cells as well as the suppression of the immune response.

1.2 Research approach

The approach used to test the hypothesis stated in 1.1 was to address each of the objectives in three different in vivo models known to be affected by TCDD. The models tested include the anti-CD3, P815 tumor allograft rejection, and DO11.10 adoptive transfer models.

Anti-CD3 is a widely used tool to study T cell activation and apoptosis in vitro and in vivo. Anti-CD3 causes activation of both CD4+ and CD8+ T cells by binding to the T cell receptor at the epsilon portion of the CD3 molecule. Previous studies in our laboratory have shown that the percent of CD4+ T cells present in the lymph nodes of anti-CD3-treated mice was significantly decreased by TCDD treatment (Prell et al., 1995). In addition, the lymph node cells from TCDD- and anti-CD3-treated mice showed characteristics of apoptosis.

We also evaluated the effects of TCDD on cell death of T cells in the P815 tumor allograft rejection model. Previous studies in our laboratory have shown that TCDD causes a decrease in the percent and number of both CD4+ and CD8+ T cells in P815-injected mice (Kerkvliet et al., 1996). In vivo depletion of the CD4+ T cells in P815-treated mice caused suppression of the CTL and alloantibody responses. The degree and timecourse of suppression by anti-CD4 was similar to that observed in mice treated with TCDD. Results from several experiments have led to the hypothesis that TCDD interferes with the activation of CD4+ T cells, which leads to downstream inhibition of the CTL and alloantibody responses (Kerkvliet et al., 1996). However, one limitation of this model is the inability to detect or identify the antigen-specific CD4+ T cells.

In the adoptive-transfer model, we could artificially enhance the frequency of OVA-specific CD4+ T cells by adoptively-transferring spleen cells from DO11.10 transgenic mice, specific for a peptide of ovalbumin ($OVA_{323-339}$), into non-transgenic syngeneic recipients (Kearney et al., 1994). We could follow the activation of the antigen-specific CD4+ T cells by flow cytometry using an anti-TCR antibody specific for the transgenic cells. The effector portion of this response, OVA-specific antibody production is dependent on the activation of the CD4+ T cells and is suppressed by TCDD. Therefore, using this model, we could determine the effects of TCDD on activated antigen-specific CD4+ T cell death and also relate the impact of T cell death to the immune response.

Using these three models, the number of the T cells (both CD4+ and CD8+ for the anti-CD3 and P815 models and CD4+ T cells for the DO11.10 adoptive transfer model) and cell death was monitored using flow cytometry. Annexin v, a protein that binds to phosphatidylserine, and 7-aminoactinomycin D (7-AAD) were used to detect cell death in specific populations of T cells. In addition, other proteins important in different apoptosis pathways were assessed by either flow cytometry or RT-PCR. Finally, the use of mice genetically altered in their susceptibility to apoptosis or the use of antibodies to block cell death or enhance survival of T cells were utilized to determine their impact on the decrease of T cells and on the overall immune response in the different models. All of these methods will be described in detail either in the materials and methods chapter (chapter 3) or in the results chapter (chapter 4).

1.3 Goal of Research

Despite nearly 30 years of research, the mechanism by which TCDD causes immunotoxicity remains unknown. Understanding how chemicals

affect the immune system is important in maintaining the health and well-being of organisms. The ultimate goal of these studies was to help elucidate a potential mechanism by which TCDD induces immunotoxicity. Specifically, we attempted to determine if cell death is one potential mechanism by which TCDD suppresses T cell function.

Chapter 2

Literature Review

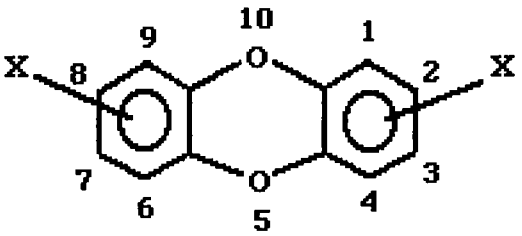
2.1 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)

2.1.1 Description

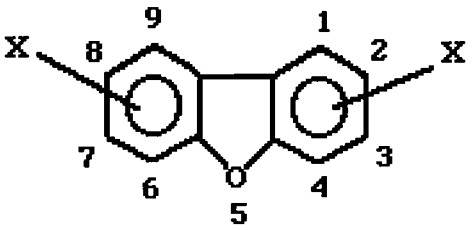
Halogenated aromatic hydrocarbons (HAH) consist of a large class of chemicals, which include chlorinated dioxins, furans, and biphenyls (Figure 2.1). There are at least 75 different congeners of the polychlorinated dibenzo-*p*-dioxins, and the most toxic is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). These congeners differ in the chlorine substitution on the aromatic rings, and the planar arrangement of these molecules appears to be the most toxic. Many of these chemicals show a similar toxicity spectrum and mediate most of their effects by binding to the cytosolic aryl hydrocarbon receptor (AhR) (reviewed in Webster and Commoner, 1994).

In the 1920s the mass production of polychlorinated biphenyls (PCBs) led to their use in transformers, capacitors, carbonless copy paper, plasticizers and numerous other applications (Webster and Commoner, 1994). The potential for these compounds to harm both the environment and humans was not realized until decades later. Subsequently the production of PCBs was halted. On the other hand, polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are formed as unwanted byproducts of herbicide production, combustion of chlorine-containing products such as chemical waste, hospital waste and sewage sludge, paper bleaching, metal production, and fossil fuel and wood combustion. Therefore, even though PCDDs and PCDFs are not manufactured per se, they are deposited in the environment as a result of these processes (Safe, 1990; Webster and Commoner, 1994; Zook and Rappe, 1994).

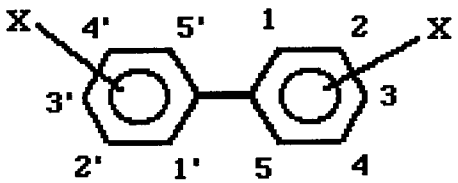
Figure 2.1 Structure of halogenated dioxins, furans, and biphenyls



Dibenzodioxins



Dibenzofurans



Biphenyls

PCDDs and PCDFs are of enviromental concern due to their chemical properties (Zook and Rappe, 1994). These chemicals have low vapor pressure, low water solubility, high lipophilicity and long-term chemical stability. They tend to partition into the soil and sediment, where they can remain for many years. For example, the environmental half-life of TCDD is approximately ten years. Since these chemicals are very lipophilic, they tend to bioaccumulate in the fat of animals; thus the major route of exposure of humans to dioxin is through the food chain. Because of the continued production of these unwanted byproducts, the relative stability of these chemicals in the environment, and the potential for human exposure, it is important to study the toxicities induced by exposure to these chemicals.

2.1.2 Toxicity of TCDD

The toxicity of TCDD varies dramatically between species. For example, the half-life of TCDD in mice is approximately 10-15 days, in rats is 12-31 days, and in humans is 5-10 years (DeVito and Birnbaum, 1994). Some species are particularly sensitive to the effects of TCDD where as others are relatively resistant. To illustrate, the LD_{50} (dose required to cause lethality in 50% of the animals treated) is 0.6 μg/kg for guinea pigs, whereas hamsters are about 1000-10,000 times less sensitive (LD_{50} > 3000 μg/kg). The differences in sensitivity among different species has been shown to be related to whether they possess a high or low affinity AhR (DeVito and Birnbaum, 1994).

Exposure to TCDD causes a wide range of toxicities, depending on the dose administered and the sensitivity of the exposed organism. In laboratory animals, exposure to TCDD has been shown to cause a wasting syndrome, thymic atrophy, chloracne, hepatomegaly, reproductive toxicity, and teratogenicity (reviewed in DeVito and Birnbaum, 1994). Interestingly,

one of the most sensitive targets for the toxic effects of TCDD is the immune system. (TCDD-induced immunotoxicity will be discussed in detail in the next section). In addition, TCDD was originally characterized as a tumor promoter. However, current evidence suggests that TCDD is a complete carcinogen (both promotes and initiates tumor formation).

In addition to the overt toxicities associated with exposure, TCDD causes a variety of biochemical effects that may underlie its toxicity. TCDD exposure causes alterations in the expression of several growth factors (e.g., TGF-β and EGFR) (Abbott et al., 1992; Dohr et al., 1994), hormones (e.g., estrogen, testosterone, thyroid, and vitamin A) and their receptors (estrogen, glucocorticoid, and insulin receptors) (Barsotti et al., 1979; Lin et al., 1991; DeVito et al., 1992; DeVito and Birnbaum, 1994). Such changes could lead to effects on growth and differentiation of cells, which may result in toxicity.

Another well documented biochemical effect of TCDD exposure is alterations in enzyme activity. TCDD has been shown to upregulate the level and activity of cytochrome P450 1A1, 1A2, UDP-glucuronyltransferase (UGT) and glutathione-S-transferase (GST) (Lucier et al., 1993; DeVito and Birnbaum, 1994). These enzymes are regulated at the transcriptional level by the ligand-AhR complex binding to consensus sequences in the promoter regions of these genes.

2.1.3 Mechanism of Dioxin Action

The AhR is a cytoplasmic receptor with a wide tissue distribution that mediates most if not all the toxic effects of TCDD (Whitlock, 1993; Hankinson, 1995). Ligands for the AhR include, but are not limited to PAHs, HAHs, and aromatic amines (Poland and Knutson, 1982). TCDD is considered to be the "prototypic" ligand for the AhR because it possesses the greatest affinity for the AhR. The endogenous ligand for the AhR has not yet been identified, however lipophilic substances in plants (Whitlock,

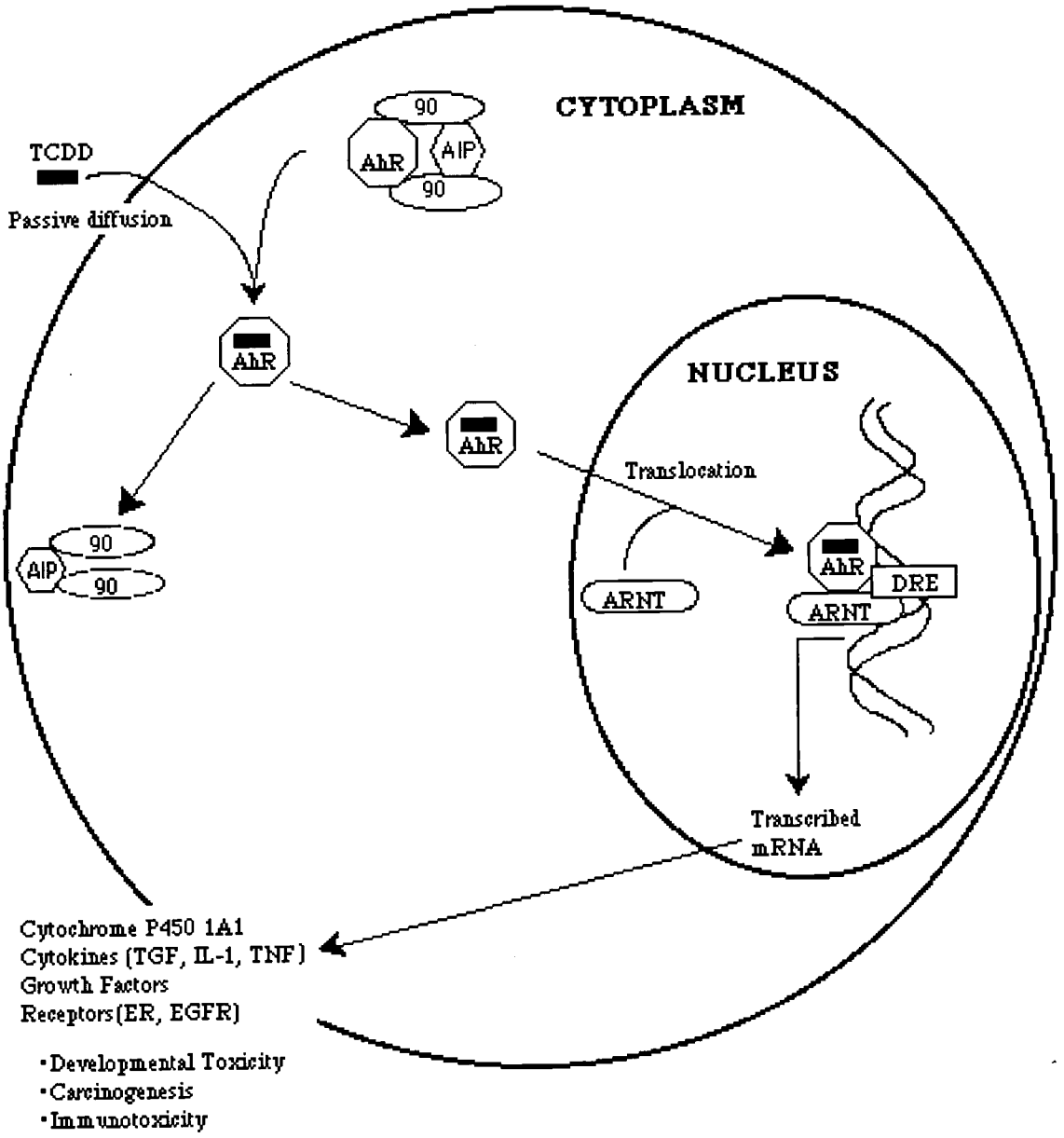
1993), bilirubin (Phelan et al., 1998), and metabolites of tryptophan (Heath-Pagliuso et al., 1998) have been shown to bind to the AhR in vitro, and represent potential endogenous ligands.

The AhR is a basic helix-loop-helix protein located in the cytosol and acquires a high affinity for DNA upon ligand binding (Whitlock, 1993; Hankinson, 1995). In the cytoplasm, the AhR is complexed with a number of other proteins, including two HSP90s (Perdew, 1988), and an AhR interacting protein (AIP) (Ma and Whitlock, 1997), that together form the AhR complex (Figure 2.2). This complex dissociates after ligand binding, allowing the liganded-AhR to translocate into the nucleus where it complexes with another protein called AhR nuclear translocator (ARNT). ARNT was originally thought to aid in the translocation of the ligand-bound AhR into the nucleus, but more recent studies have indicated that this protein is only present in the nucleus and that liganded AhR can enter the nucleus in ARNT-deficient cells (Safe, 1986; Whitlock, 1993). Therefore, ARNT appears to bind to the liganded AhR to form a heteromeric protein that can bind to DNA and activate gene transcription. This heteromeric protein complex binds to specific sequences of DNA called dioxin responsive elements or DREs. The core sequence of the DRE is 5'-GCGTG-3' (Whitlock, 1993). As an example, the Cyp 1A1 gene, which is strongly induced by TCDD, contains five DREs in its upstream promoter region. Several other genes, such as GST, UGT, ER, IL-1β, and IL-2 and have been identified to contain at least one DRE in the upstream regulatory region and therefore are potentially susceptible to regulation by TCDD (Lai et al., 1996).

Although the majority of evidence suggests that TCDD mediates its action via AhR-dependent gene transcription, not all of the consequences of TCDD's action appear to be attributable to gene activation. For example, TCDD has been shown to cause: 1) an altered phosphorylation of proteins within 15 minutes of exposure (Snyder et al., 1993), 2) an increase in intracellular calcium concentrations within 2 minutes of exposure

Figure 2.2: The Ah receptor model. A schematic of the Ah receptor (AhR) model is shown. The AhR ligand (TCDD) diffuses into the cell where it binds to the AhR. Upon ligand binding, two heat shock proteins (HSP) 90 and Ah receptor inteacting protein (AIP) dissociate from the AhR. The TCDD:AhR complex enters the nucleus where it binds to another protein, AhR nuclear translocator protein (ARNT). This heteromeric protein complex binds to DNA at specific sequences known as dioxin response elements (DRE), and alters transcription of genes such as cytochrome P450 1A1 (CYP1A1). The alteration of gene transcription of several genes such as growth factors, growth factor receptors, and cytokines may mediate the toxicity of TCDD and other AhR ligands.

Figure 2.2

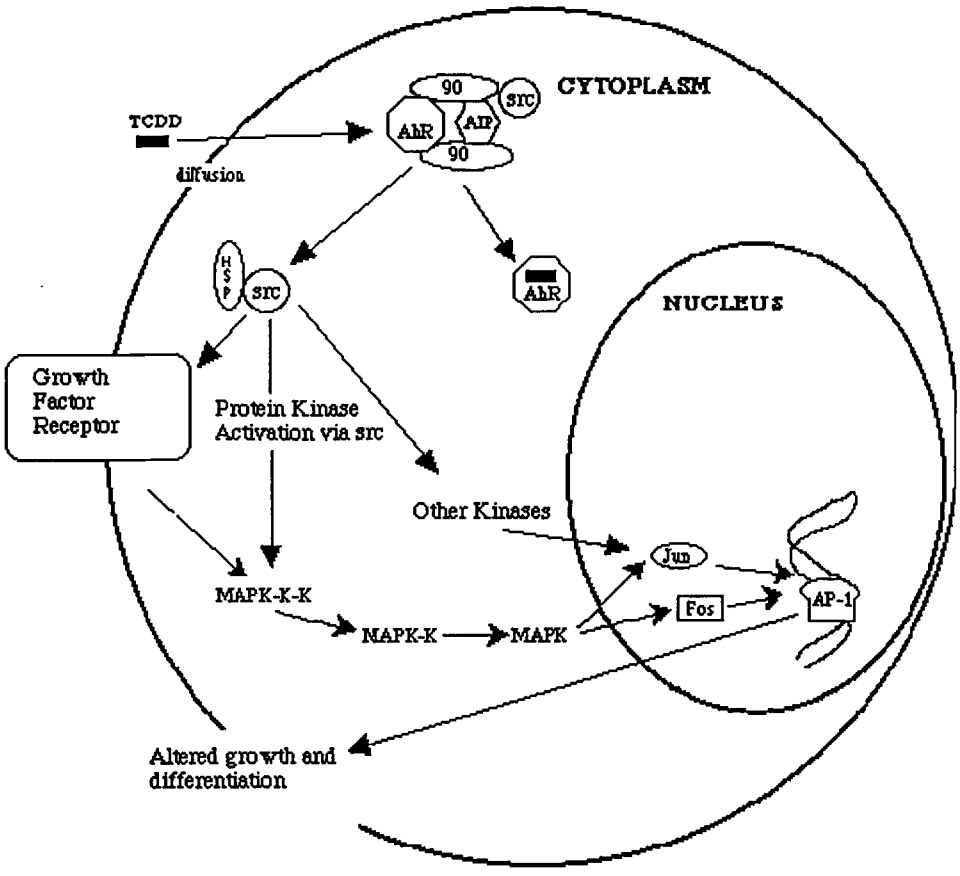

CYTOPLASM
90
AhR
AIP
90
TCDD
Passive diffusion
AhR
AhR
NUCLEUS
Translocation
90
AIP
90
ARNT
AhR
DRE
ARNT
Transcribed
mRNA
Cytochrome P450 1A1
Cytokines (TGF, IL-1, TNF)
Growth Factors
Receptors(ER, EGFR)
•Developmental Toxicity
•Carcinogenesis
•Immunotoxicity

(Matsumura, 1994), and 3) a rise in JunD mRNA within 15 minutes of exposure (Puga et al., 1992). Because these events occur too rapidly to be accounted for by increased gene transcription, Matsumura (1994) postulated that they occur as a result of altered second messanger systems within the cell (Figure 1.3). The proteins that bind to the unliganded AhR have been shown to possess some intrinsic kinase activity which affect intracellular signaling pathways (Enan and Matsumura, 1996). For example, heat shock proteins contain src kinase activity, which causes the activation of the MAP-kinase pathway and the subsequent activation of nuclear transcription factors, including AP-1, which causes an increase in JunD mRNA and other "immediate early" (IE) genes (reviewed in Matsumura, 1994).

Initial evidence that the AhR mediates most of the toxic effects of TCDD came from the discovery of genetic polymorphisms in the AhR gene (Poland and Glover, 1990). The different alleles confer different binding affinities of the AhR to its ligands. The C57Bl/6 mouse, which has AhR^{bb} allele, is considered to be the prototypic "responsive" strain. The DBA/2 mouse, which has the AhR^{dd} allele, is considered to be the prototypic "nonresponsive" strain. The concentration of dioxin need to elicit a similar degree of toxicity is much higher for the AhR^{dd} strain (reviewed in Kerkvliet and Burleson, 1994). Studies using congenic mice (mice that are genetically similar except at the Ah locus) have also revealed that mice with the low affinity AhR have a decreased sensitivity to TCDD, suggesting that sensitivity to TCDD segregates with the AhR and is not significantly influenced by other genetic factors. Furthermore, recent studies have shown that AhR knockout mice are resistant to the toxic manifestations of TCDD, even at doses 10 times higher than the LD_{50} (Fernandez-Salguero et al., 1996; Gonzalez and Fernandez-Salguero, 1998).

Figure 2.3: DRE-independent mechanism of TCDD-induced toxicity. A diagram representing the hypothetical DRE-independent protein phosphorylation pathway is shown. The AhR ligand TCDD diffuses into the cell and binds to the AhR. After ligand binding, the AhR dissociates from its complex consisting of HSP90, AIP, and protein kinase c-src. Following dissociation, the c-src kinase is activated and then activates other intracellular kinases, including c-ras and the mitogen activated protein kinase (MAPK) cascade. These activated kinases phosphorylate transcription factors like AP-1, which then alters the transcription of "immediate early" (IE) genes that control the growth and differentiation of cells.

Figure 2.3


CYTOPLASM
90
src
AhR
Arp
90
TCDD
diffusion
HSP
src
AhR
NUCLEUS
Growth
Factor
Receptor
Protein Kinase
Activation via src
Other Kinases
Jun
Fos
AP-1
MAPK-K-K
MAPK-K
MAPK
Altered growth and
differentiation

2.2 Immunotoxicity of TCDD

2.2.1 Introduction

The main function of the immune system is to maintain the health of an organism, and as such, alterations in immune function can have serious health consequences (Kerkvliet, 1994). For example, suppression of immune function can increase the incidence and severity of infectious diseases and cancer. Alternatively, enhancement of immune function can lead to autoimmunity or allergies. Both of these types of immune alterations have been shown to be caused by exposure to toxicants. Because of the importance of the immune system to an organism, it is of value to determine how toxic compounds affect immune function.

The immune system is very sensitive to the toxic effects of TCDD (Kerkvliet and Burleson, 1994). TCDD impacts immune function in a variety of different species and scenarios at doses lower than those required to elicit overt toxicity. TCDD-treated animals have an increased susceptibility to viral and parasitic infections in addition to neoplastic disease. In models of host resistance or infection, TCDD increases mortality in animals infected with *Salmonella*, *E. coli*, and *Streptococcus pneumonia*, to name a few. These effects are observed at low doses of TCDD and most likely result from suppression of immune function. However, it remains a possibility that TCDD affects non-immunological parameters that can also reduce the capacity for host resistance (Kerkvliet and Burleson, 1994).

Many different types of immune responses have been reported to be suppressed by TCDD. For example, the antibody response to sheep red blood cells (SRBC) is very sensitive to TCDD; the ED_{50} for this response is 0.7 μg/kg in mice (reviewed in Kerkvliet, 1995). However, other antibody responses, such as to TNP-Ficoll and TNP-LPS, are not as sensitive to TCDD and require a 10-fold higher dose to elicit the same response. The difference in sensitivities of these antibody responses may be related to the

different cellular interactions that are necessary for the response to occur. As an example, the antibody response to SRBC is dependent on T helper cells, whereas the antibody response for TNP-LPS is not, suggesting that T lymphocyte-dependent responses are more sensitive to the suppressive effects of TCDD.

Exposure to TCDD has also been shown to suppress T cell-mediated responses, including the delayed type hypersensitivity responses to oxazalone (Clark et al., 1981). In addition, Hinsdill et al (1980) reported that Swiss mice fed 50 ppb TCDD for six weeks showed suppressed contact sensitization to dinitrofluorobenzene. Several laboratories have also reported that the cytotoxic T lymphocyte (CTL) response to P815 allogeneic tumor cells is suppressed by TCDD (Clark et al., 1981; Kerkvliet et al., 1990a); the ED50 for this response is 7.5 ug/kg TCDD (De Krey and Kerkvliet, 1995).

2.2.2 Immunotoxicity in Humans

The ability of TCDD and other HAH to suppress immune function in humans has not been definitively proven. In 1979, PCB consumption of contaminated oil in Taiwan led to increased rates of infections and decreased serum IgA and IgM titers (Lu and Wu, 1985). In exposed people, the overall percentage of T cells was decreased, but there appeared to be an increase in the suppressor CD8+ T cell population. In addition, the delayed type hypersensitivity response was also decreased in exposed people. In vitro assays to determine immune cell function did not reveal decreased function, but instead the lymphoproliferative responses to mitogen were increased in exposed humans. Alternatively, humans exposed to polybrominated biphenyls (PBB) had decreased T cell numbers, decreased in vitro proliferation to mitogens, but increased DTH response (Bekesi et al., 1979).

There have been a few reports of altered immune status in humans exposed to high levels of TCDD via environmental exposures. In Seveso, Italy, children exposed to TCDD from the explosion of a herbicide factory were examined for alterations in immune parameters (Mocarelli et al., 1986). Despite over half the children tested exhibiting signs of chloracne, no abnormalities in serum Ig levels, complement or lymphoproliferative responses were reported. However, in a study conducted six years after the explosion, a rise in level of complement, increased peripheral blood lymphocytes, and an increase in lymphoproliferative responses correlated with the incidence of chloracne (Mocarelli et al., 1986). However, these changes did not reflect increased health problems. People exposed to TCDD in Times Beach, MO also had altered immune parameters, including increased T lymphocytes and serum IgA, but had no alteration in lymphoproliferative responses (Webb et al., 1989). Similar to Seveso, exposed people did not report any increases in clinical diseases associated with exposure to TCDD.

There are many complicating factors associated with determining immunotoxic effects of exposure to HAH in humans. For instance, a lack of adequate data on actual exposure, testing immune parameters long after the exposure occurred and using immunological assays which do not consistently show suppression in animal models (such as mitogen responses) can contribute to the difficulty in determining immunotoxic effects in humans.

2.2.3 Potential Targets for TCDD-Induced Immunotoxicity

Although the immune system is profoundly suppressed in TCDD-treated laboratory animals, the exact mechanism by which TCDD causes suppression of immune responses is unknown. In addition, the proximal cellular target(s) for TCDD in the immune system has yet to be discovered.

As discussed in the following section, there are several possible immune targets for TCDD-induced immunosuppression.

2.2.3.1 Thymus

One of the hallmarks of exposure to TCDD is atrophy of the thymus. It occurs in all species tested at doses lower than that required to elicit overt toxicity (Poland and Knutson, 1982; Kerkvliet, 1994; Kerkvliet and Burleson, 1994). However, the role of thymic atrophy in mediating TCDD-induced immune suppression is not clear. In young animals, neonatal thymectomy reduces T cell function and produces a fetal wasting syndrome. Similarly, exposure to TCDD during the fetal period of life has dramatic effects on T cell function (reviewed in Kerkvliet, 1994). In contrast, in adult animals, thymic atrophy induced by TCDD may not impact immune function (Tucker et al., 1986; Kerkvliet and Brauner, 1987) because the role of the thymus in the adult immune system is questionable. Thymectomy of adult animals does not alter the quality or quantity of T cells in the periphery, except after long delay. Furthermore, a direct relationship between the effects of TCDD on the thymus and TCDD-induced suppression of immune function has not been established (Silkworth and Antrim, 1985; Kerkvliet and Brauner, 1990). Finally, TCDD suppresses immune responses at doses below the level that induce thymic atrophy.

The mechanism by which TCDD induces thymic atrophy is under intense investigation. TCDD has been hypothesized to cause: 1) apoptosis of the thymocytes directly (McConkey et al., 1988; Kamath et al., 1997), 2) alterations in thymocyte differentiation (Kerkvliet and Brauner, 1990; Holladay et al., 1991; Blaylock et al., 1992), 3) alterations of thymocytes themselves (Fine et al., 1990) or 4) alterations in the thymic epithelial cells (Greenlee et al., 1985). Recently, in studies using chimeric mice which expressed AhR in either hematopoietic or non-hematopoietic cells, TCDD was shown to induce thymic atrophy via a direct interaction with

hematopoietic cells and not thymic stromal cells (Staples et al., 1998b). These results suggest that TCDD induces thymic atrophy by a direct effect on immune cells and not on stromal or epithelial cells.

2.2.3.2 B Lymphocytes

As mentioned above, TCDD inhibits antibody responses to several different types of antigens, suggesting that TCDD may target B cells directly. In support of this hypothesis, initial studies to identify the direct cellular target of TCDD provided evidence that B cells are more sensitive than either T cells or macrophages. For example, B cells isolated from TCDD-treated mice were functionally compromised in in vitro antibody responses, whereas T cells and macrophages were not (Dooley and Holsapple, 1988). In addition, TCDD induced alterations in early activation of B cells, including increased tyrosine kinase activity (Clark et al., 1991a) and protein phosphorylation (Snyder et al., 1993), which could affect the differentiation of B cells. In support of this conclusion, TCDD has been shown to decrease the production of IgM from activated B cell lines and splenic B cells (Sulentic et al., 1998). However, it should be noted that this in vitro data is not necessarily supported by in vivo data. For example, TCDD suppresses T-cell dependent antibody responses at much lower doses than T cell-independent responses (Kerkvliet et al., 1990b). Therefore, these results suggest that while TCDD may affect B cells directly, T cells and/or macrophages may be more sensitive.

2.2.3.3 Macrophages and Inflammation

Macrophages are not particularly sensitive to the direct effects of TCDD. Functions such as phagocytosis, tumor cell lysis, and oxidative reactions are not suppressed by TCDD in vitro (Mantovani et al., 1980). Moreover, TCDD enhances inflammatory reactions. For example, TCDD-induced toxicity is accompanied by neutrophilia and macrophage accumulation in the skin (Puhvel and Sakamoto, 1988) and liver (Vos et

al., 1974). Such increased inflammation may be mediated by increased production of inflammatory cytokines such as TNF, IL-1, and IL-6 (reviewed in Kerkvliet, 1994). In addition, mice have increased sensitivity to endotoxin after exposure to TCDD, which was shown to be a result of increased TNF production (Clark et al., 1991b). However, the role that increased inflammation plays in the TCDD-induced suppression of immune responses has not been established.

2.2.3.4 T Lymphocytes

T lymphocytes also represent a potential target cell for the immunotoxic effects of TCDD (reviewed in Kerkvliet, 1998). Both CD4+ and CD8+ T cells are affected by TCDD. CD8+ T cell expansion and differentiation into effector cells in response to P815 tumor cells are suppressed in mice exposed to TCDD (Kerkvliet et al., 1996). Furthermore, cytokine production (IL-2, IFN-γ, and TNFα) from CD8+ T cells is also suppressed in TCDD-treated mice injected with P815 tumor cells. Likewise, CD4+ T cell function is also affected by TCDD (Lundberg et al., 1991; Tomar and Kerkvliet, 1991; Lundberg et al., 1992; Kerkvliet et al., 1996). For example, the proliferation of ovalbumin-specific CD4+ T cells isolated from TCDD-treated mice was suppressed following in vitro restimulation (Lundberg et al., 1992). Also, in vitro generation of anti-TNP antibodies to TNP-SRBC was suppressed when cultured with hapten-specific CD4+ T cells isolated from TCDD-treated mice (Tomar and Kerkvliet, 1991).

The effect of TCDD on cytokine production from either CD4+ or CD8+ T cells may depend on the nature of the antigenic stimulus. For example, TCDD has been shown to significantly suppress TNFα production mice injected with P815 tumor cells (Kerkvliet et al., 1996). In contrast, mice that were injected with SRBC had increased TNFα production when exposed to TCDD (Moos et al., 1997). Alternatively, TCDD treatment had no effect on TNFα production from mice injected with anti-CD3 (Prell et

al., 1995). Thus, TCDD can have different effects on immune functions depending on the stimulus.

Despite the large database of descriptive evidence to support TCDD affecting T cells, the underlying biochemical or molecular mechanisms that mediate these effects in T cells are unknown. However, it has been very difficult to demonstrate direct effects of TCDD on T cell function in vitro (reviewed in Kerkvliet, 1994). This could be due to the fact that either the direct cellular target for TCDD is not present in the cultures, or that factors in the culture media, such as serum, calcium concentration, and TCDD carrier conditions, could influence the results. In addition, Lawrence et al., (1996) showed that although T cells express low levels of AhR, it does not appear be functional. These results suggest that while T cells are affected by TCDD, they may not be the direct cellular target.

2.3 Mechanisms of T Cell Death

2.3.1 Characterization and Description of Cell Death

The term apoptosis, which comes from the Greek meaning "leaves falling", was coined in the early 1970's by Kerr and collegues. The term, which is also known as programmed cell death, describes the morphological changes that accompany a certain type of cell death. These highly organized changes include cellular shrinkage, membrane structure alterations, membrane blebbing, and the formation of apoptotic bodies, which contain whole organelles and fragments of the nucleus (Fawthrop et al., 1991; Schwartz and Osborne, 1993). Neighboring cells phagocytose the apoptotic bodies, which prevents the release of their cellular contents into the interstitium and the subsequent development of an inflammatory response. In addition to the morphological changes, many energy dependent biochemical events occur within apoptotic cells. These include alterations in ion flux (including Ca^{++} and K^{+}), osmotic balance, new gene

synthesis, and the hallmark of apoptosis, fragmentation of DNA into nucleosomal-sized fragments.

In contrast to apoptosis, cells can also be induced to undergo necrosis. Necrosis is characterized by cellular swelling (mostly due to changes in ion flux), membrane damage, and eventual cellular lysis. This form of cell death usually arises as a result of injury or toxicant exposure. The lysis of the cells will result in the induction of an inflammatory response and damage to neighboring cells or surrounding tissue (reviewed in Fawthrop et al., 1991; Trump and Berezesky, 1998).

There are many tools available to study or identify cell death (Darzynkiewicz et al., 1992). Apoptotic or necrotic cells can be distinguished based on the morphological characteristics described above using either microscopy or flow cytometry. The use of vital dyes, such as propidium iodide (PI), 7-aminoactinomycin D (7-AAD), or trypan blue can be used to identify necrotic cells, which have lost cell membrane integrity (Darzynkiewicz et al., 1992). In addition, measurement of DNA fragmentation by gel electrophoresis, flow cytometry, or ELISA has also been routinely used to assess apoptosis (Dolzhanskiy and Basch, 1995; Zhang et al., 1995). Alternatively, changes in the phospholipid bilayer of cell membranes can be used to identify apoptotic cells. Phosphatidylserine (PS) is normally located on the inner leaflet of cellular membranes. Once a cell receives a signal to undergo apoptosis, the PS is translocated to the outer leaflet of the cell membrane and can be detected using flow cytometry (van Engeland et al., 1998). Annexin v, a protein that recognizes PS, can be used in combination with a vital dye to distinguish viable, apoptotic, and dead cells.

2.3.2 T cell Apoptosis

A T cell may have different fates depending on the signals delivered by APC (Figure 2.4). In the context of antigen and appropriate

costimulation, the T cell will become activated and undergo clonal expansion and differentiation into effector cells (left). Alternatively, if the T cell encounters antigen without costimulation, then the T cell will enter a stage called anergy or functional nonresponsiveness (center). This T cell will not respond to that particular antigen, nor will it drive an effective immune response. Finally, if the T cell encounters antigen and receives a death signal, then the T cell will undergo apoptosis (right).

The thymus represents a good organ to study the mechanisms of T cell apoptosis because a large number of cells are eliminated by apoptosis during the process of maturation (Cohen et al., 1992). T cells that do not bind to self major histocompatibility (MHC) molecules or that respond to self antigens are deleted in the thymus. However, the mechanisms by which T cells undergo apoptosis in the thymus have not been fully elucidated. One potential mechanism involves the Bcl family of pro- and antiapoptotic proteins. T cells from mice that overexpress the antiapoptotic protein Bcl-2 are not deleted from the thymus, and as a result these mice succumb to lymphadenopathy and autoimmune disorders (Cory, 1995).

Peripheral T cells undergo apoptosis under several different conditions (Lynch et al., 1995; Boise and Thompson, 1996; Lenardo, 1996; Lenardo et al., 1999). For instance, apoptosis of peripheral T cells occurs during the down regulation of an ongoing immune response, during activation-induced cell death (AICD), and during the induction of peripheral tolerance. At the end of an immune response T cells will undergo apoptosis when the antigen has been cleared and there are reduced levels of cytokines or growth factors present. In contrast, AICD occurs during the course of an immune response when antigen concentration is high and high levels of cytokines, such as IL-2, can promote cell death. Additionally, autoreactive T cells are removed from the periphery by apoptosis in a process called peripheral tolerance.

Figure 2.4: T cell fate. A diagram showing the different fates of a T cell upon encounter with antigen presented by an antigen presenting cell. Under conditions of adequate costimulation the T cell becomes activated (left). If the T cell is presented antigen without costimulation, the T cell will become anergic or nonresponsive (center). Finally, if the T cell receives a death signal in addition to antigen, then the T cell will undergo apoptosis (right).

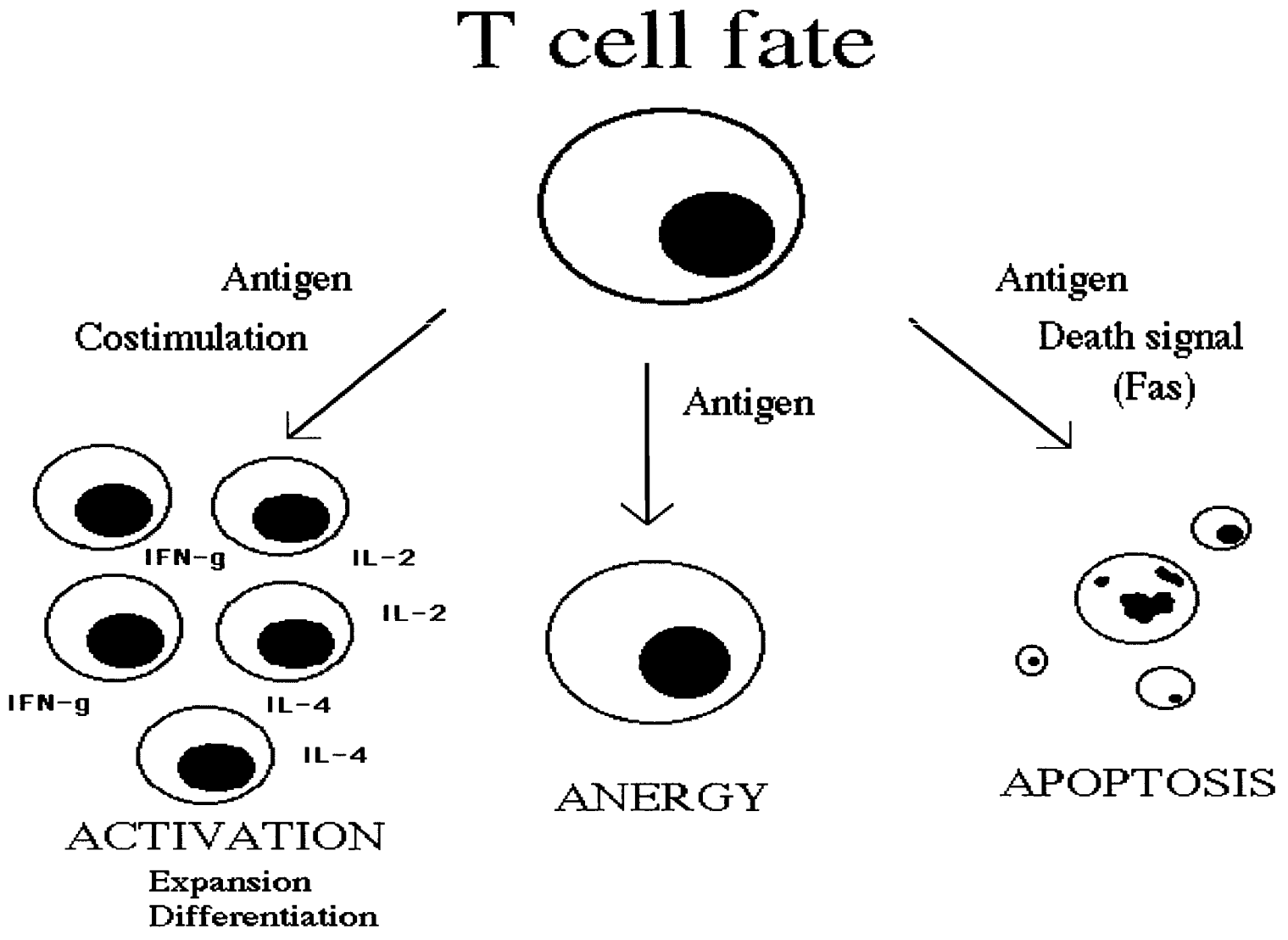

T cell fate
Antigen
Costimulation
Antigen
Antigen
Death signal
(Fas)
IFN-g
IL-2
IL-2
IFN-g
IL-4
IL-4
ACTIVATION
Expansion
Differentiation
ANERGY
APOPTOSIS

Figure 2.4

The tumor necrosis factor receptor (TNFR) and TNF families have been shown to be critical in the decision between activation and apoptosis (Ware et al., 1996). TNFRI, Fas, DR3, DR4, and DR5 are members of the TNFR family that mediate apoptosis. For example, Fas/Fas ligand (FasL) interaction is critical in the down regulation of an immune response, in AICD, and in the induction of peripheral tolerance (Crispe, 1994; Lenardo et al., 1999). Fas is expressed on virtually all cells, is upregulated upon activation, and contains an intracellular sequence known as the death domain (Nagata and Golstein, 1995). FasL is expressed on activated T cells and dendritic cells, and has also been shown to be expressed in liver, intestine, testes, and eye (Nagata and Golstein, 1995). Upon ligand binding to Fas, Fas associated death-domain-containing protein (FADD/Mort1) binds to the death domain. Pro-caspase 8 (FLICE) and cap3 then bind to FADD. This complex is known as the death-inducing signaling complex (DISC) (Peter and Krammer, 1998). Pro-caspase 8 is cleaved at the DISC and the active form dissociates from the complex and initiates the caspase cascade and the execution phase of apoptosis (Peter and Krammer, 1998). The importance of the Fas/FasL pathway in peripheral tolerance is best demonstrated in mice with spontaneous mutations in either Fas or FasL, lpr or gld, respectively (Nagata and Golstein, 1995). Despite normal selection of T cells in the thymus, T cells from these mice do not undergo apoptosis upon encounter with self antigens in the periphery. Therefore, these mice succumb to autoimmune disease between 3-6 months of age.

Another family of molecules that regulates the decision between activation and death is the Bcl family, which contains both proapoptotic and antiapoptotic members (reveiwed in Reed, 1994; Cory, 1995). The proapoptotic members, including bax, bid, and bad, create pores in the mitochondria, which leads to the release of cytochrome c and other mitochondrial products important in apoptosis induction. On the other hand, bcl-2 and bcl-x_L block apoptosis by inhibiting the release of cytochrome c from the mitochondria. In addition, these two proteins can

also block the activation of certain caspases, such as caspase 3. The bcl family members hetero- or homodimerize and the relative ratios of the proapoptotic to antiapoptotic members will determine the susceptibility of a cell to undergo apoptosis. Over expression of bcl-2 or bcl-x_L can block the down regulation of an immune response and can also inhibit apoptosis induced by growth factor withdrawal (Lenardo et al., 1999).

2.3.3 TCDD and Apoptosis

Exposure to TCDD produces a variety of toxic responses by unknown mechanism(s). One of the possible mechanisms by which TCDD exerts it toxic effects is through the induction of apoptosis. The literature is replete with inconsistent data regarding whether exposure to TCDD leads to apoptosis. For example, TCDD has been reported to cause embryonic toxicity in medaka, which has been causally linked with apoptosis in the embryonic vasculature (Cantrell et al., 1998). In contrast, in rainbow trout sac fry, exposure to TCDD did not cause apoptosis in vascular tissues, but was associated with craniofacial abnormalities and decreased heart size and blood flow (Hornung et al., 1999). Furthermore, TCDD is well known for its ability to promote tumor formation. For example, Christensen et al. (1999) showed that chronic TCDD exposure promotes the expression of bcl-2 and bcl-xL in liver tumors and Stinchcombe et al. (1995) reported that diethlynitrosamine (DEN)-initiated liver tumors promoted by TCDD showed evidence of decreased apoptosis. In contrast, breast cancer cells exposed to TCDD showed increased apoptosis (Wang et al., 1997). Therefore, it appears that TCDD has different effects on apoptosis in different species or types of tumor cells.

Apoptosis has been proposed as a mechanism by which TCDD induces thymic atrophy. McConkey et al. (1988) reported that immature rat thymocytes treated with TCDD in vitro increased their intracellular calcium concentration, which was followed by DNA fragmentation and

decreased cell viability. Unfortunately, these results have not been reproduced by other laboratories (Blaylock et al., 1992). In vivo, TCDD has also been reported to cause apoptosis of thymocytes (Kamath et al., 1997). However, thymocytes displayed an apoptotic phenotype only after in vitro culture. Fas/FasL interaction has been proposed as a mechanism by which TCDD causes thymic atrophy (Rhile et al., 1996; Kamath et al., 1999). In support of this conclusion, thymic atrophy was not evident in lpr or gld mice at low doses of TCDD. However, at higher doses of TCDD, these mice had an equivalent degree of thymic atrophy. In addition, TCDD upregulated FasL mRNA expression in thymocytes, and sera from TCDD-treated mice killed Fas-positive target cells (Kamath et al., 1999). However, the results from these studies have not been reproduced by other laboratories. In fact, Silverstone et al. (1994) did not find any evidence to support the hypothesis that TCDD-induced thymic atrophy is mediated by apoptosis. Moreover, TCDD induced thymic atrophy in bcl-2 transgenic mice which are defective in apoptosis, strongly suggesting that TCDD induces thymic atrophy via a non-apoptotic mechanism (Staples et al., 1998a). Thus, it appears that TCDD may only induce thymocyte apoptosis at very high doses and after in vitro culture and may not be a relevant mechanism for the thymic atrophy induced in vivo.

There have been relatively few studies published that have evaluated the effects of TCDD on apoptosis of peripheral T cells. In a study by Prell et al. (1995), TCDD caused a decrease in CD4+ T cells in anti-CD3-treated mice. In addition, lymph node cells from anti-CD3- and TCDD-treated mice showed signs of apoptosis, as measured by light scatter characteristics using flow cytometry. Histological examination of lymph node cells from mice several days after treatment with TCDD and anti-CD3 also showed signs of necrosis, suggesting that this dosing regime may be overtly toxic to the mice. A recent study by Pryputiniewicz et al. (1998), reported that TCDD increased apoptosis of antigen-activated but not resting lymph node cells from mice treated with anti-CD3 in complete Freund's

adjuvant (CFA). The TCDD-induced increase in apoptosis, which was only detected after in vitro culture with media or anti-CD3, was correlated with a decrease in the proliferation of the cells upon in vitro restimulation. From these results, the authors conclude that TCDD decreased the responsiveness of T cells to anti-CD3/CFA by inducing the cells to undergo apoptosis.

Based on these results, we addressed the hypothesis that exposure to TCDD causes a decrease in T cell numbers by inducing or augmenting cell death. In addition, since functional T cells are important in the development of many immune responses, the induction of cell death in T cells could result in suppression of the overall immune response in TCDD-treated mice. Therefore, we were also interested in determining whether the decrease in T cell numbers was an underlying cause of TCDD-induced immune suppression.

Chapter 3

The Effect of Exposure to TCDD on T Cell Death in Anti-CD3-treated Mice

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Nancy I. Kerkvliet

3.1 Introduction

Exposure to TCDD results in reduced numbers of CD4+ and CD8+ T cells in both the spleen and lymph nodes in several models of immune activation (Lundberg et al., 1991; Lundberg et al., 1992; Prell et al., 1995; Kerkvliet et al., 1996). Previous studies in our laboratory have reported the effects of TCDD on T cells exposed to anti-CD3, a molecule which leads to a rapid and transient T cell activation and results in a massive release of cytokines that have been shown to cause body weight loss (cachexia), hypothermia, diarrhea, and hypomotility (Hirsch et al., 1989; Alegre et al., 1991; Ferran et al., 1991a; Ferran et al., 1991b). Unexpectedly, exposure to TCDD increased the proliferation and cycling of T cells 24 hours after in vivo activation with anti-CD3 (Neumann et al., 1993). Paradoxically, TCDD enhanced the toxicity of anti-CD3, as measured by a greater and more prolonged decrease in body weight at 72 hours after activation, as well as altered the production of IL-6, IFN-γ, and GM-CSF (Prell et al., 1995). Subsequently, TCDD was shown to significantly decrease the percent of CD4+, but not CD8+ T cells in anti-CD3-treated animals. The forward and side scatter characteristics of the lymph node cells from TCDD- and anti-CD3- treated animals resembled those of apoptotic cells (i.e., decreased forward scatter and increased side scatter) (Prell et al., 1995). Based on these results, we hypothesized that the TCDD-induced decrease in CD4+ T cells was caused by increased cell death. CD8+ T cells were also examined in the same experiments described below and the results are presented in section 3.3.2.

3.2 Materials and Methods

3.2.1 Animals

Male C57Bl/6J, B6.MRL-Fas^{lpr}, and B6Smn.C3H-Fasl^{gld} mice were purchased from Jackson Labs (Bar Harbor, ME) at 6-8 weeks of age. All animals were housed in front of laminar flow units and were fed standard rodent chow and tap water *ad libitum*.

3.2.2 Reagents

All cell culture reagents were purchased from GibcoBRL (Grand Island, NY) unless otherwise stated. Fetal bovine serum (FBS) was purchased from Hyclone (Ogden, UT). The hamster anti-murine CD3 mAb, 145-2C11 (ATCC, Rockville, MD), was purified from culture supernatant using Protein A sepharose Fast Flow columns (Pharmacia, Piscataway, NJ) and diluted to 1 mg/ml in phosphate buffered saline (PBS). HamsterIgG and ratIgG (Cappel/Organon Teknika, West Chester, PA) were used as a nonactivating control antibodies. Anti-TNF neutralizing antibody (MP6-XT22) was a generous gift from Dr. Anthony Vella. Phycoerythrin (PE)-labeled anti-CD4 (clone RM4-5), PE-labeled anti-Fas (clone Jo2), Fitc-labeled anti-CD4 (clone GK1.5), biotinylated-anti-CD4 (clone RM4-5) and biotinylated-anti-CD8 (clone 53-6.7) were purchased from Pharmingen (San Diego, CA). Tri-color anti-CD8 (clone CT-CD8a) was purchased from Caltag (Burlingame, CA). Steptavidin conjugated to Red613 and Red613-labeled anti-CD8 (clone 53-6.7) were purchased from GibcoBRL.

3.2.3 Animal treatments

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1.5 μg/ml. A vehicle control solution was prepared in a similar manner. Animals were given a single dose of 15 μg/kg body weight by gavage. Mice were injected with 25 μg of anti-CD3 or hamsterIgG Ab per rear footpad one day after treatment with

vehicle or TCDD. In some experiments, animals were injected with 1 mg of anti-TNF or ratIgG control antibody one hour prior to exposure to TCDD. Animals were killed at various times after anti-CD3 injection by CO_2 overdose and the inguinal, popliteal, axillary, and superficial axillary lymph nodes were harvested and pooled.

3.2.4 Preparation of lymph node cells

A single cell suspension was prepared by pressing the lymph nodes between the frosted ends of two microscope slides. The cell suspension was washed once and resuspended in HBSS supplemented with 5% FBS and 20mM HEPES buffer (HBSS-5) and enumerated using a Coulter counter (Coulter Electronics, Hialeah, FL).

3.2.5 Flow cytometry

Lymph node cells (1-2 x 10^6) were incubated on ice in 96-well V bottom plates (Costar, Corning, NY) in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Non-specific binding of the antibodies to Fc receptors was blocked by the addition of 30 μg of ratIgG, hamsterIgG, or 1% normal mouse serum (Jackson Immunoresearch Labs, Inc., West Grove, PA) for 15 min on ice. Subsequently, the cells were stained for 10 min on ice with the specific antibodies mentioned above for CD4, CD8, or Fas. Non-specific fluorescence was measured using appropriately labeled isotype-matched IgGs. Data were collected on at least 50,000 freshly stained cells by listmode aquisition using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using Winlist software (Verity Software House, Topsham, ME).

3.2.6 Flow cytometric analysis of apoptosis

After surface staining, the cells were stained for apoptosis using 7-aminoactinomycin D (7-AAD) from Calbiochem (La Jolla, CA) and FITC- or PE-labeled annexin v from R&D systems (Minneapolis, MN) following the

manufacturer's protocol. Briefly, the cells were resuspended in 1X binding buffer (HEPES buffered saline, 25mM $CaCl_2$) and stained with 0.1 μg labeled annexin v and 2 μg 7-AAD. Data were collected and analyzed as described above. This method allows for the discrimination of viable, apoptotic, and dead cells (Fig 3.1).

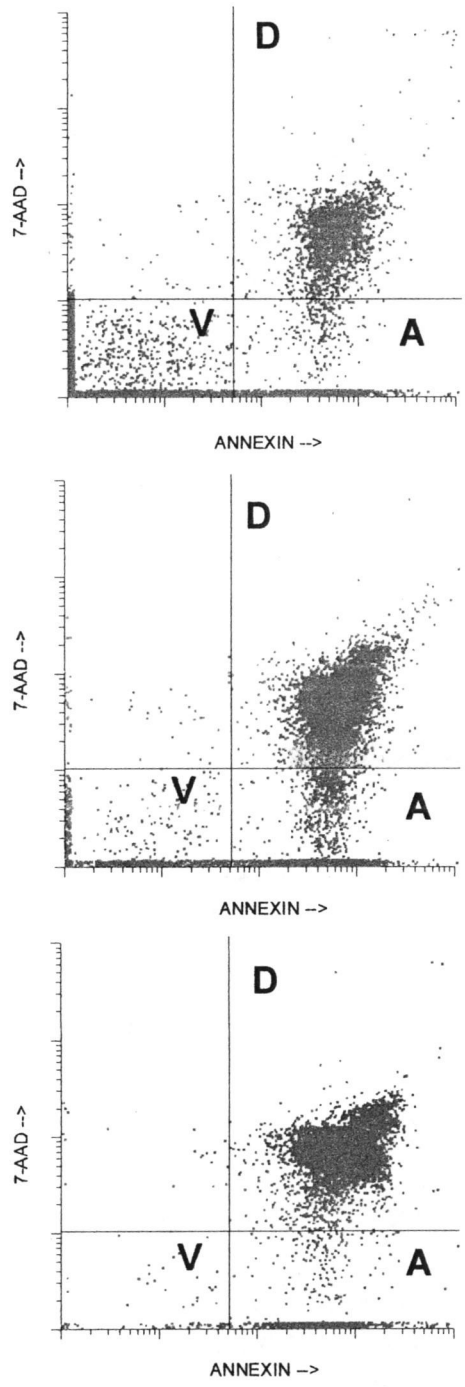
3.2.7 RNA preparation and reverse transcription-PCR

Total RNA was obtained by lysis of lymph node cells from anti-CD3-treated mice in 4 M guanidine-thiocyanate as previously described (Kerkvliet et al., 1996). cDNA was synthesized using RNA extracted from $5x10^6$ lymph node cells cells using oligo(dt) primers (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (GibcoBRL, Grand Island, NY). PCR amplification of cDNA was performed using Taq polymerase (Boehringer Mannheim, Indianapolis, IN), specific gene oligonucleotide primers (see below), and deoxynucleotides for 30-50 cycles using an Ericomp thermocycler (San Diego, CA). DNA products were separated by agarose gel electorphoresis and visulaized using ethidium bromide. All cDNA sample were initially analyzed for β_2m to determine relative amounts and integrity of mRNA/cDNA. Samples displaying low levels of β_2m gene expression or degraded DNA amplification products were discarded.

Primers for β_2m and FasL were obtained using RightPrimer (BioDisk Software, San Fransisco, CA) to span introns of the respective genes to allow for discrimination between amplified DNA and genomic or cDNA. The primer sequences used were: β_2m, 5'-ATGGCTCGCTCGGTGACCCT and 3'-TCATGATGCTTGATCACATG; and FasL, 5'-TGTCACCACTACCACCGCCA and 3'-TCTATACCCGGGTGTCGTCGA.

Figure 3.1: Representative histograms for discrimination of viable, apoptotic, and dead cells using annexin v and 7-AAD. Thymocytes from C57Bl/6 mice were cultured in vitro for 16 hours with either vehicle-control (PBS) or 1 μM dexamethasone (Sigma, St. Louis, MO). An aliquot of control thymocytes were also incubated at 65°C for 2 min to induce necrosis. Thymocytes were stained for apoptosis using PE-annexin v and 7-AAD. The number corresponds to the percent of thymocytes in each labeled region.

Figure 3.1



Control
V=66%
A=23%
D=11%

Dexamethasone
V=5%
A=60%
D=35%

Necrosis
V=0%
A=4%
D=96%

V = Viable
A = Apoptotic
D = Dead

3.2.8 Statistical analysis

Results are presented as mean ± SEM of 3-6 animals/group unless stated otherwise. Analysis of variance modeling was performed using Statview Statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using Fisher's least significant difference multiple comparison t test or Student's T test. Values of $p \leq 0.05$ were considered statistically significant.

3.3 Results

3.3.1 Investigations on CD4+ T cells

3.3.1.1 The effect of TCDD on the percent and number of CD4+ T cells from anti-CD3-treated mice

As shown in Figure 3.2, exposure to TCDD caused a significant decrease in both the percentage (Fig 3.2A) and number (Fig 3.2B) of CD4+ T cells in the draining lymph nodes of mice injected 72-96 hours previously with anti-CD3. Our results confirm the results of Prell et al. (1995).

3.3.1.2 Analysis of PS expression on anti-CD3-activated CD4+ T cells

The possible role of apoptosis in the TCDD-induced decrease in CD4+ T cells was investigated using FITC-labeled annexin v to detect phosphatidylserine (PS) expression on the surface of CD4+ T cells. Since PS expression is an early event in the process of apoptosis, occurring prior to the fragmentation of DNA (van Engeland et al., 1998), this method should maximize the ability to detect apoptotic cells in vivo. Dead cells also stain positive for annexin v, so to differentiate apoptotic from dead cells, a viability dye (such as propidium iodide or 7-AAD) can be used. Thus, when cells are stained with annexin and 7-AAD, they can be identified as

Figure 3.2: Effect of TCDD on the percent and number of CD4+ T cells in the lymph nodes of anti-CD3-treated mice. C57Bl/6 mice were treated by gavage with either vehicle (open symbols) or TCDD (15 μg/kg) (filled symbols) one day prior to the injection of 50 μg anti-CD3 (circles) in the rear foot pads. Lymph nodes were harvested as described in Materials and Methods, and the percentage (A) and number (B) of CD4+ T cells were analyzed by flow cytometry. Control hamster IgG-treated (square) mice are represented as time zero. Data represent mean ± SEM of six animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 3.3: The effect of TCDD on cell death of the CD4+ T cells from the lymph nodes of anti-CD3-treated mice. Mice were treated as described in Figure 3.2. Lymph nodes were harvested at various times after anti-CD3 administration as described in Materials and Methods. The percent of CD4+ T cells that were annexin-/7-AAD- (A), annexin+/7-AAD- (B), and annexin+/7-AAD+ (C) was determined by flow cytometry in vehicle- (open circle) and TCDD- (closed circle) treated mice. Control hamster IgG-treated (square) mice are represented as time zero. Data represent mean ± SEM of six animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 3.2

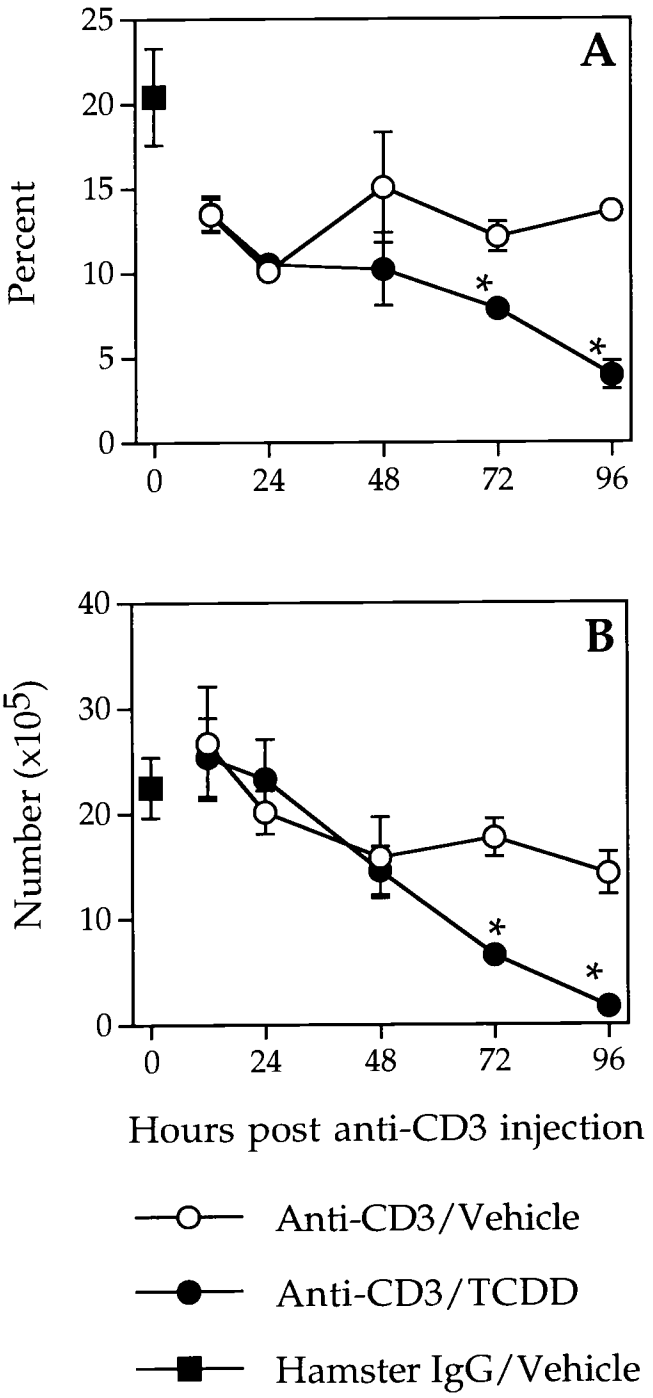
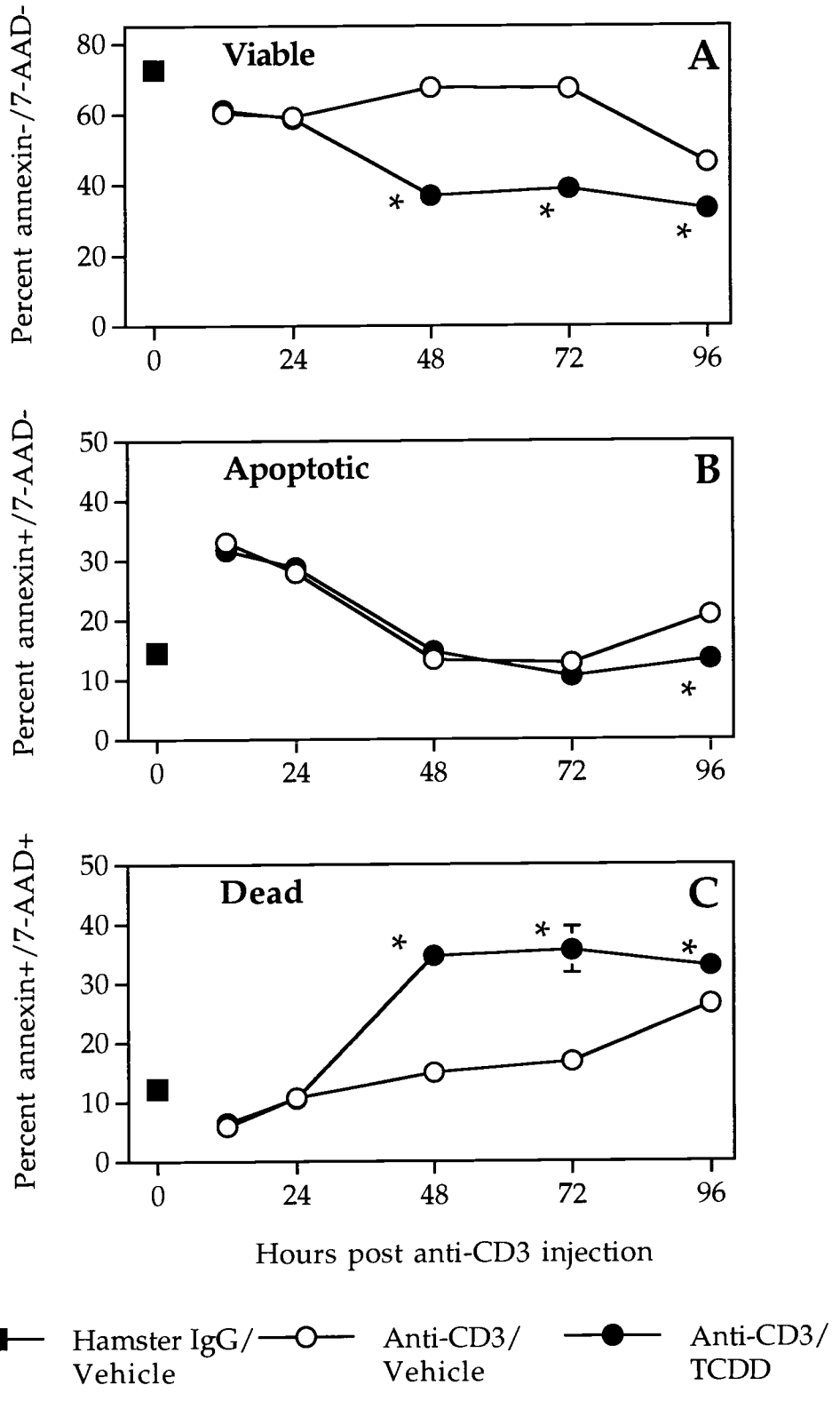

A
B
Percent
Number (x10^5)
0
5
10
15
20
25
30
40
0
24
48
72
96
*
Hours post anti-CD3 injection
Anti-CD3/Vehicle
Anti-CD3/TCDD
Hamster IgG/Vehicle

Figure 3.3


Percent annexin-/7-AAD-
Viable
A
80
60
40
20
0
0
24
48
72
96
Percent annexin+/7-AAD-
Apoptotic
B
50
40
30
20
10
0
Percent annexin+/7-AAD+
Dead
C
Hours post anti-CD3 injection
Hamster IgG/ Vehicle
Anti-CD3/ Vehicle
Anti-CD3/ TCDD

viable (annexin-/7-AAD-), apoptotic (annexin+/7-AAD-), or dead (annexin+/7-AAD+).

As shown in Figure 3.3, anti-CD3 increased the percentage of apoptotic CD4+ T cells at 12 and 24 hours after activation (Fig 3.3B), which correlated with a decrease in the percentage of viable CD4+ T cells (Fig 3.3A). Exposure to TCDD further decreased the percentage of viable CD4+ T cells at 48-96 hours after treatment with anti-CD3. This decrease in viability correlated with an increase in the percentage of dead T cells (Fig 3.3C). Surprisingly, TCDD did not increase the percentage of T cells staining positive for apoptosis, and in fact, a significant decrease was observed at 96 hours after anti-CD3 treatment (Fig 3.3B). Although the degree of annexin staining was variable in three subsequent experiments, in no case did TCDD cause an increase in apoptosis (data not shown). Thus, it appears that TCDD may cause increased cell death of CD4+ T cells, but the role of apoptosis in this cell death is unclear.

3.3.1.3 The response of Fas-deficient mice to anti-CD3 and TCDD

Since apoptosis is difficult to measure in vivo, alternative approaches were used to reveal whether apoptosis is involved in CD4+ T cell deletion from TCDD- and anti-CD3-treated mice. To this end, we used mice that are defective in apoptosis, the B6.MRL-Fas^{lpr} mice (referred to as lpr). These mice do not express Fas, a molecule important in the induction of apoptosis. For example, activation of the Fas pathway has been shown to be involved in anti-CD3-induced apoptosis of T cells in vitro (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). In addition, Fas/FasL interaction is a primary mechanism for peripheral deletion of autoreactive T cells and is involved in the down regulation of an ongoing immune response (Singer and Abbas, 1994; Van Parijs et al., 1996). If Fas is involved in TCDD-induced CD4+ T cell death, then TCDD should not be able to enhance anti-CD3-induced deletion of CD4+ T cells in lpr mice. As shown in Figure 3.4, both lpr and wildtype mice had a 10-12% reduction in

original body weight at 48 and 72 hours after anti-CD3 treatment, suggesting that the T cells from lpr mice can respond to anti-CD3 stimulation. TCDD did not have any effect on the anti-CD3-induced reduction in body weight at any timepoint in either wildtype or lpr mice.

As shown in Figure 3.5A and 3.5B, there was no difference in the percent or number of CD4+ T cells from wildtype or lpr mice at 72 hours after anti-CD3 injection alone. Interestingly, TCDD significantly decreased the percent and number of CD4+ T cells from wildtype, but not lpr, animals at 72 hours after anti-CD3 treatment (Fig 3.5A and 3.5B). These same trends were evident at 48 hours after activation (data not shown). These results suggest that the Fas pathway may be important in TCDD-enhanced deletion of CD4+ T cells from anti-CD3-treated mice.

3.3.1.4 The response of FasL-deficient mice to anti-CD3 and TCDD

To further test the role of Fas in TCDD-enhanced CD4+ T cell deletion, we treated FasL-deficient B6Smn.C3HFasl^{gld} (referred to as gld mice) with anti-CD3 and TCDD. As shown in Figure 3.6, anti-CD3 injection induced only a 5-7% reduction in original body weight at 24-48 hours after exposure in gld mice as compared to a 10-12% reduction observed in wildtype mice. Therefore, it appears that the gld mice may not be as responsive to anti-CD3 treatment as wildtype mice.

As shown in Figure 3.7A and 3.7B, at 72 hours after anti-CD3 treatment, the percent and number of CD4+ T cells was slightly higher in the gld mice. Interestingly, TCDD significantly decreased the percent and number of CD4+ T cells from wildtype, but not gld mice. These same trends were also evident at 48 hours after activation (data not shown). These results also suggest that the Fas pathway may be important in TCDD-enhanced deletion of CD4+ T cells from anti-CD3-treated mice.

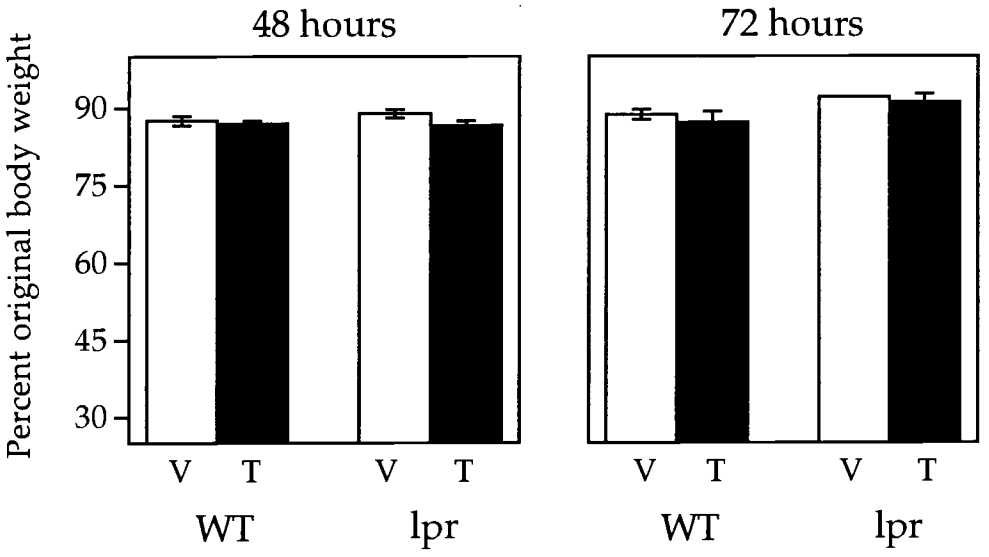
Figure 3.4: The effect of Fas-deficiency on body weight alterations induced by anti-CD3. Wildtype C57Bl/6 and Fas deficient B6.MRL-Fas^{lpr} mice were treated as described in Fig. 3.2. Body weight was monitored at 48 and 72 hours after anti-CD3 injection, and is presented as percent original body weight. Data represent mean + SEM of four mice per group.

Figure 3.5: The effect of the disruption of the Fas pathway on the TCDD-induced decrease of CD4+ T cells from the lymph nodes of anti-CD3-treated mice. Wildtype C57Bl/6 and Fas deficient B6.MRL-Fas^{lpr} mice were treated as described in Figure 3.2. Lymph nodes were harvested at 72 hours after anti-CD3 injection as described in Materials and Methods. The percent (A) and number (B) of the CD4+ T cells were determined by flow cytometry. Data represent mean $\pm$ SEM of four animals per group. Significant differences ($p \leq 0.05$) between the vehicle- and TCDD-treated groups are indicated by *.

Figure 3.6: The effect of FasL-deficiency on body weight alterations induced by anti-CD3. Wildtype C57Bl/6 and FasL deficient B6Smn.C3H-Fasl^{gld} mice were treated as described in Fig. 3.2. Body weight was monitored at 48 and 72 hours after anti-CD3 injection, and is presented as percent original body weight. Data represent mean + SEM of three mice per group.

Figure 3.7: The effect of the disruption of the FasL pathway on the TCDD-induced decrease of CD4+ T cells from the lymph nodes of anti-CD3-treated mice. Wildtype C57Bl/6 and FasL deficient B6Smn.C3H-Fasl^{gld} mice were treated as described in Figure 3.2. Lymph nodes were harvested at 72 hours after anti-CD3 injection as described in Materials and Methods. The percent (A) and number (B) of the CD4+ T cells were determined by flow cytometry. Data represent mean $\pm$ SEM of three animals per group. Significant differences ($p \leq 0.05$) between the vehicle- and TCDD-treated groups are indicated by *.

Figure 3.4


48 hours
72 hours
Percent original body weight
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75
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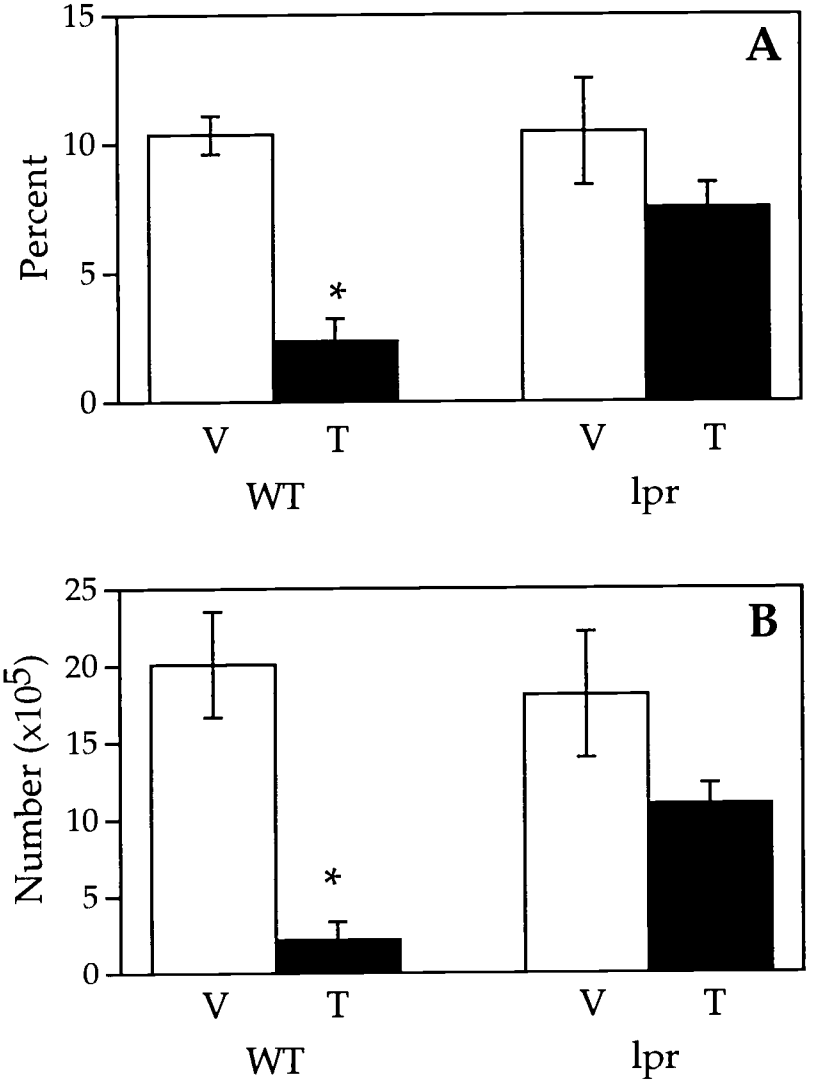
T
WT
lpr
WT
lpr

Figure 3.5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Percent
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WT
lpr
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Number (x10^5)
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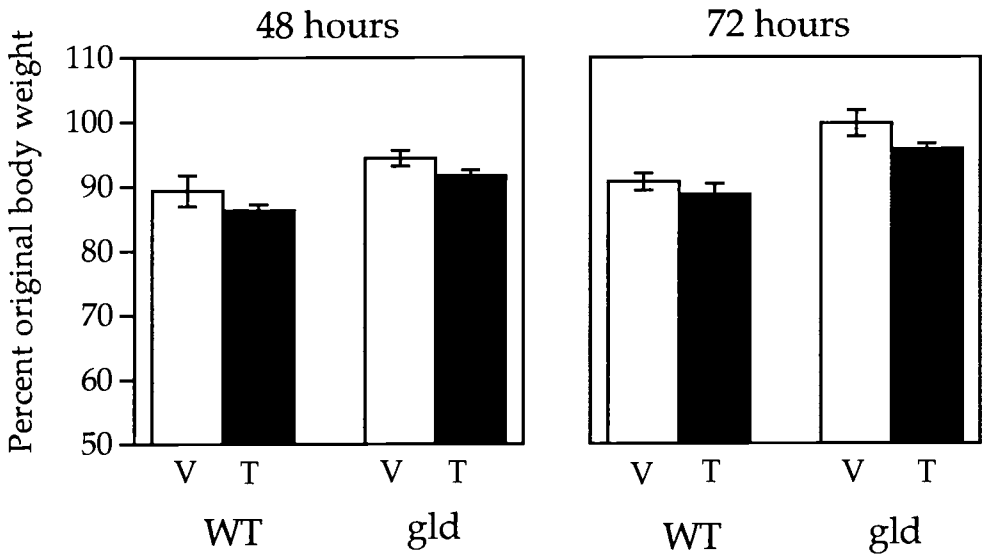
V
T
V
T
WT
lpr

Figure 3.6


48 hours
72 hours
Percent original body weight
110
100
90
80
70
60
50
V
T
V
T
WT
gld
V
T
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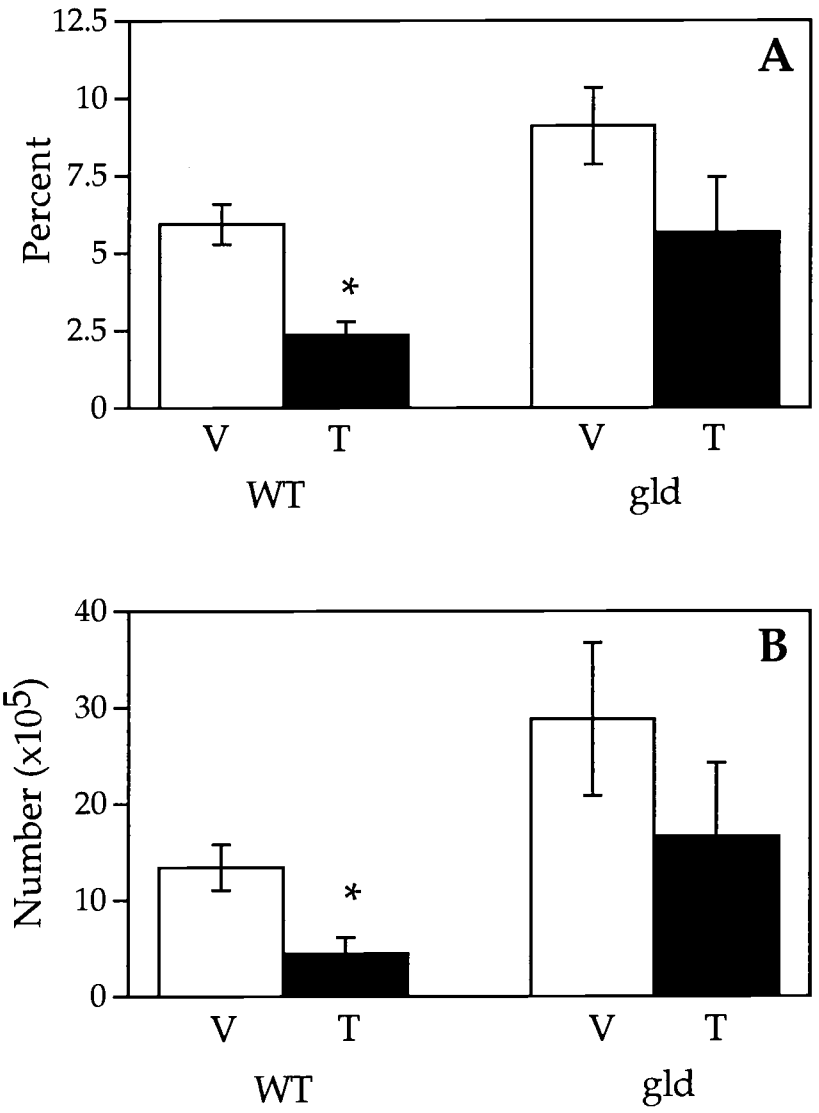
T
WT
gld

Figure 3.7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10
7.5
5
2.5
0
Percent
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V
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V
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WT
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40
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20
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0
Number (x10^5)
B
*
V
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V
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WT
gld

3.3.1.5 The effect of TCDD on Fas expression

Since Fas/FasL interaction may be important in the TCDD-enhanced decrease of CD4+ T cells, we wanted to determine if TCDD altered the expression of Fas on the CD4+ T cells. As shown in Figure 3.8, anti-CD3 treatment alone upregulated the expression of Fas on the CD4+ T cells at all time points tested, although by 72 hours the level of expression had decreased slightly. Surprisingly, treatment of mice with TCDD did not alter the anti-CD3-induced upregulation of Fas at any timepoint tested (Fig 3.8). Thus, it would appear that TCDD does not modulate the Fas pathway via enhanced Fas expression.

On the other hand, TCDD could affect the expression of FasL on the T cells, thereby influencing the Fas pathway. However, our attempts to evaluate the effects of TCDD on FasL expression were unsuccessful. We did not detect any FasL protein on the cell surface using flow cytometry (data not shown). This result is not too surprising since FasL is rapidly cleaved from the cell surface after expression (Nagata and Golstein, 1995). In addition, we also attempted to measure FasL messanger RNA levels by RT-PCR. However, we were not able to obtain consistent results and thus, were not able to interpret the findings (data not shown). Therefore, the effect of TCDD on FasL expression remains to be determined.

3.3.1.6 The involvement of the TNF pathway in the TCDD-induced decrease of CD4+ T cells

Another pathway important in cell death is the TNF pathway. TNF, an inflammatory cytokine produced in response to anti-CD3, can induce cells to become activated or undergo apoptosis depending on which intracellular signaling pathway (i.e., NFκB or caspases, respectively) is activated (Bazzoni and Beutler, 1996; Ware et al., 1996). We blocked TNF activity in anti-CD3-treated mice using a neutralizing anti-TNF antibody (MP6-XT22) (Vella et al., 1995). Animals were injected intraperitoneally with 1 mg of MP6-XT22 one hour before exposure to TCDD. This dose of

Figure 3.8

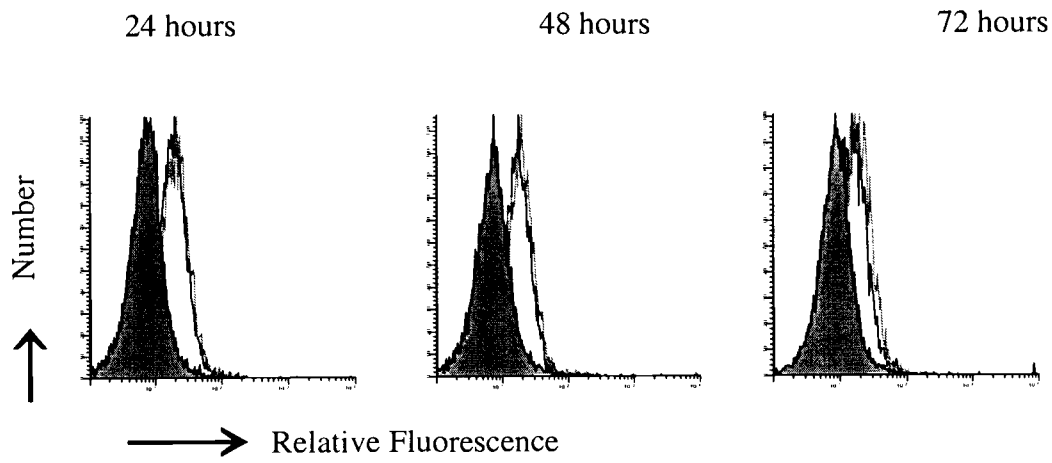


Figure 3.8: The effect of TCDD on Fas expression on the CD4+ T cells from the lymph nodes of anti-CD3-treated mice. Mice were treated as described in Figure 3.2. Fas expression was analyzed by flow cytometry at 24, 28, and 72 hours after anti-CD3 treatment. Representative histograms are shown for vehicle- (black solid line) or TCDD-treated (gray solid line) mice injected with anti-CD3 and the hamster IgG control group (filled histogram). Data are representative of three different experiments.

antibody was chosen because it ablated plasma TNF levels in anti-CD3-treated animals as measured by the L929 bioassay (data not shown).

As shown in Figure 3.9A and 3.9B, anti-TNF treatment significantly increased the percent and number of CD4+ T cells in anti-CD3-treated mice as compared to rat IgG control mice at 48 hours after activation. In contrast, anti-TNF had no effect on the TCDD-induced decrease in the percent and number of CD4+ T cells at 48 hours after anti-CD3 administration (Fig 3.9A and 3.9B). The same trends were also evident at 72 hours after anti-CD3 treatment (data not shown). These data suggest that TNF may play a role in the anti-CD3-induced decrease of CD4+ T cells, but does not appear to be involved in TCDD's augmentation of cell deletion.

3.3.2 Investigations on CD8+ T cells

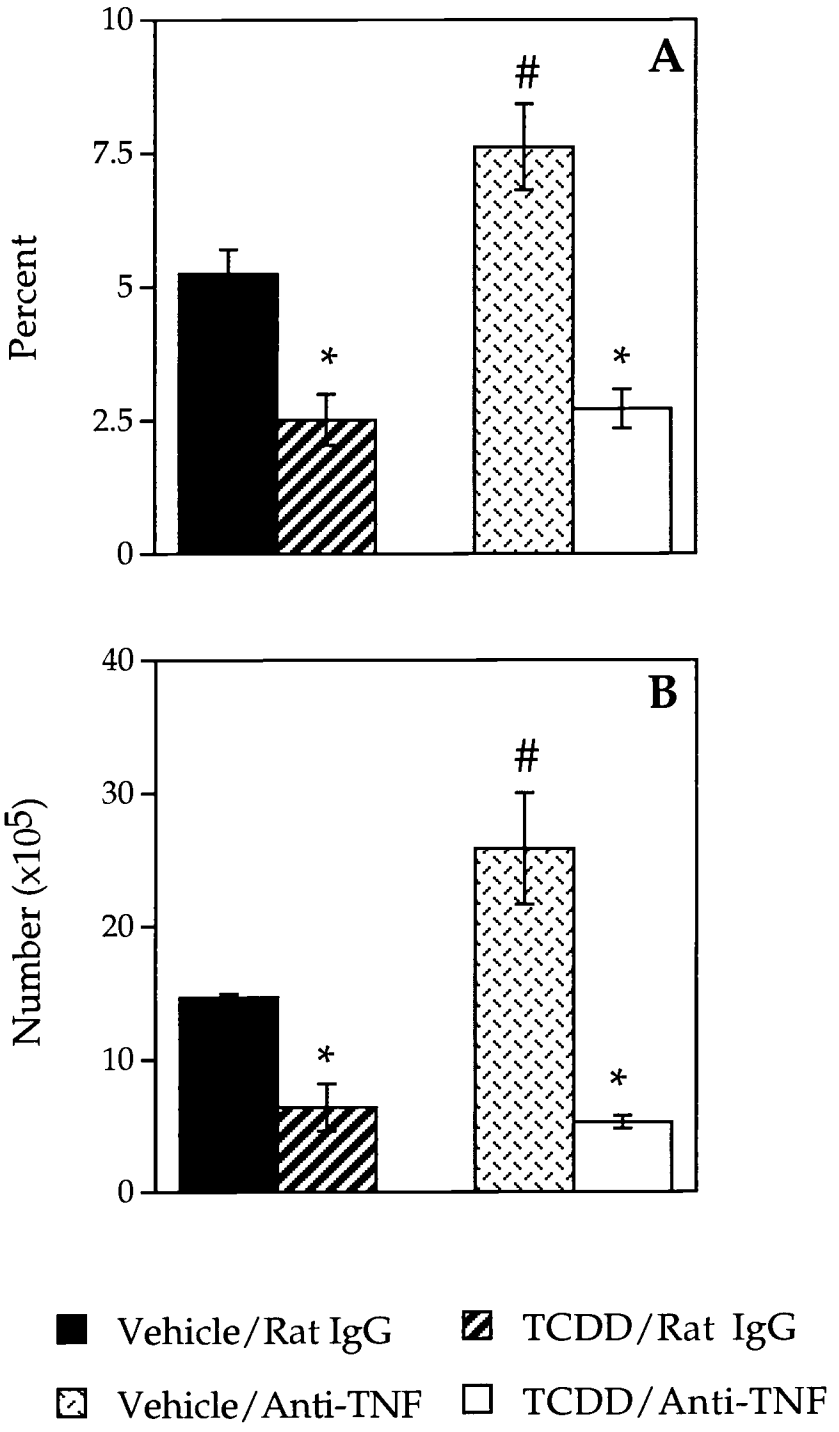
3.3.2.1 The effect of TCDD on the percent and number of CD8+ T cells from anti-CD3-treated mice

Although the studies reported by Prell et al. (1995) showed no effect of TCDD on the percent of CD8+ T cells from anti-CD3-treated mice at any time point tested, it remains a possibility that TCDD might affect the number of CD8+ T cells. Therefore, we also evaluated the effects of TCDD on CD8+ T cells from anti-CD3-treated mice in the same experiments presented in section 3.3.1 of this dissertation, which were driven by the effect of TCDD on CD4+ T cells.

As shown in Figure 3.10, anti-CD3 administration alone caused dramatic fluctuations in the percent (Fig 3.10A) and number (Fig 3.10B) of CD8+ T cells. At 48 hours after anti-CD3 injection, the percent and number of CD8+ T cells transiently increased approximately three-fold as compared to 24 hours. Interestingly, TCDD treatment did not affect the transient

Figure 3.9: The effects of anti-TNF antibody treatment on the TCDD-induced decrease of CD4+ T cells. C57Bl/6 mice were injected ip with 1 mg of either anti-TNF or rat IgG antibody prior to treatment with either vehicle or TCDD (15 μg/kg). One day later mice were injected with either 50 μg of anti-CD3 or hamster IgG in the rear foot pads. Lymph nodes were harvested at 48 hours after anti-CD3 injection and processed as described in Materials and Methods. The percent (A) and number (B) of CD4+ T cells were determined by flow cytometry. Data represent mean $\pm$ SEM of three animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$). # Indicates statistically significant difference from rat IgG-treated group ($p \leq 0.05$).

Figure 3.9


A
B
Percent
Number (x10^5)
10
7.5
5
2.5
0
40
30
20
10
0
#
*
Vehicle/Rat IgG
TCDD/Rat IgG
Vehicle/Anti-TNF
TCDD/Anti-TNF

increase in CD8+ T cells at 48 hours. In contrast to previous reports (Prell et al., 1995), exposure to TCDD caused a significant decrease in the percent of CD8+ T cells 96 hours after exposure to anti-CD3 (Fig 3.10A). In addition, the number of CD8+ T cells was significantly decreased in TCDD-treated mice at 72-96 hours after anti-CD3 injection (Fig 3.10B). Thus, similar to the CD4+ T cells, TCDD may act on CD8+ T cells to produce excess cell death.

3.3.2.2 Analysis of PS expression on anti-CD3-activated CD8+ cells

As shown in Figure 3.11A and 3.11B, anti-CD3 caused an early decrease in the percent of viable CD8+ T cells with a corresponding increase in the percent of apoptotic CD8+ cells. Furthermore, exposure to TCDD further decreased the percentage of viable CD8+ T cells which correlated with an increase in the percent of dead, but not apoptotic cells at 48-96 hours after anti-CD3 administration (Fig 3.11C). Thus, similar to the CD4+ T cells, TCDD may also cause increased cell death of CD8+ T cells.

3.3.2.3 The response of CD8+ T cells from Fas and FasL deficient mice to anti-CD3 and TCDD

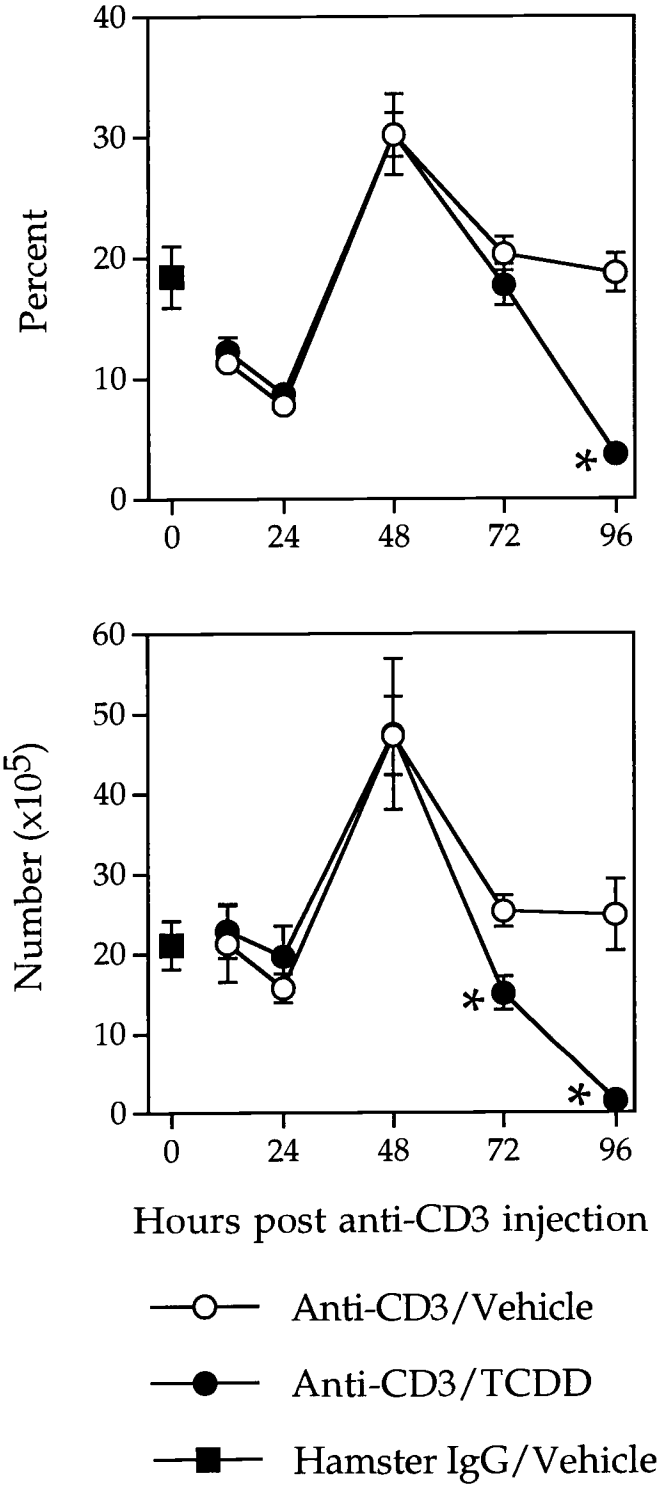
Although the experiments using the Fas and Fas ligand deficient mice were designed specifically to monitor the effects of Fas/FasL interaction on the TCDD-induced decrease of CD4+ T cells, we also followed the CD8+ T cells in the these same experiments. As shown in Figure 3.12, the same increase in percent and number of CD8+ T cells was observed at 48 hours as compared to 72 hours in both wildtype and lpr mice injected with anti-CD3 alone. Contrary to the previous study, in this experiment TCDD exposure caused a significant decrease in the percent and number of CD8+ T cells at both 48 and 72 hours after activation in wildtype mice. Interestingly, TCDD did not significantly alter the percent or number of CD8+ T cells in lpr mice, suggesting that lpr mice are not as sensitive to TCDD.

Figure 3.10: The effect of TCDD exposure on the percent and number of CD8+ T cells from the lymph nodes of anti-CD3-treated mice. C57Bl/6 mice were treated by gavage with either vehicle (open symbols) or TCDD (15 μg/kg) (filled symbols) one day prior to the injection of 50 μg anti-CD3 (circles) in the rear foot pads. Lymph nodes were harvested, as described in Materials and Methods, and the percentage (A) and number (B) of CD8+ T cells were analyzed by flow cytometry. Control hamster IgG-treated (square) mice are represented as time zero. Data represent mean ± SEM of six animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 3.11: The effect of TCDD on cell death of the CD8+ T cells from the lymph nodes of anti-CD3-treated mice. Mice were treated as described in Figure 3.10. The percent of CD8+ T cells that were annexin-/7-AAD- (A), annexin+/7-AAD- (B), and annexin+/7-AAD+ (C) was determined by flow cytometry in Vehicle- (open circle) and TCDD- (closed circle) treated mice. Control hamster IgG-treated (square) mice are represented as time zero. Data represent mean ± SEM of six animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 3.12: The effect of Fas deficiency on the TCDD-induced decrease in CD8+ T cells from the lymph nodes of anti-CD3 treated mice. Wildtype C57Bl/6 and Fas deficient B6.MRL-Fas^{lpr} mice were treated with vehicle or TCDD (15 μg/kg) by gavage one day prior to the injection of 50 μg of anti-CD3 in the rear foot pads. Lymph nodes were harvested at 48 and 72 hours after anti-CD3 injection as described in Materials and Methods. The percent and number of the CD8+ T cells were determined by flow cytometry. Data represent mean ± SEM of four animals per group Significant differences between the vehicle- and TCDD-treated groups are indicated by * ($p \leq 0.05$).

Figure 3.10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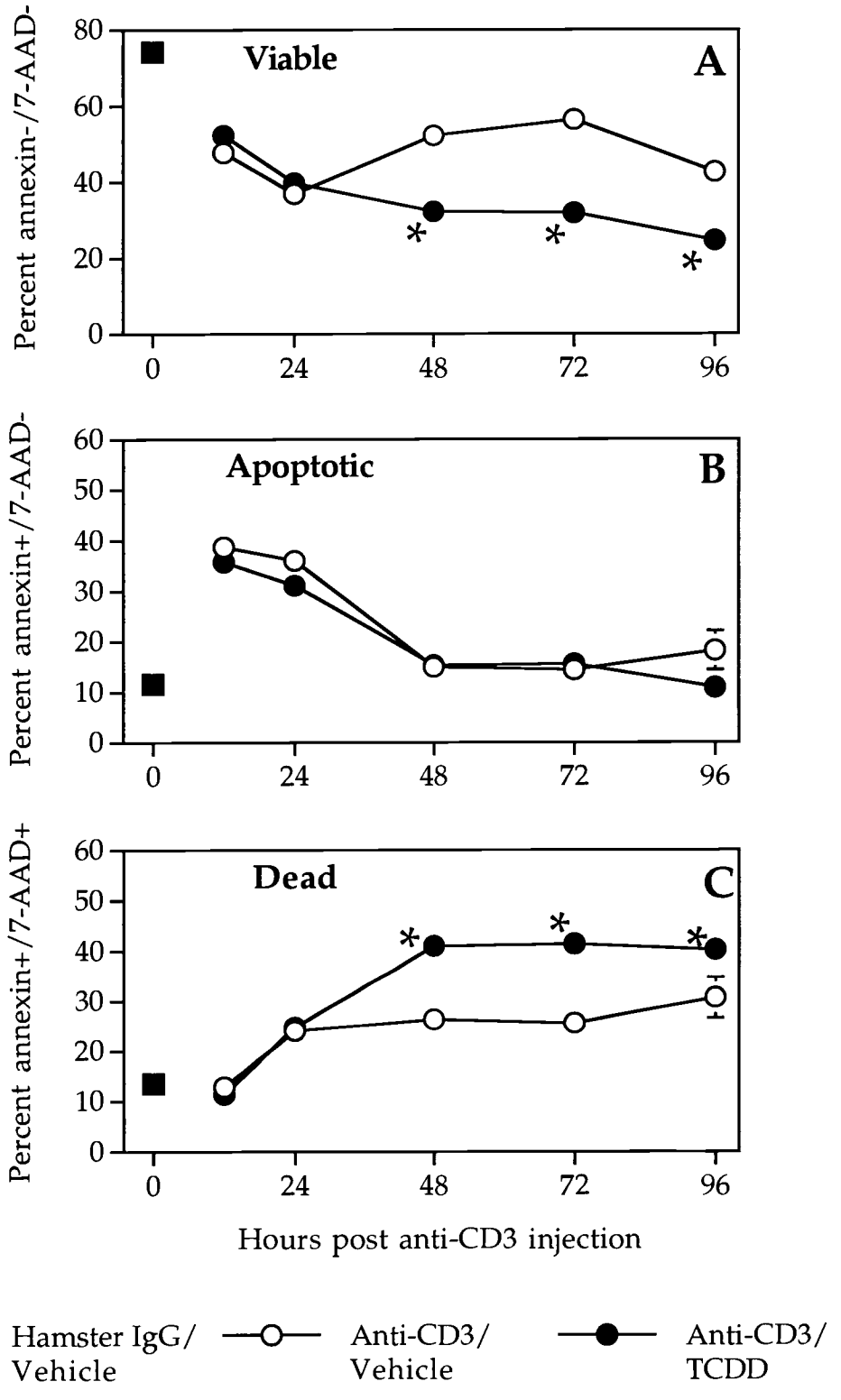
30
20
10
0
0
24
48
72
96
*
Number (x10^5)
60
50
40
30
20
10
0
0
24
48
72
96
*
*
Hours post anti-CD3 injection
Anti-CD3/Vehicle
Anti-CD3/TCDD
Hamster IgG/Vehicle

Figure 3.11


Viable
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Apoptotic
B
Dead
C
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Percent annexin+/7-AAD-
Percent annexin+/7-AAD+
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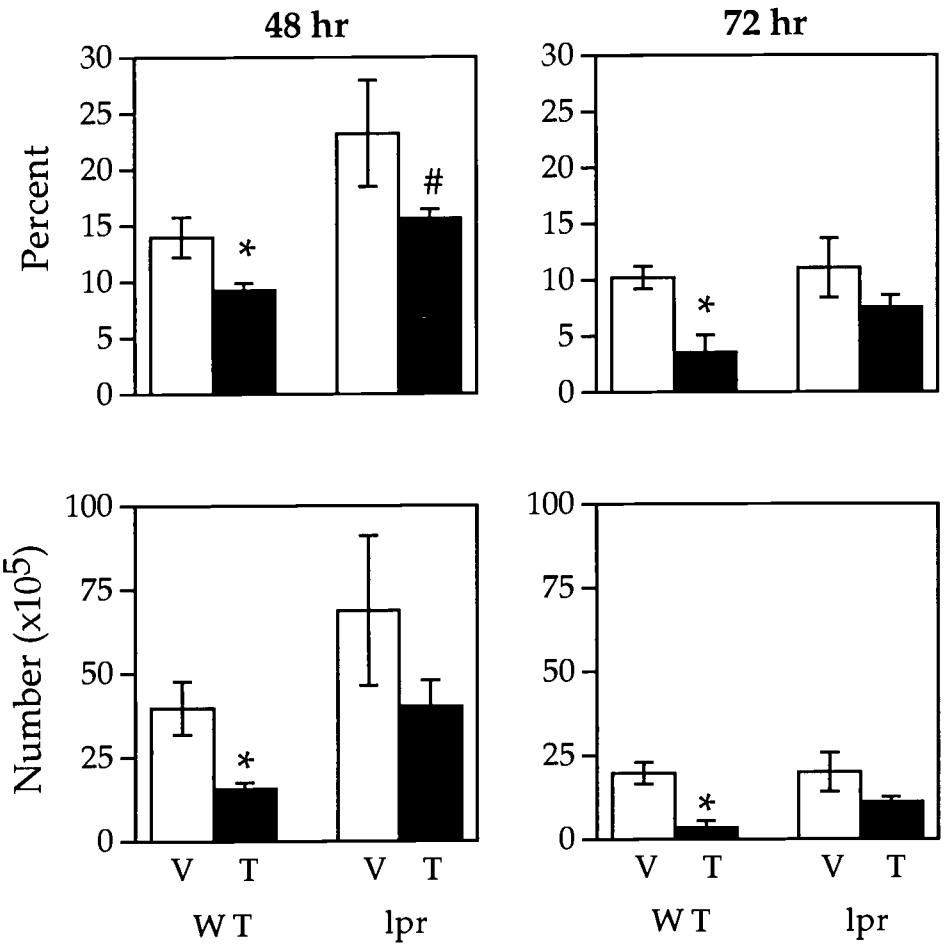
10
20
30
40
50
60
80
0
24
48
72
96
Hours post anti-CD3 injection
Hamster IgG/ Vehicle
Anti-CD3/ Vehicle
Anti-CD3/ TCDD

Figure 3.12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lpr

We also assessed CD8+ T cells in FasL deficient (gld) mice treated with anti-CD3 and TCDD. As shown in Figure 3.13, the percent (Fig 3.13A) and number (Fig 3.13B) of CD8+ T cells in gld mice increased at 48 hours after anti-CD3 injection as compared to 24 or 72 hours. These data are similar to the anti-CD3-induced effect observed in wildtype mice (see Figure 3.10). Exposure to TCDD did not affect the number of the CD8+ T cells in gld mice. Again, these results corroborate the findings using the Fas deficient mice and further support the conclusion that the TCDD-induced alterations in CD8+ T cells might be mediated by Fas/FasL interaction.

3.3.2.4 The involvement of the TNF pathway in anti-CD3-induced changes in CD8+ T cells

Results generated in the experiments evaluating the role of the TNF pathway in the TCDD-induced decreases in CD8+T cell number were difficult to interpret because of variability in the control response. For example, anti-CD3 did not cause as dramatic an increase in the number of CD8+ T cells at 48 hours after exposure (Fig 3.14). In addition, TCDD did not cause a similar increase in cell number at 48 hours, although the values were not significantly different between vehicle- and TCDD-treated mice at that timepoint. Treatment of mice with anti-TNF appeared to have an effect on further increasing the percent and number of CD8+ T cells from vehicle-treated mice at 48 hours, however anti-TNF did not affect the percent and number of CD8+ T cells from TCDD-treated mice at any timepoint tested (Fig 3.14A and 3.14B).

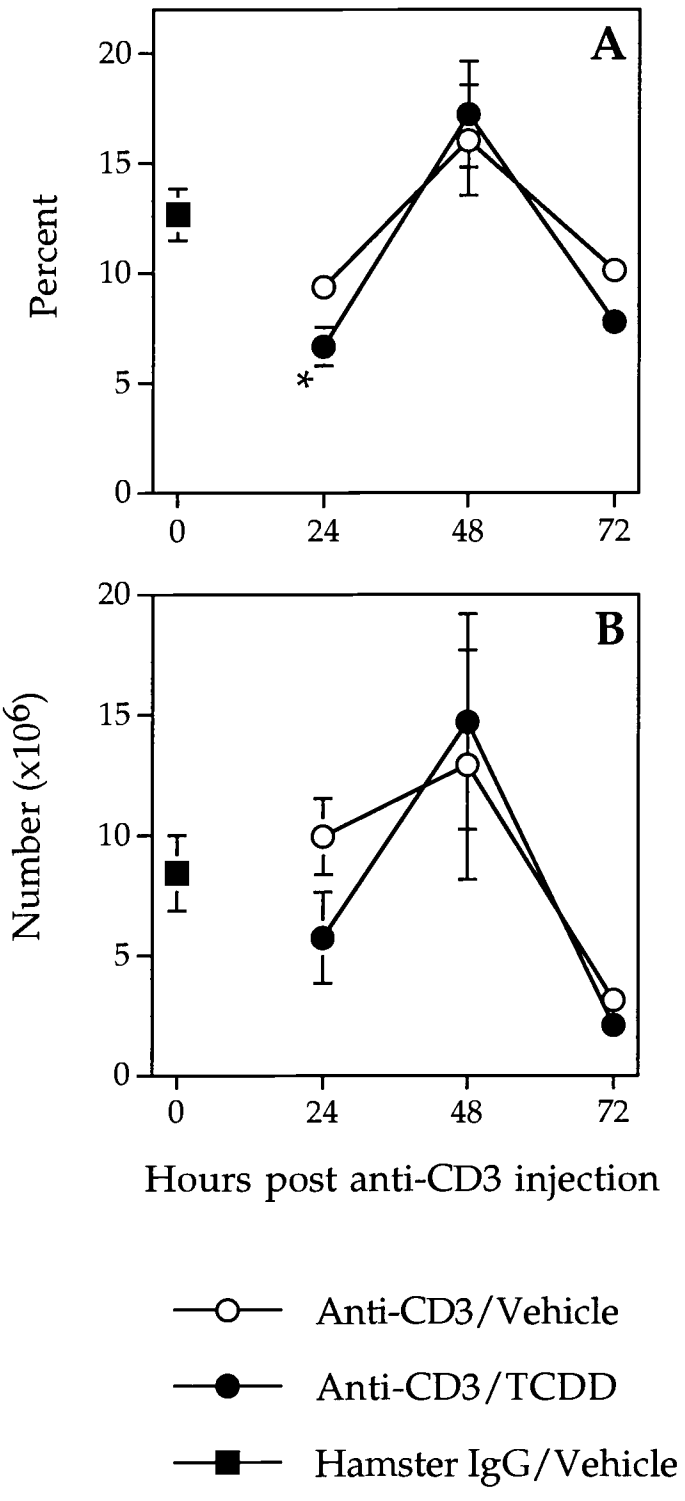
3.3.4 Summary

Taken together, our results suggest that both CD4+ and CD8+ T cells are susceptible to TCDD-enhanced deletion from anti-CD3-treated mice.

Figure 3.13: The effect of FasL deficiency on the TCDD-induced decrease in CD8+ T cells from the lymph nodes of anti-CD3 treated mice. FasL deficient B6Smn.C3H-Fasl^{gld} mice were treated with vehicle or TCDD (15 μg/kg) by gavage one day prior to the injection of 50 μg of anti-CD3 in the rear foot pads. Lymph nodes were harvested at various times after anti-CD3 injection as described in Materials and Methods. The percent (A) and number (B) of the CD8+ T cells were determined by flow cytometry. Data represent mean ± SEM of three animals per group Significant differences between the vehicle- and TCDD-treated groups are indicated by * ($p \leq 0.05$).

Figure 3.14: The effects of anti-TNF antibody treatment on the anti-CD3-induced alterations of CD8+ T cells. C57Bl/6 mice were injected ip with 1 mg of anti-TNF (triangles) or rat IgG (circles) antibody prior to treatment with Vehicle (open symbols) or TCDD (15 μg/kg) (closed symbols). One day later mice were injected with either 50 μg of anti-CD3 or hamster IgG (squares) in the rear foot pads. Lymph nodes were harvested at 24-72 hours after anti-CD3 injection and processed as described in Materials and Methods. The percent (A) and number (B) of CD8+ T cells were determined by flow cytometry. Data represent mean ± SEM of three animals per group. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 3.13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72
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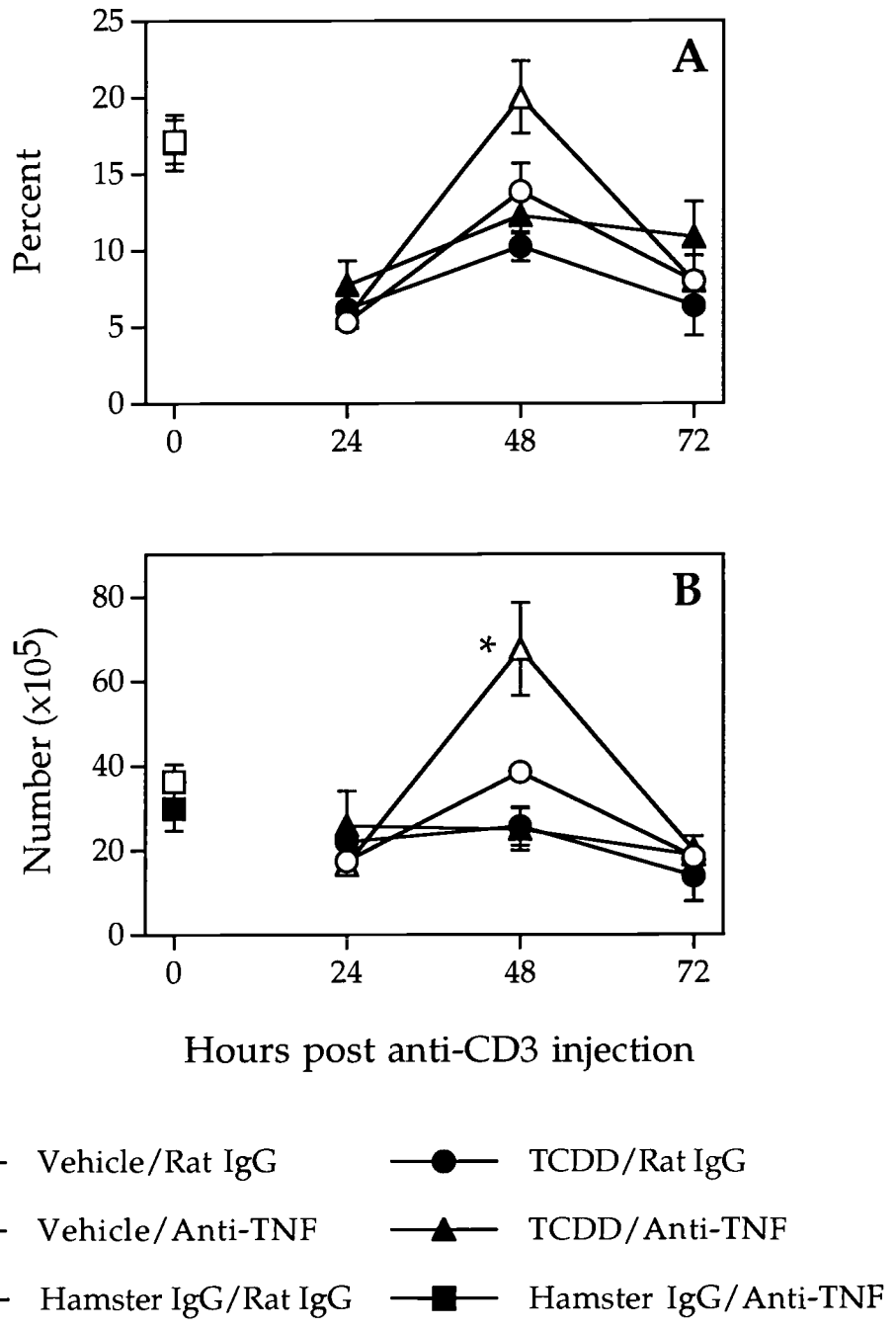
Hours post anti-CD3 injection
Anti-CD3/Vehicle
Anti-CD3/TCDD
Hamster IgG/Vehicle

Figure 3.14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24
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72
*
Hours post anti-CD3 injection
Vehicle/Rat IgG
TCDD/Rat IgG
Vehicle/Anti-TNF
TCDD/Anti-TNF
Hamster IgG/Rat IgG
Hamster IgG/Anti-TNF

Although apoptosis was difficult to detect in T cells examined directly out of the animal, the studies using the Fas and FasL deficient mice suggest that apoptosis via Fas/FasL interaction may play a role in TCDD-induced T cell deletion. However, the exact mechanism by which TCDD affects the Fas pathway remains to be elucidated.

3.4 Discussion

Anti-CD3, a molecule which binds to the T cell receptor, has been widely used as a tool to study T cell activation in vitro and in vivo. The in vivo administration of anti-CD3 leads to rapid and transient T cell activation which results in a massive release of cytokines, including IL-2, IFN-γ, IL-3, IL-6, TNFα, and GM-CSF, which have been shown to cause body weight loss (cachexia), hypothermia, diarrhea, and hypomotility (Hirsch et al., 1989; Alegre et al., 1991; Ferran et al., 1991a; Ferran et al., 1991b). In addition, anti-CD3 has also been shown to cause apoptosis of T cells in vitro (Brunner et al., 1995; Ju et al., 1995) and in vivo (Hirsch et al., 1988; Tucek-Szabo et al., 1996). Previous studies in our laboratory have reported that TCDD significantly decreased the percent of CD4+, but not CD8+ T cells in anti-CD3-treated animals (Prell et al., 1995). In addition, the forward and side scatter characteristics of the lymph node cells from TCDD- and anti-CD3-treated animals resembled those of apoptotic cells (i.e., decreased forward scatter and increased side scatter). The objective of the present studies was to determine whether the TCDD-induced loss in T cells was caused by increased apoptosis.

Annexin v is a sensitive flow cytometric method to determine apoptosis. Annexin v is a protein which binds to phosphatidylserine (PS), a membrane phospholipid that is translocated from the inner leaflet to the outer leaflet of cellular membranes in response to several apoptotic stimuli, including growth factor withdrawal, irradiation, and death

receptor engagement (van Engeland et al., 1998). The expression of PS on the cell surface is an early event in the process of apoptosis, occurring prior to the fragmentation of DNA. Annexin v can also be used with several other fluorescently-labeled antibodies, allowing for the determination of apoptosis within a specific population of cells. In addition, annexin v, when used simultaneously with a viability dye, can distinguish viable, apoptotic, and dead cells.

In our study, anti-CD3 caused an increase in the percent of apoptotic (annexin+/7-AAD-) CD4+ and CD8+ T cells. However, exposure to TCDD did not increase the percent of apoptotic CD4+ or CD8+ T cells as compared to vehicle controls. In addition, the percent of dead (annexin+/7-AAD+) CD4+ and CD8+ T cells was not consistently altered by TCDD. In two of four experiments TCDD significantly increased the percentage of dead T cells, while in two experiments, TCDD did not alter the percentage of dead T cells. These results indicate that there is not a strong correlation between the decreased numbers of CD4+ and CD8+ T cells following TCDD exposure and cell death as measured by annexin staining.

The lack of correlation could reflect enhanced migration of the T cells out of the lymph nodes instead of increased apoptosis. However, the this interpretation seems unlikely since anti-CD3 causes T cell deletion by apoptosis in vivo (Hirsch et al., 1988; Tucek-Szabo et al., 1996). Another possible explanation for the lack of correlation could be that the amount of annexin staining detected in a single experiment might be influenced by several factors, such as the kinetics of apoptosis induction, the length of time an apoptotic cell remains in the lymph nodes, and the phagocytosis of apoptotic cells. Alternatively, annexin staining may not be sensitive enough to detect the relatively small effect of TCDD in addition to anti-CD3's effect.

Our results contrast with a recent report that TCDD increased apoptosis of antigen activated but not resting lymph node cells from mice treated with anti-CD3 plus complete Freund's adjuvant (CFA)

(Pryputniewicz et al., 1998). In these studies, the TCDD-induced decrease in cell proliferation correlated with an increase in apoptotic lymph node cells. There are several differences between the studies by Pryputniewicz et al (1998) and our studies which may account for these dissimilar results. First, the authors only detected increased apoptosis after in vitro culture of the lymph node cells and suggested that this culture period was necessary to prevent the rapid phagocytosis of apoptotic cells by neighboring cells. However, we were unable to detect increased apoptosis in TCDD-treated mice, even after in vitro culture with media for 24 hours. A second difference between the studies was the dose of TCDD. They used a higher dose of TCDD than we did (50 μg/kg as compared to 15 μg/kg, respectively). Since we have previously shown that a 15 μg/kg dose of TCDD causes increased necrosis in the lymphoid organs of anti-CD3-treated mice (Prell et al., 1995), it is possible that a higher dose of TCDD results in even more necrosis and therefore may confound their results. Third, the authors did not report the concentration of anti-CD3 used in their studies and it is possible that the dose used was different than ours. Higher doses of anti-CD3 are more toxic to the animals and may lead to a greater amount of apoptosis in T cells (Tucek-Szabo et al., 1996). Finally, their unconventional approach of including anti-CD3 in CFA may have altered the response.

Since our annexin data was inconclusive, alternative approaches were used to examine the role of apoptosis in anti-CD3- and TCDD-treated mice. For example, the Fas pathway has been shown to be one of the major mechanisms by which anti-CD3 causes apoptosis in vitro (Brunner et al., 1995; Ju et al., 1995) and is a mechanism by which autoreactive peripheral T cells are deleted in vivo (Singer and Abbas, 1994; Nagata and Golstein, 1995; Van Parijs et al., 1996). Fas, a member of the TNFR family, contains a death domain which is essential for the delivery of the death signal. Fas is expressed on many cell types and is upregulated upon activation (Nagata and Golstein, 1995). FasL, a member of the TNF family, is a protein that is

expressed on activated T cells and dendritic cells and in "immune privileged" areas of the body such as the eye and testes (Nagata and Golstein, 1995). Ligand binding to Fas initiates a series of protein-protein interactions and enzyme cleavage that results in the activation of caspases, the effectors of apoptosis (Peter and Krammer, 1998; Scaffidi et al., 1998; Lenardo et al., 1999).

We used Fas deficient B6.MRL-Fas^{lpr} and FasL deficient B6Smn.C3H-Fasl^{gld} mice which are defective in apoptosis (Reap et al., 1995). If TCDD enhanced T cell apoptosis via Fas, then these mice should not show T cell deletion. Unexpectedly, lpr mice responded to anti-CD3 in similar manner as wildtype mice, as evidenced by a similar reduction in body weight and an equivalent decrease in the percent and number of CD4+ and CD8+ T cells. One possible explanation for why lpr mice were sensitive to anti-CD3-induced T cell deletion is that the lpr mutation is leaky and some level of Fas expression and signaling can occur in lpr animals (Yamagiwa et al., 1996). However, we did not detect any Fas expression on the T cells in the lpr animals, making this explanation unlikely. These results are consistent with a report which showed that T cells from lpr mice are capable of being activated by anti-CD3 and induced to undergo apoptosis (Tucek-Szabo et al., 1996). These results also suggest that anti-CD3 does not use the Fas pathway to delete T cells in vivo. It is possible that other apoptotic signaling pathways can compensate for the defect in lpr mice and cause T cell deletion (Sytwu et al., 1996).

In contrast to lpr mice, gld mice were less sensitive to anti-CD3. They did not lose as much body weight in response to anti-CD3 as compared to wildtype mice, and there was no decline in the percent or number of CD4+ T cells after anti-CD3 treatment. These results suggest that FasL is necessary for anti-CD3-induced T cell deletion.

Despite the difference in the responses of the lpr and gld mice to anti-CD3 alone, the TCDD-enhanced deletion of CD4+ and CD8+ T cells was not evident in either lpr or gld mice. These results suggest that

TCDD-enhanced deletion of T cells from anti-CD3-treated mice may be mediated by Fas/FasL interaction. This conclusion would be consistent with a recently reported Fas-mediated mechanism of TCDD-induced thymic atrophy (Rhile et al., 1996; Kamath et al., 1999).

Since Fas expression is upregulated on T cells following activation stimuli, one way TCDD could cause death is to increase Fas expression. However, when we examined CD4+ and CD8+ T cells following anti-CD3 treatment, there was no difference in the expression between vehicle- and TCDD-treated mice. Therefore, enhanced Fas expression does not appear to be the mechanism of TCDD-induced T cell deletion.

Alternatively, FasL expression could be increased by exposure to TCDD. Unfortunately, we could not detect FasL expression on the T cells by flow cytometry. However, this result was not surprising since FasL protein is transiently expressed after activation and is rapidly cleaved from the cell surface after expression (Nagata and Golstein, 1995). We also attempted to look at FasL expression by RT-PCR. However, we were unable to detect consistent levels of FasL message in anti-CD3-treated T cells or total lymph node cells. Interestingly, a recent report showed that TCDD increased FasL message levels in thymocytes (Kamath et al., 1999). Thus, although it remains to be formally demonstrated, it is possible that TCDD might also increase FasL mRNA in peripheral T cells.

Another important factor induced in response to anti-CD3 is the inflammatory cytokine, TNF (Alegre et al., 1991; Ferran et al., 1991; Prell et al., 1995). TNF has been shown to cause apoptosis of T cells both in vitro (Zheng et al., 1995) and in vivo (Speiser et al., 1996). Likewise, TCDD has been shown to influence TNF levels in several different systems (Clark et al., 1991; Kerkvliet et al., 1996; Moos et al., 1997). We examined the potential role of TNF in TCDD-induced T cell deletion by treating mice with an antibody that inhibits TNF activity. Interestingly, anti-TNF treatment increased the percent and number of CD4+ T cells in anti-CD3-treated mice, but was unable to prevent the deletion of CD4+ cells

from TCDD-treated mice. These data suggest that TCDD enhanced CD4+ T cell deletion is independent of TNF. Due to an inconsistent control response in the CD8+ T cells, the effect of anti-TNF on these cells remains to be determined.

In conclusion, we have shown that the TCDD-induced deletion of CD4+ and CD8+ T cells from anti-CD3-treated mice may be caused by increased cell death. In addition, our results show that Fas/FasL interaction may play a role in the deletion of both CD4+ and CD8+ T cells from TCDD- and anti-CD3-treated mice, thus providing suggestive evidence that apoptosis is involved in TCDD-induced T cell deletion. In contrast, the TNF pathway does not appear to mediate the TCDD-enhanced deletion of CD4+ T cells from anti-CD3-injected mice. Taken together, these results suggest that TCDD might influence aspects of the Fas pathway (such as FasL expression or proteins involved in the signaling cascade like FLICE and FADD), which may be responsible for TCDD-induced T cell deletion.

Chapter 4

The Effect of Exposure to TCDD on T Cell Apoptosis in Mice Injected with P815 Allogeneic Tumor Cells

Authors:

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Nancy I. Kerkvliet

4.1 Introduction

Our laboratory has previously shown that the P815 tumor allograft rejection model is sensitive to the suppressive effects of TCDD (Kerkvliet et al., 1990a; De Krey and Kerkvliet, 1995; Kerkvliet et al., 1996). The cytotoxic T lymphocyte (CTL) response, alloantibody response, and cytokine production (IL-2, IFN-γ, and TNF) are suppressed in P815-injected animals treated with TCDD. In addition, TCDD exposure has been shown to cause a decrease in the number of CD4+ T cells on days 8 and 10 of the response. Furthermore, exposure to TCDD resulted in a significant decrease in the number of CD8+ T cells on days 9 and10 of the response as compared to vehicle-treated mice (Kerkvliet et al., 1996). Therefore, we tested the hypothesis that TCDD causes the decrease in CD4+ and CD8+ T cells by increased apoptosis.

4.2 Materials and Methods

4.2.1 Animals

Male C57Bl/6J mice were purchased from Jackson Labs (Bar Harbor, ME) at 6-8 weeks of age. All animals were housed in front of laminar flow units and were fed standard rodent chow and tap water *ad libitum*.

4.2.2 Reagents

All cell culture reagents were purchased from GibcoBRL (Grand Island, NY) unless otherwise stated. Fetal bovine serum (FBS) was purchased from Hyclone (Ogden, UT). Phycoerythrin (PE)-labeled anti-CD4 (clone RM4-5), PE-labeled anti-Fas (clone Jo2), Fitc-labeled anti-CD4 (clone GK1.5), biotinylated-anti-CD4 (clone RM4-5) and biotinylated-anti-CD8 (clone 53-6.7) were purchased from Pharmingen (San Diego, CA). Tri-color anti-CD8 (clone CT-CD8a) was purchased from Caltag (Burlingame, CA).

Steptavidin conjugated to Red613 and Red613-labeled anti-CD8 (clone 53-6.7) were purchased from GibcoBRL.

4.2.3 Animal treatment

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1.5 μg/ml. A vehicle control solution was prepared in a similar manner. Animals were given a single dose of 15 μg/kg body weight by gavage. Mice were injected intaperitoneally (ip) with viable P815 allogeneic tumor cells ($1x10^7$) one day after treatment with vehicle or TCDD. Animals were killed by CO_2 overdose and the spleens were harvested on different days relative to antigen injection.

4.2.4 Preparation of spleen cells

A single cell suspension was prepared by pressing the spleen between the frosted ends of two microscope slides. The cell suspension was washed once and resuspended in HBSS supplemented with 5% FBS and 20mM HEPES buffer (HBSS-5) and erythrocytes were depleted by hypotonic lysis. The cells were then washed in HBSS-5 and enumerated using a Coulter counter (Coulter Electronics, Hialeah, FL).

4.2.5 Flow cytometry

Spleen cells ($1\text{-}2 \times 10^6$) were incubated on ice in 96-well V bottom plates (Costar, Corning, NY) in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Non-specific binding of the antibodies to Fc receptors was blocked by the addition of 30 μg of ratIgG (Jackson Immunoresearch Labs, Inc., West Grove, PA) for 15 min on ice. Subsequently, the cells were stained for 10 min on ice with the specific antibodies mentioned above for CD4 and CD8. Non-specific fluorescence was measured using appropriately labeled isotype-matched IgGs. Data were collected on at least 50,000 freshly stained cells by listmode aquisition using a Coulter XL flow cytometer

(Coulter Electronics, Hialeah, FL) and analyzed using Winlist software (Verity Software House, Topsham, ME).

4.2.6 Flow cytometric analysis of apoptosis

After surface staining, the cells were stained for apoptosis using 7-aminoactinomycin D (7-AAD) from Calbiochem (La Jolla, CA) and FITC- or PE-labeled annexin v from R&D systems (Minneapolis, MN) following the manufacturer's protocol. Briefly, the cells were resuspended in 1X binding buffer (HEPES buffered saline, 25mM $CaCl_2$) and stained with 0.1 μg labeled annexin v and 2 μg 7-AAD. Data were collected and analyzed as described above. This method allows for the discrimination of viable, apoptotic, and dead cells (see Figure 3.1).

4.2.7 Statistical analysis

Results are presented as mean $\pm$ SEM of 3-6 animals/group unless stated otherwise. Analysis of variance modeling was performed using Statview Statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using Fisher's least significant difference multiple comparison t test or Student's T test. Values of $p \leq 0.05$ were considered statistically significant.

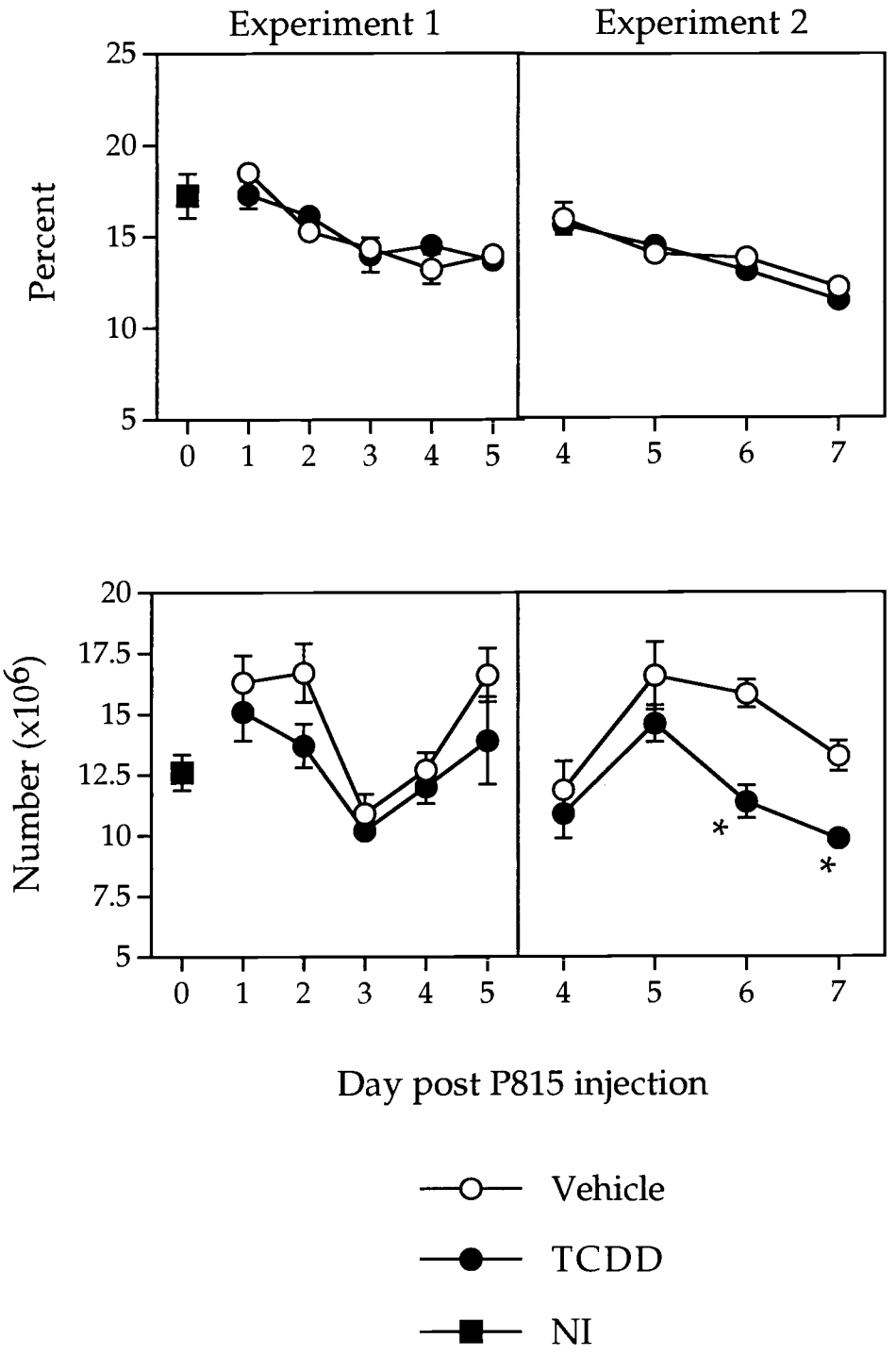
4.3 Results

4.3.1 Investigations on CD4+ T cells

As shown in Figure 4.1, the percent of CD4+ T cells from TCDD-treated mice injected with P815 tumor cells was not different from vehicle-treated mice on days 1-7. Interestingly, TCDD exposure caused a decrease in the total number of splenic CD4+ T cells on days 6 and 7 after P815 injection. These results are similar to those reported by Kerkvliet et al (1996).

Figure 4.1: The effect of TCDD on the percent and number of CD4+ T cells in the spleen of P815-treated mice. Male C57Bl/6 mice were gavaged with either vehicle (open circle) or TCDD (15 μg/kg) (closed circle) one day prior to the injection of $1x10^7$ live P815 tumor cells into the peritoneal cavity. Spleens were removed at various timepoints after antigen exposure, as described in Materials and Methods, and the percent (A) and number (B) of CD4+ T cells were analyzed by flow cytometry. Non-injected (NI) mice are represented as time zero. Data represent mean ± SEM of five to six mice per group per timepoint. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 4.1


Experiment 1
Experiment 2
Percent
25
20
15
10
5
0 1 2 3 4 5 4 5 6 7
Number (x10^6)
20
17.5
15
12.5
10
7.5
5
0 1 2 3 4 5 4 5 6 7
*
*
Day post P815 injection
Vehicle
TCDD
NI

In order to determine whether the TCDD-induced decrease in CD4+ T cell number was a result of increased cell death, we measured PS expression using annexin v, as previously described. As shown in Table 4.1, TCDD did not alter the percentage of cells undergoing apoptosis on days 1-4 after antigen injection. Unexpectedly, on days 5-7 after P815 injection, TCDD significanly decreased the percentage of CD4+ T cells expressing an apoptotic phenotype. In these experiments spleen cells were depleted of RBC by water lysis. This technique also removes dead cells and therefore the percentage of spleen cells that were positive for 7-AAD was low (<5%). However, when CD4+ T cells were analyzed for apoptosis from spleens depleted of RBC using ammonium chloride treatment (ACT), which does not remove dead cells, there was no effect of TCDD on the percentage of dead cells (data not shown). Therefore, these data suggest that the decrease in CD4+ T cells in TCDD-treated mice was not caused by increased apoptosis.

4.3.2 Investigations on CD8+ T cells

We also monitored the percent and number of CD8+ T cells in the same experiments shown in section 4.3.2. As shown in Figure 4.2, TCDD caused a decrease in the percent of CD8+ T cells on day 1, and 5-7 relative to antigen exposure. Similarly, exposure to TCDD caused a significant decrease in the number of CD8+ T cells on days 2, 6, and 7 after P815 injection. These results are similar to those reported by Kerkvliet et al (1996).

We also examined the effect of TCDD exposure on inducing the CD8+ T cells from P815-treated animals to undergo apoptosis. As shown in Table 4.2, there was a significant decrease in the percentage of CD8+ T cells expressing an apoptotic phenotype in TCDD-treated animals on days 1-9 after P815 injection. In addition, treatment with TCDD had no effect on the

Table 4.1. TCDD exposure decreases the percentage of CD4+ T cells staining positive for apoptosis.[a]

Day After P815[b]	Vehicle	TCDD
1	11.2 ± 0.6[c]	10.0 ± 0.5
2	13.7 ± 0.8	11.8 ± 0.3
3	11.4 ± 0.7	11.1 ± 0.5
4	11.5 ± 0.7	9.8 ± 0.7
5	10.8 ± 0.4	8.3 ± 0.5*
6	10.3 ± 1.0	6.9 ± 0.7*
7	19.7 ± 1.3	14.1 ± 1.0*

[a] Apoptosis of the CD4+ T cells is designated by cells which are positive for annexin v and exclude 7-AAD.

[b] Male C57Bl/6J mice were gavaged with either peanut oil vehicle or TCDD (15 μg/kg) one day prior to injection of 1×10^7 P815 tumor cells. The spleens were harvested at various times after immunization and assayed for apoptosis as described in Materials and Methods.

[c] Data represent mean ± SE of 5 - 6 animals per group. Significant differences ($p \leq 0.05$) between vehicle- and TCDD-treated mice are indicated by an asterisk. The data represent two separate experiments.

percentage of dead CD8+ T cells. These data suggest that the TCDD-induced decrease in CD8+ T cells is not caused by enhanced apoptosis.

Table 4.2. TCDD exposure decreases the percentage of CD8+ T cells staining positive for apoptosis.[a]

Day After P815[b]	Vehicle	TCDD
1	11.6 ± 0.5[c]	9.3 ± 0.3*
2	12.2 ± 0.4	10.0 ± 0.4*
3	9.6 ± 0.5	7.3 ± 0.4*
4	10.2 ± 0.6	5.8 ± 0.4*
5	11.6 ± 0.9	6.0 ± 0.8*
6	12.8 ± 1.5	3.4 ± 0.3*
7	21.3 ± 0.8	5.1 ± 0.1*
9	21.9 ± 1.0	12.6 ± 0.7*

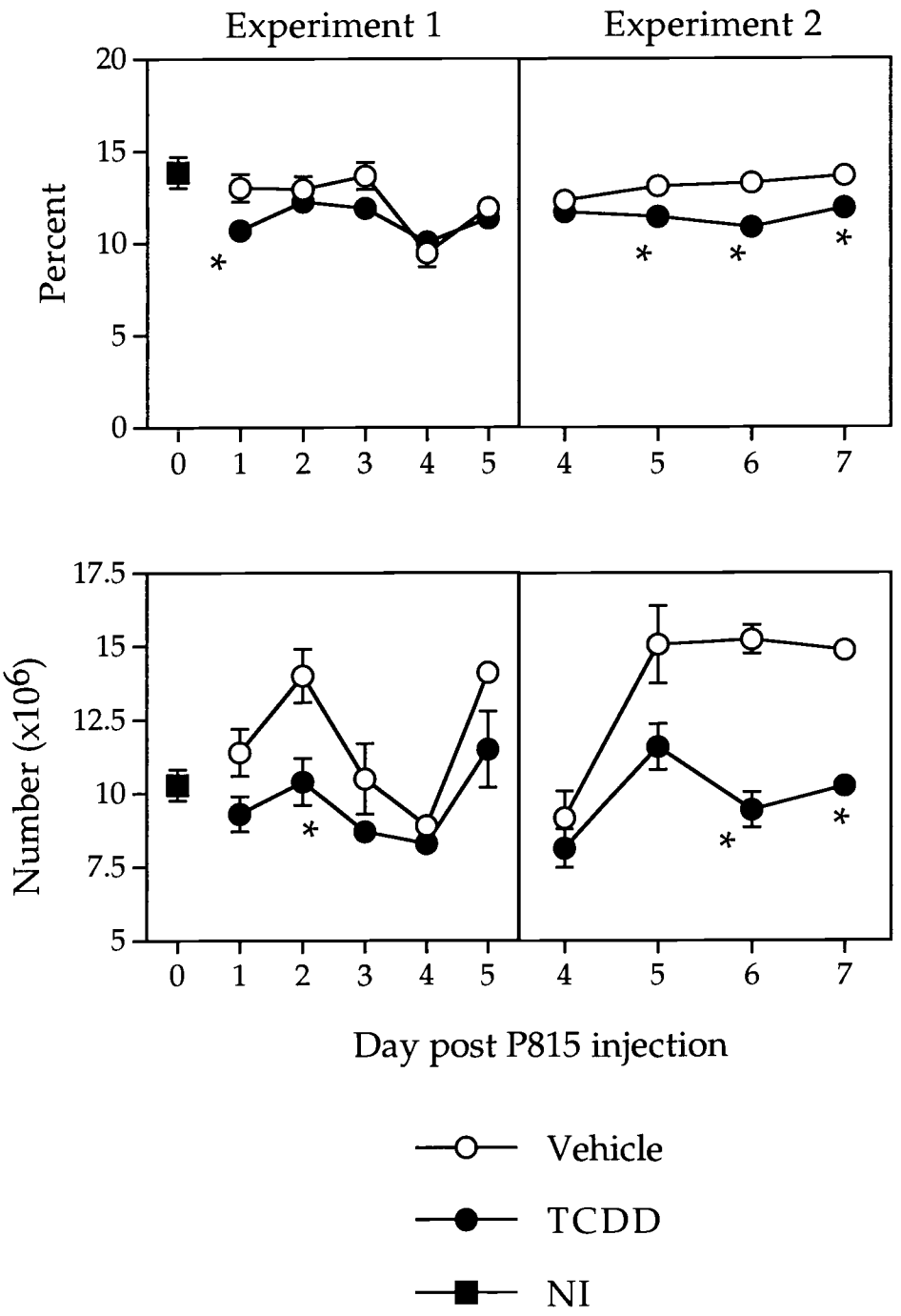
[a] Apoptosis of the CD8+ T cells is designated by cells which are positive for annexin v and exclude 7-AAD.
[b] Male C57Bl/6J mice were gavaged with either peanut oil vehicle or TCDD (15 μg/kg) one day prior to injection of 1×10^7 P815 tumor cells. The spleens were harvested at various times after immunization and assayed for apoptosis as described in Materials and Methods.
[c] Data represent mean ± SE of 3 - 6 animals per group. Significant differences ($p \leq 0.05$) between vehicle- and TCDD-treated mice are indicated by an asterisk. The data represent three separate experiments.

4.4 Discussion

Previous studies in our laboratory have shown that exposure to TCDD suppresses the cytotoxic T lymphocyte (CTL) and alloantibody responses to P815 allogeneic tumor cells and decreases the number of splenic CD4+ and CD8+ T cells (Kerkvliet et al., 1990; De Krey and Kerkvliet, 1995; Kerkvliet et al., 1996). The objective of the present studies was to determine whether the TCDD-induced decrease in the number of CD4+ and CD8+ T cells was due to increased apoptosis.

Figure 4.2: The effect of TCDD on the percent and number of CD8+ T cells in the spleen of P815-treated mice. Male C57Bl/6 mice were gavaged with either vehicle (open circle) or TCDD (15 μg/kg) (closed circle) one day prior to the injection of $1x10^7$ live P815 tumor cells into the peritoneal cavity. Spleens were removed at various timepoints after antigen exposure, as described in Materials and Methods, and the percent (A) and number (B) of CD8+ T cells were analyzed by flow cytometry. Non-injected (NI) mice are represented as time zero. Data represent mean ± SEM of five to six mice per group per timepoint. * Indicates statistically significant difference from vehicle-treated group ($p \leq 0.05$).

Figure 4.2


Experiment 1
Experiment 2
Percent
20
15
10
5
0
0 1 2 3 4 5 4 5 6 7
Number (x10^6)
17.5
15
12.5
10
7.5
5
0 1 2 3 4 5 4 5 6 7
Day post P815 injection
Vehicle
TCDD
NI

Previous studies in our laboratory have demonstrated that CD4+ T cells are essential for the development of both the CTL and alloantibody responses (Kerkvliet et al., 1996). Interestingly, these studies also showed that the timeframe in which CD4+ T cells are required for generation of the CTL response correlated with the window of sensitivity of the response to TCDD. Therefore, it was afterulated that TCDD causes immune suppression by altering the activation of the CD4+ T cells. When we examined the presence of CD4+ T cells in the spleens of P815 injected mice, we found that exposure to TCDD decreased the number of cells during the later days of the response. Surprisingly, the TCDD-induced decrease in the number of CD4+ T cells did not correlate with enhanced apoptosis, as measured by annexin v and 7-AAD. In fact, TCDD actually decreased the percent of apoptotic CD4+ T cells during the later days of the response. Thus, the TCDD-induced decrease in CD4+ T cells does not appear to be mediated by an increase in apoptosis. It is important to keep in mind, however, that these results were generated by gating on the entire population of CD4+ T cells, not only the antigen-specific cells. Since the population of CD4+ T cells responding to P815 is very small, TCDD-induced changes occuring specifically in these cells may not be detectable when all CD4+ T cells are analyzed together. Therefore, the effect of TCDD on activated antigen-specific CD4+ T cell apoptosis remains to be elucidated.

TCDD exposure also results in a decrease in CTL activity in P815-treated mice, which correlates with a reduced population of CTL effectors (CTL_E) (Kerkvliet et al., 1996). One potential mechanism by which TCDD exposure suppresses CTL_E development could be through the induction of apoptosis rather than activation of CD8+ T cells. However, this explanation seems unlikely since the percentage of apoptotic CD8+ T cells was actually less in TCDD-treated mice as compared to vehicle-treated mice. The inability of TCDD to enhance apoptosis of CD8+ cells was also supported by studies conducted using mice treated with superantigen

(SEA). SEA activates Vβ3+ T cells which are subsequently deleted by apoptosis (McCormack et al., 1993; McCormack et al., 1994; Vella et al., 1997). Exposure to TCDD failed to augment, and instead delayed, the deletion of CD8+Vβ3+ T cells following injection of SEA (Prell et al., 2000). Therefore, these results indicate that enhanced apoptosis is not involved in TCDD-induced suppression of the CD8+ T cells.

There are alternative mechanisms by which TCDD could suppress the CTL response. For example, we have recently shown that TCDD suppresses the number of activated precursor CTL (CTL_P*) (Oughton and Kerkvliet, 1999). In addition, we have also shown that the CTL response in TCDD-treated mice can be restored if the CD8+ T cells receive the appropriate signals, such as B7-costimulation (Prell and Kerkvliet, 1997) and IL-2 (Prell et al., 2000), during the time of CTL activation. Therefore, these results indicate that the TCDD-induced suppression of the CTL response is most likely mediated by a lack of activation of the CTL_P. However, the exact mechanism by which TCDD inhibits the activation of CTL_P has not been fully elucidated.

Chapter 5

The Effect of Exposure to TCDD on Ovalbumin-specific DO11.10 CD4+ T Cell Apoptosis in Adoptively-transferred Mice

Authors:

Erica A. Dearstyne

David M. Shepherd

Nancy I. Kerkvliet

5.1 Introduction

The results from the studies performed in the P815 tumor allograft rejection model did not support the hypothesis that TCDD induces CD4+ T cells to undergo apoptosis following activation. However, these results were generated by analyzing the entire population of CD4+ T cells, not just the antigen specific cells. Because antigen-specific cells occur at a frequency that is below the limits of detection, changes occurring in the antigen-specific population may not be apparent when the entire population of T cells is evaluated.

In order to overcome the problem, we used the ovalbumin-specific DO11.10 T cell receptor (TCR) transgenic (Tg) mice. In these mice, all the T cells are specific for a peptide of ovalbumin ($OVA_{323-339}$). The response to OVA peptide is not normal in intact DO11.10 mice, presumably due to the high frequency of antigen-specific cells present (Kearney et al., 1994). Therefore, an adoptive transfer system has been reported in which a fixed number of the transgenic T cells are injected into a nontransgenic, syngeneic recipient (Pape et al., 1997). The activation of the Tg T cells can then be followed in the recipient by flow cytometry using the clonotype specific KJ1-26 anti-Tg TCR antibody (Haskins et al., 1983).

Our laboratory has used the DO11.10 adoptive transfer model to examine the effects of TCDD on the activation of ovalbumin (OVA)-specific $CD4^+$ T cells. We have shown that although the antibody response to OVA was suppressed in TCDD-treated mice, very few alterations in the activation of the OVA-specific $CD4^+$ T cells were observed (Shepherd et al., 2000). Specifically, TCDD did not affect the expression of activation markers or the clonal expansion of OVA-specific CD4+ T cells. Interestingly, TCDD decreased the number of the OVA-specific $CD4^+$ T cells on days 5-10 after immunization with OVA/CFA. From these results, we hypothesized that TCDD increases apoptosis of the activated

antigen-specific CD4+ T cells, thereby leading to fewer numbers present in the spleen.

5.2 Materials and Methods

5.2.1 Animals

Balb/c mice (male and female) were purchased from either Jackson Labs or B & K Universal Incorporated (Kent, WA) at 6-8 weeks of age. DO11.10 transgenic mice (a generous gift of Dr. Marc Jenkins, University of Minnesota Medical School) were bred in our pathogen-free animal facility according to the National Research Council guidelines and were used at 3-6 months of age. All animals were housed in front of laminar flow units and were fed standard rodent chow and tap water *ad libitum*.

5.2.2 Reagents

All cell culture reagents were purchased from GibcoBRL (Grand Island, NY) unless otherwise stated. Fetal bovine serum (FBS) was purchased from Hyclone (Ogden, UT). Biotinylated-KJ1-26 antibody and KJ1-26 hybridoma was provided by Dr. Marc Jenkins. KJ1-26 antibody was purified from culture supernatant using Protein A sepharose Fast Flow columns and biotinylated using N-hydroxysuccinimido-biotin from Sigma. Phycoerythrin (PE)-labeled anti-CD4 (clone RM4-5), PE-labeled anti-Fas (clone Jo2), Fitc-labeled anti-CD4 (clone GK1.5), biotinylated-anti-CD4 (clone RM4-5) and biotinylated-anti-CD8 (clone 53-6.7) were purchased from Pharmingen (San Diego, CA). Tri-color anti-CD8 (clone CT-CD8a) was purchased from Caltag (Burlingame, CA). Steptavidin conjugated to Red613 and Red613-labeled anti-CD8 (clone 53-6.7) were purchased from GibcoBRL. OX40 expression was assessed using soluble OX40L:huIg fusion protein (generous gift from Dr. Andrew Weinberg, Earl A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Medical

Center). Anti-OX40 agonistic antibody was generously provided by Dr. Andrew Weinberg.

5.2.3 Animal treatments

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1.5 μg/ml. A vehicle control solution was prepared in a similar manner. Animals were given a single dose of 15 μg/kg body weight by gavage. In some experiments, both the donor DO11.10 and recipient Balb/c mice were gavaged with TCDD in order to provide exposure to TCDD throughout the entire adoptive transfer procedure. It was subsequently determined experimentally that exposure of the DO11.10 mice to TCDD was not necessary for the suppression of the response. Therefore in many experiments only Balb/c recipient mice were gavaged with TCDD. For the dose response experiments, both donor DO11.10 and recipient Balb/c mice were gavaged with a single dose of 15, 5, 0.5, or 0 μg/kg TCDD.

5.2.4 DO11.10 adoptive transfer model

The protocol for the adoptive transfer of DO11.10 T cells has been previously described (Kearney et al., 1994), with slight modifications. Briefly, spleen cells from DO11.10 mice were harvested, processed as described below, pooled, and the percentage of CD4+KJ1-26+ cells determined by flow cytometry. Balb/c recipient mice (sex and age matched) were intravenously (iv) injected with $5x10^6$ CD4+KJ1-26+ T cells (total spleen cells injected $\approx 2x10^7$) in a volume of 0.5 ml. The adoptively-transferred mice were rested for two days and then immunized ip with 2 mg OVA emulsified in CFA in a volume of .25 ml. In two experiments, vehicle- or TCDD-treated adoptively-transferred Balb/c mice were injected with 100 μg anti-OX40 or control ratIgG antibody/injection on day 0 and +3 relative to OVA/CFA injection. In a separate set of experiments, vehicle- or TCDD-treated adoptively-transferred mice were

injected with 0.5 μg/injection recombinant mouse IL-12 (R&D Systems, Minneapolis, MN) on days -1, 0, and +1 relative to OVA/CFA injection. Mice were sacrificed and the spleens were harvested at various times after antigen exposure.

5.2.5 CFSE-labeling of DO11.10 splenocytes

CFSE (5-(and 6)-carboxyfluorscein diacetate, succinimidyl ester) was used to monitor the proliferation of OVA-specific DO11.10 cells in vivo (Lyons and Parish, 1994). The dye freely diffuses into cells and becomes fluorescent and non-permeable upon cleavage of the carboxyl groups by cellular esterases. Upon cell division, CFSE is equally divided among daughter cells, thereby decreasing the fluorescence by half with each division. It is possible to follow up to eight discrete cell divisions (Fig 5.1). Spleen cells from DO11.10 mice were labeled with 10 μM CFSE (Molecular Probes, Eugene, OR) at room temperature in the dark for 8 min. After labeling, the cells were adoptively-transferred into syngeneic Balb/c hosts as described above.

5.2.6 Preparation of spleen cells

A single cell suspension was prepared by pressing the spleen between the frosted ends of two microscope slides. The cell suspension was washed once and resuspended in HBSS supplemented with 5% FBS and 20mM HEPES buffer (HBSS-5) and enumerated using a Coulter counter (Coulter Electronics, Hialeah, FL). Erythrocytes were depleted by ammonium chloride. The cells were then washed in HBSS-5 and enumerated using a Coulter counter.

5.2.7 Flow cytometry

Spleen cells (1-2 x 10^6) were incubated on ice in 96-well V bottom plates (Costar, Corning, NY) in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Non-specific binding of the antibodies to Fc receptors

Figure 5.1

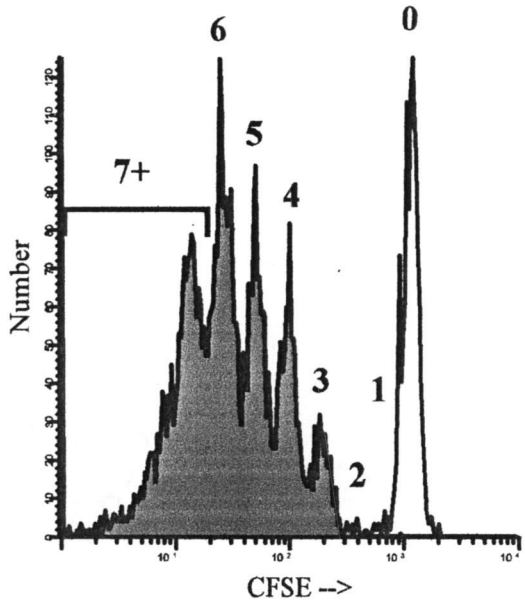


Figure 5.1: Histogram of CFSE-labeled DO11.10 splenocytes. DO11.10 splenocytes were labeled with CFSE as described in Materials and Methods. The unfilled histogram represents CFSE-labeled cells that have not undergone cell division. The filled histogram represents labeled cells after several rounds of division. The number of cell divisions each cell has undergone is indicated by the numbers above the peaks.

was blocked by the addition of 30 μg of ratIgG, hamsterIgG, or 1% normal mouse serum (Jackson Immunoresearch Labs, Inc., West Grove, PA) for 15 min on ice. Subsequently, the cells were stained for 10 min on ice with the specific antibodies mentioned above for CD4, CD8, KJ1-26, or Fas and the fusion protein sOX40L:huIg. Non-specific fluorescence was measured using appropriately labeled isotype-matched IgGs. Data were collected on at least 50,000 freshly stained cells by listmode aquisition using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using Winlist software (Verity Software House, Topsham, ME).

5.2.8 Flow cytometric analysis of apoptosis

After surface staining, the cells were stained for apoptosis using 7-aminoactinomycin D (7-AAD) from Calbiochem (La Jolla, CA) and FITC- or PE-labeled annexin v from R&D systems (Minneapolis, MN) following the manufacturer's protocol. Briefly, the cells were resuspended in 1X binding buffer (HEPES buffered saline, 25mM $CaCl_2$) and stained with 0.1 μg labeled annexin v and 2 μg 7-AAD. Data were collected and analyzed as described above. This method allows for the discrimination of viable, apoptotic, and dead cells (see Figure 3.1).

5.2.9 Enzyme-linked immunosorbent assays (ELISA)

For detection of OVA-specific antibodies, enzyme immunoassay plates (Costar, Cambridge, MA) were coated overnight at 4°C with 1 mg/ml of chicken OVA in PBS, blocked with 3% BSA in PBS for 60 min at 37°C,washed, and incubated overnight at 4°C with serial dilutions (1:10 to 1:100,000) of plasma. Plates were then washed and incubated with a 1:5000 dilution of biotinylated anti-mouse IgM and IgG (Southern Biotechnology, Birmingham, AL). The secondary biotinylated Abs were complexed with avidin-peroxidase and visualized with 2,2'-azino[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) as a substrate.

Absorbance was read at 405 nm using a Bio-tek model EL309 automated plate reader (Bio-Tek Instruments, Winooski, VT).

For detection of cytokines, whole spleen cells ($10x10^6$) were suspended in 1 ml of complete RPMI medium containing 10% FBS with or without OVA (10μM), and incubated for 24 h. Cytokines present in culture supernatents were measured by sandwich ELISA based on noncompeting pairs of anti-IL-2 (JES6-1A12 and JES6-5H4), anti-IFN-γ (R4-6A2 and XMG1.2), or anti-IL-4 (11B11 and BVD6-24G2). Capture and biotinylated detection antibody pairs and their respective standards were purchased from PharMingen, except for the IL-4 standard which was obtained from Genzyme (Cambridge, MA). ELISA were performed according to manufacturer's directions, with known amounts of recombinant murine cytokines used to generate standard curves for comparison.

5.2.10 Isolation of OVA-specific CD4+ T cells

Splenic OVA-specific CD4+ T cells were isolated from vehicle- or TCDD-treated adoptively-transferred Balb/c mice 3 or 4 days after immunized with OVA/CFA. Spleen cells were harvested and processed as described above. Total CD4+ T cells were isolated using Mouse CD4 Cellect T cell columns (Cytovax Biotechnologies Inc., Edmonton, Alberta, Canada) following manufacturer's protocol. Briefly, spleen cells were incubated with cell reagent (monoclonal rat anti-mouse Ly2 (CD8a) clone: YTS 169.4) for 30 min on ice and then placed over prepared columns and 10-15 ml of eluant was collected. The cells were washed and enumerated as described above. Cell purity and viability was typically ≥ 90% as determined by flow cytometry.

OVA-specific (KJ1-26+) CD4+ T cells were positively selected from the isolated CD4+ T cells using magnetic bead separation following manufacturer's protocol (Miltenyi Biotech, Irvine, CA). Briefly, CD4+ T

cells were coated with biotinylated KJ1-26 mAb, bound to streptavidin-coated magnetic beads, and separated out by adherence over a Mini-MACS magnetic column. OVA-specific CD4+ T cells were eluted from the column, washed, and enumerated as described. Cell purity was typically ≥ 65% and cell viability was ≥ 90%.

5.2.11 RNA preparation and reverse transcription-PCR

Total RNA was obtained by lysis of OVA-specific CD4+ T cells using TRIzol reagent (GibcoBRL, Grand Island, NY), following manufacturer's protocol. cDNA was synthesized using RNA extracted from .5-1x10^6 OVA-specific CD4+ T cells using oligo(dt) primers (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (GibcoBRL, Grand Island, NY). PCR amplification of cDNA was performed using Taq polymerase (Boehringer Mannheim, Indianapolis, IN), specific gene oligonucleotide primers (see below), and deoxynucleotides for 30-50 cycles using an Ericomp thermocycler (San Diego, CA). DNA products were separated by agarose gel electorphoresis and visualized using ethidium bromide. All cDNA sample were initially analyzed for β_2m to determine relative amounts and integrity of mRNA/cDNA. Samples displaying low levels of β_2m gene expression or degraded DNA amplification products were discarded.

Primers for β_2m and FasL were obtained using RightPrimer (BioDisk Software, San Francisco, CA) to span introns of the respective genes to allow for discrimination between amplified DNA and genomic or cDNA. The primer sequences used were: β_2m, 5'-ATGGCTCGCTCGGTGACCCT and 3'-TCATGATGCTTGATCACATG; and FasL, 5'-TGTCACCACTACCACCGCCA and 3'-TCTATACCCGGGTGTCGTCGA.

5.2.12 Statistical analysis

Results are presented as mean ± SEM of 3-6 animals/group unless stated otherwise. Analysis of variance modeling was performed using Statview Statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using Fisher's least significant difference multiple comparison t test or Student's T test. Values of $p \leq 0.05$ were considered statistically significant.

5.3 Results

5.3.1 Descriptive studies

5.3.1.1 The effect of TCDD on total spleen cell number from adoptively-transferred mice injected with OVA/CFA

As shown in Figure 5.2, injection of adoptively-transferred mice with OVA/CFA resulted in an increase in total spleen cell number over time. Exposure to TCDD significantly decreased spleen cell number on days 5 and 7 after OVA/CFA injection. A similar, the decrease in spleen cell number in TCDD-treated mice was also evident in a separate study at day 10 after immunization (data not shown). The decrease in spleen cell number in TCDD-treated mice has since been reproduced in at least six separate experiments. The significant decrease in spleen cell number reflected a TCDD-induced decrease in the number of Mac1+ cells (which also coexpress Gr1), CD11c^{hi} cells, and B220+ cells in the spleen (Fig 5.3).

5.3.1.2 The effect of TCDD on the percent and number of OVA-specific CD4+ T cells in adoptively-transferred mice injected with OVA/CFA

Clonal expansion of antigen-specific T cells is one characteristic of an adaptive immune response and results in the production of many activated T cells that are capable of driving an immune response. In mice that were adoptively-transferred but not injected with antigen, the

Figure 5.2: Effect of TCDD on the total number of spleen cells from adoptively-transferred mice. The number of spleen cells from vehicle- (open circle) and TCDD- (filled circle) treated mice were enumerated as described in Materials and Methods. Data represent mean ± SEM of five mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.3: The effect of TCDD on the splenic phenotypes in adoptively-transferred mice. Spleen cells from vehicle- (open bar) and TCDD- (filled bar) treated mice were immunophenotyped by flow cytometric analysis for CD8, B220, Mac1, and CD11c expression. Data represent mean ± SEM of four or five mice per group. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.2

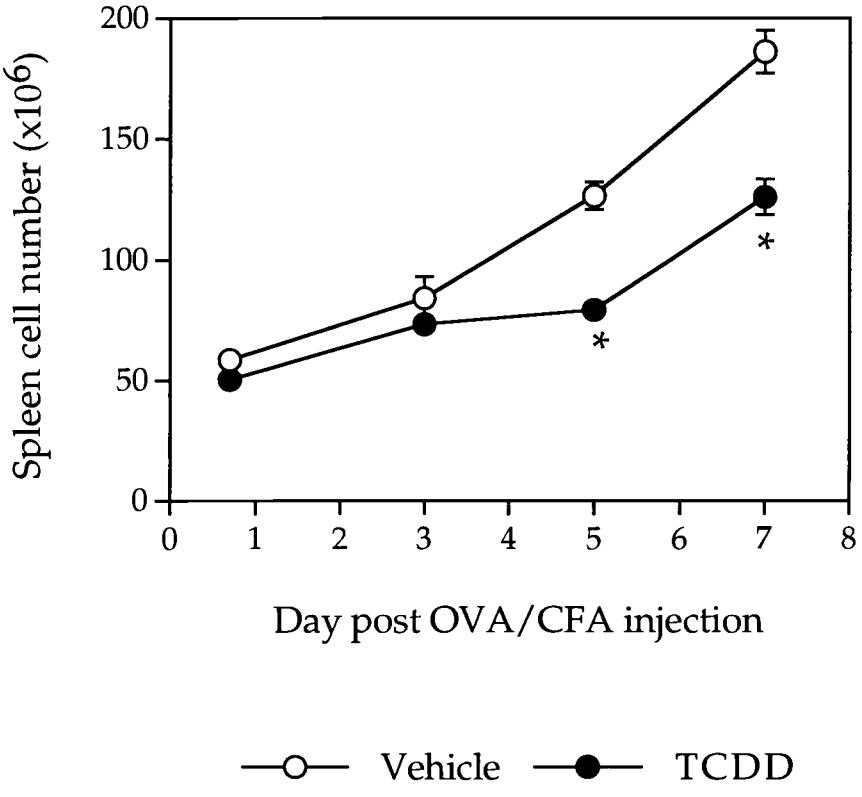
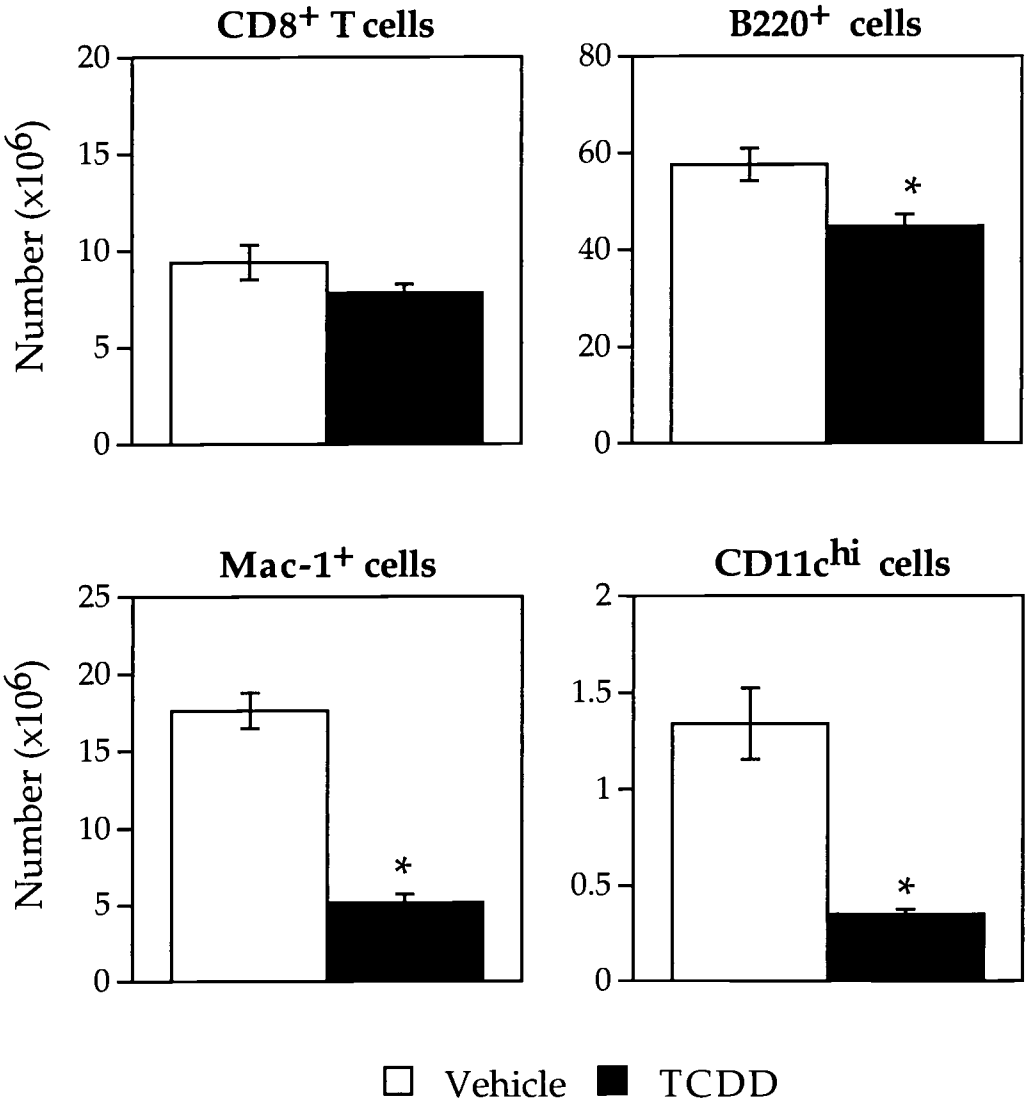

Spleen cell number (x10⁶)
200
150
100
50
0
0
1
2
3
4
5
6
7
8
*
*
Day post OVA/CFA injection
Vehicle
TCDD

Figure 5.3


CD8+ T cells
B220+ cells
Mac-1+ cells
CD11chi cells
Number (x10^6)
Number (x10^6)
20
15
10
5
0
80
60
40
20
0
25
20
15
10
5
0
2
1.5
1
0.5
0
*
*
*
Vehicle
TCDD

frequency of OVA-specific CD4+ T cells in the spleen was maintained at 0.3-0.5% of total spleen cells for at least two weeks. It has been shown previously that these cells can remain in the spleen for at least four weeks (Kearney et al., 1994). As shown in Figure 5.4, the percent (Fig 5.4A) and number (Fig 5.4B) of OVA-specific CD4+ T cells from vehicle-treated mice increased by approximately 4-6 fold in the spleen three days after injection of OVA/CFA, and slowly declined through day 7. In adoptively-transferred mice treated with TCDD, the percent and number of OVA-specific cells increased to the same extent as in the vehicle-treated mice on day 3. However, these cells declined much more rapidly in the TCDD-treated mice, such that the percent and number of OVA-specific CD4+ T cells returned to baseline levels on day 5 after antigen exposure. The changes observed in the OVA-specific CD4+ T cells were specific to this population, as similar fluctuations were not observed in the non-OVA-specific CD4+ T cell population in the same mice (Figure 5.5).

The maximal decrease in the percent and number of OVA-specific CD4+ T cells induced by TCDD was observed on day 5 after OVA/CFA exposure. Thus, we chose day 5 for a dose-response experiment. As shown in Figure 5.6A and 5.6B, the percent and number of OVA-specific CD4+ T cells were dose-dependently decreased on day 5 after OVA/CFA injection. Significant suppression in both the percent and number of OVA-specific CD4+ T cells was observed at doses as low as 5 μg/kg TCDD.

5.3.1.3 The effect of TCDD on PS expression in adoptively-transferred mice injected with OVA/CFA

In order to determine if the decline in OVA-specific CD4+ T cells in TCDD-treated mice was due to increased cell death, we measured PS expression on the antigen-specific T cells using annexin v in conjunction with 7-AAD. As shown in Figure 5.7, the percent of apoptotic OVA-specific CD4+ T cells was significantly elevated in TCDD-treated mice on days 3 and 5 after antigen exposure. In addition, TCDD treatment also caused an

Figure 5.4: Effect of TCDD on the percent and number of activated OVA-specific CD4+ T cells. The percent (A) and number (B) of OVA-specific $CD4^+$ T cells from vehicle- (open circle) and TCDD- (filled circle) treated mice were measured by flow cytometry. OVA-specific CD4+ T cells were identified as cells that fell with in the viable gate and stained positively for both CD4 and KJ1-26 (Tg-TCR). Data represent mean ± SEM of five mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.5: Effect of TCDD on the percent and number of splenic non-OVA-specific CD4+ T cells. The percent (A) and number (B) of non-OVA-specific $CD4^+$ T cells from vehicle- (open circle) and TCDD- (filled circle) treated mice were measured by flow cytometry. Non-OVA-specific CD4+ T cells were identified as cells that fell with in the viable gate and stained positively for CD4 but not KJ1-26 (Tg-TCR). Data represent mean ± SEM of five mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.6: Effect of different doses of TCDD on the percent and number of activated OVA-specific CD4+ T cells. The percent (A) and number (B) of OVA-specific $CD4^+$ T cells from mice treated with 0, 0.5, 5, or 15 μg/kg TCDD were determined on day 5 relative to antigen exposure as described in Figure 5.4 legend. Data represent mean ± SEM of six mice per group. Significant differences between the groups are represented by different letters.

Figure 5.7: Effect of TCDD exposure on the percent of OVA-specific CD4+ T cells positive for apoptosis. The percent of OVA-specific or non-OVA-specific CD4+ T cells displaying an apoptotic or dead phenotype was determined in adoptively-transferred mice treated with vehicle (open symbols) or 15 μg/kg TCDD (closed symbols) by flow cytometry as described in Materials and Methods. Data represent mean ± SEM of five mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.4

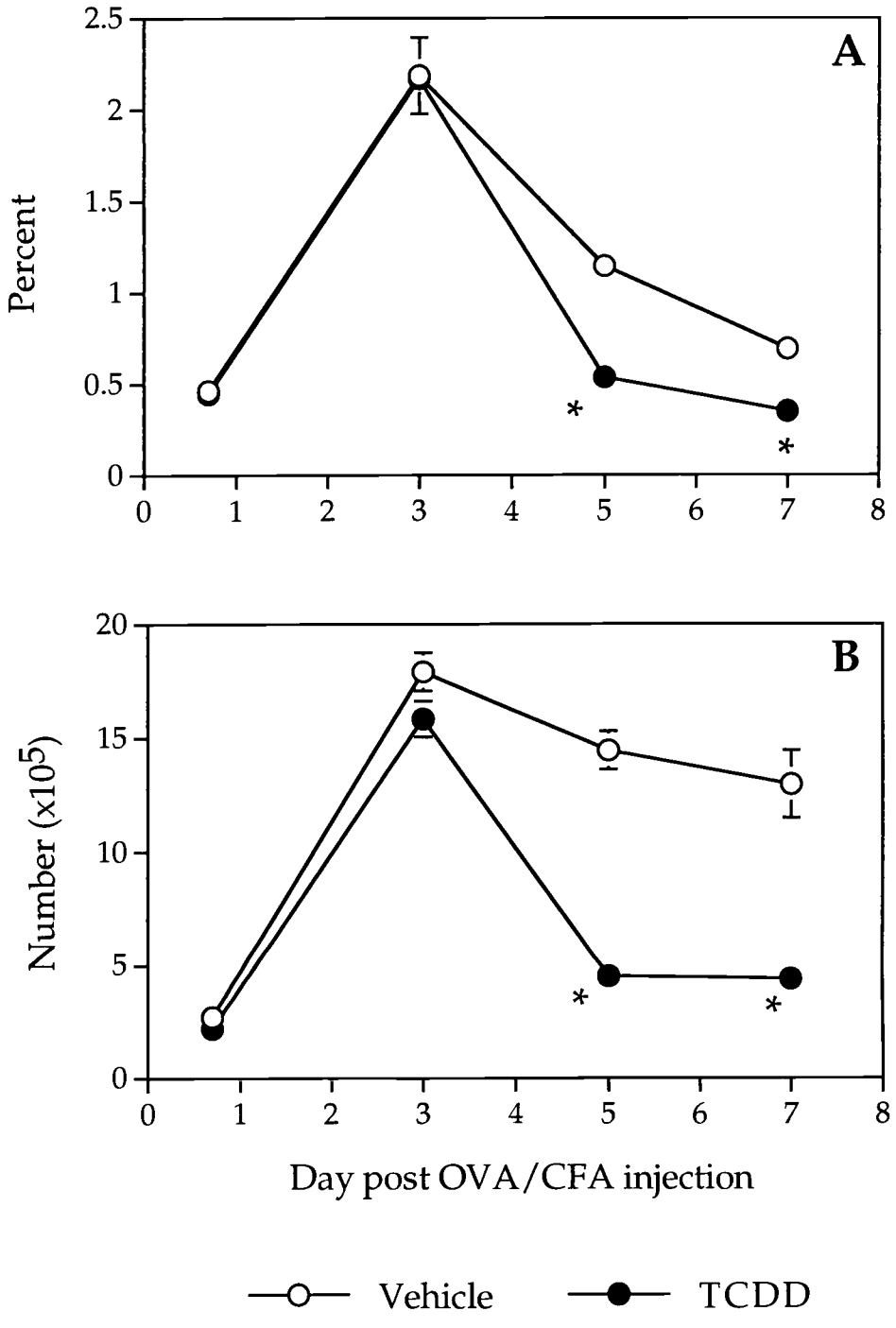

A
B
Percent
Number (x10^5)
Day post OVA/CFA injection
Vehicle
TCDD
*
*
*
*

Figure 5.5

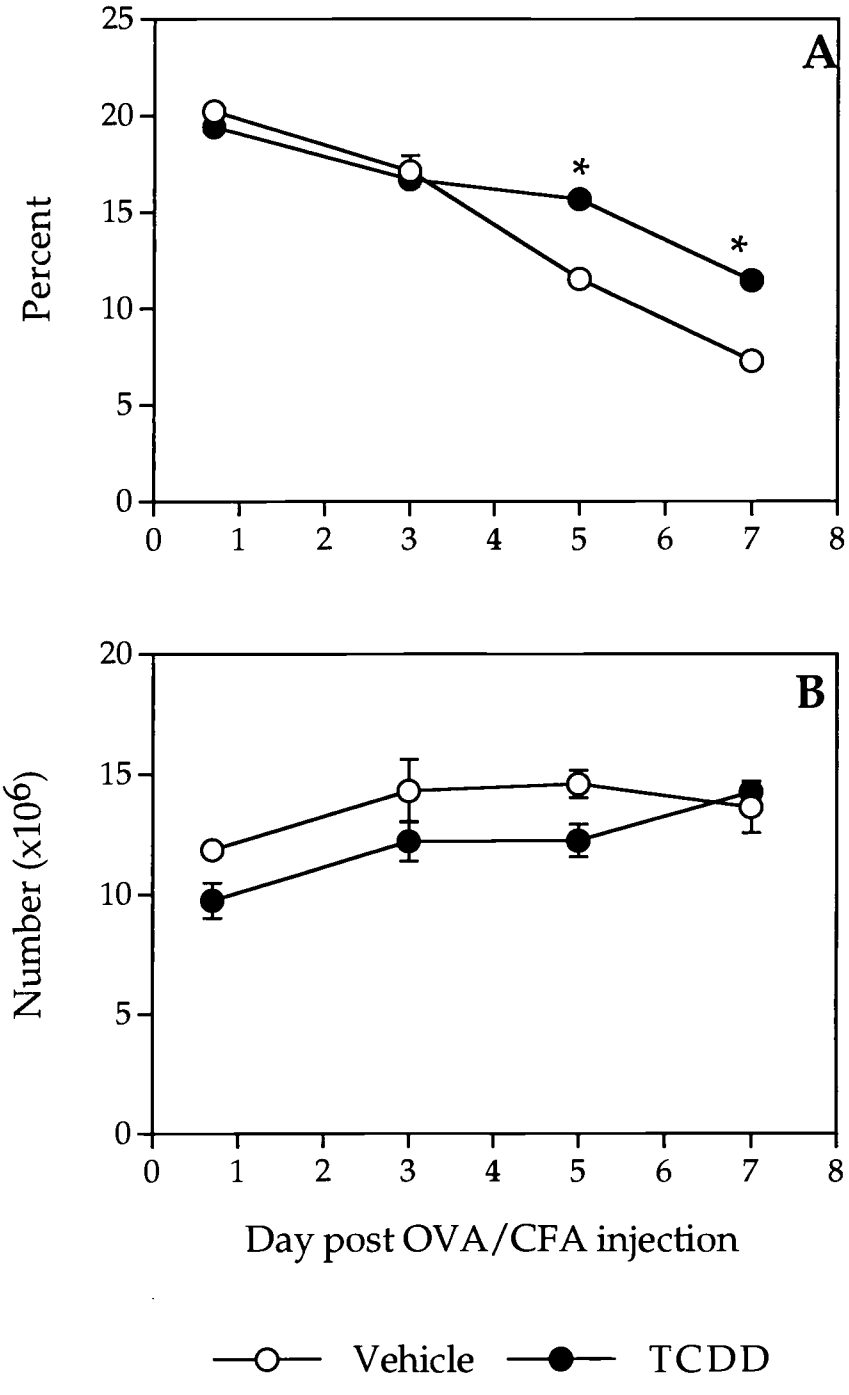

A
B
Percent
Number (x10⁶)
Day post OVA/CFA injection
Vehicle
TCDD
*
*

Figure 5.6

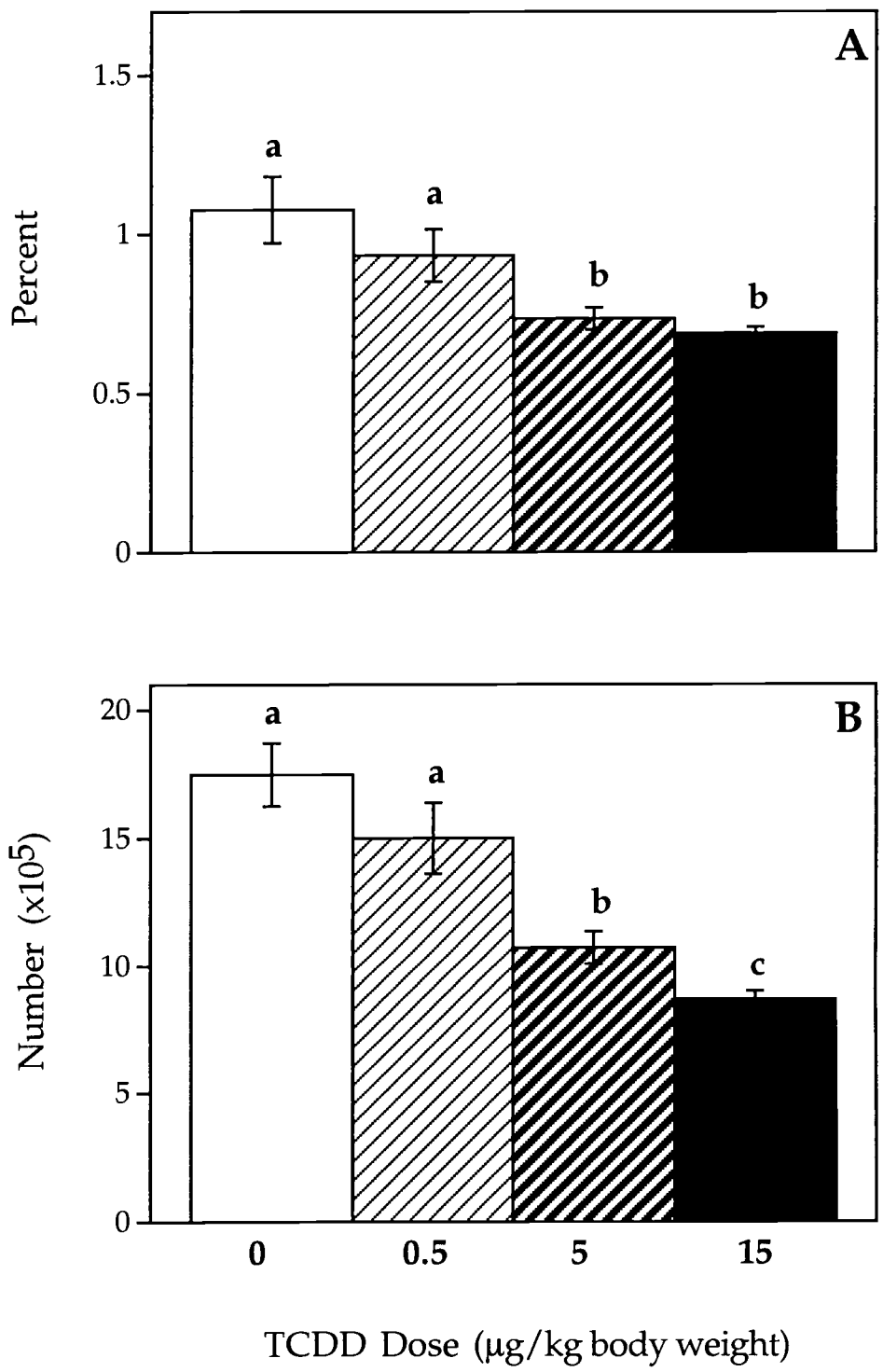
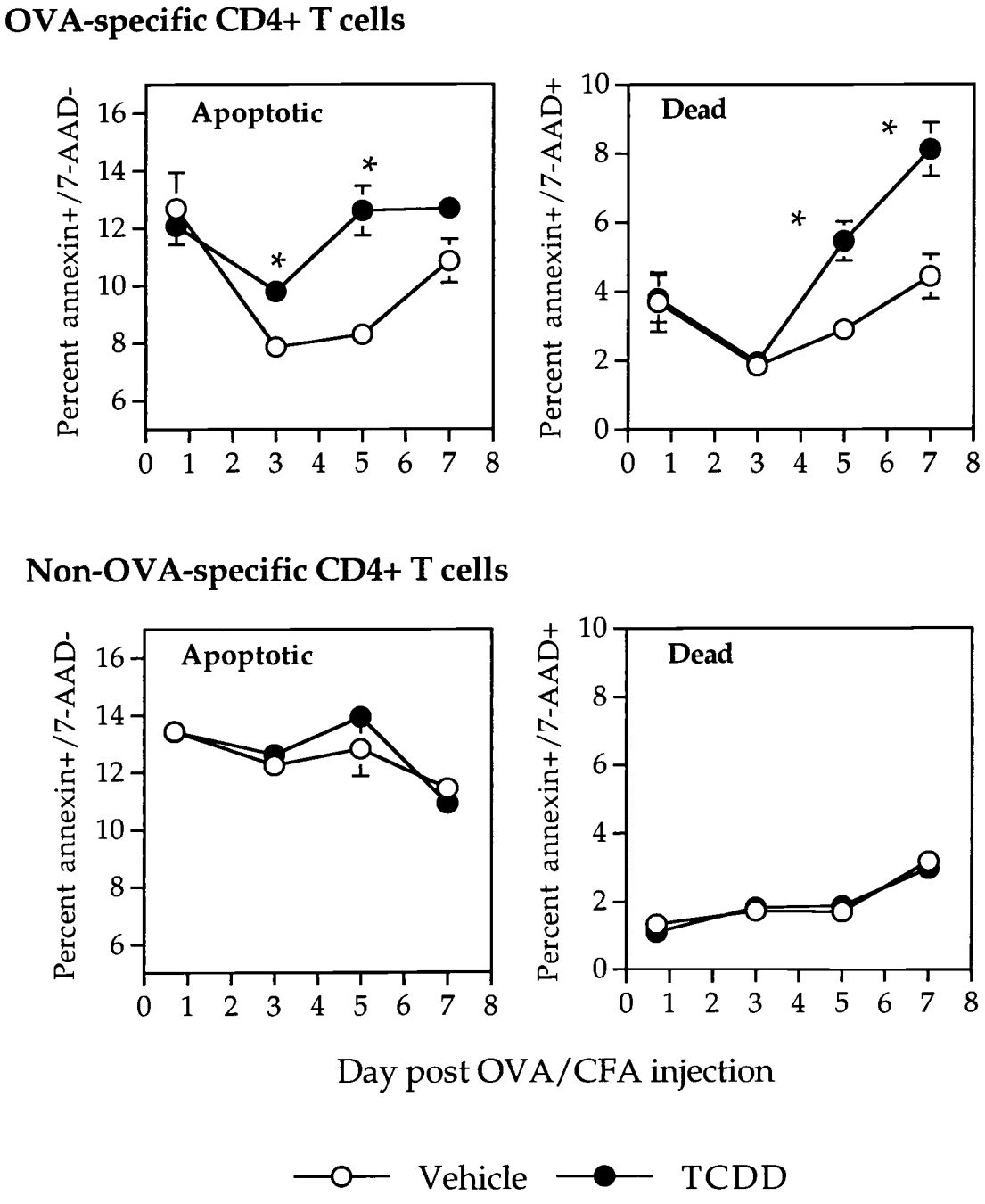

A
1.5
1
0.5
0
Percent
a
a
b
b
B
20
15
10
5
0
Number (x10^5)
a
a
b
c
0
0.5
5
15
TCDD Dose (µg/kg body weight)

Figure 5.7


OVA-specific CD4+ T cells
Apoptotic
Dead
Percent annexin+/7-AAD-
Percent annexin+/7-AAD+
Non-OVA-specific CD4+ T cells
Apoptotic
Dead
Percent annexin+/7-AAD-
Percent annexin+/7-AAD+
Day post OVA/CFA injection
Vehicle
TCDD

increase in the percent of dead OVA-specific CD4+ T cells on days 5 and 7 after activation. The changes in annexin staining induced by both antigen injection and TCDD treatment were specific to the OVA-specific T cells, as similar changes were not observed in the non-OVA-specific T cell population (Fig 5.7). These results suggest that TCDD may cause the decline in OVA-specific CD4+ T cells at least in part by increased cell death.

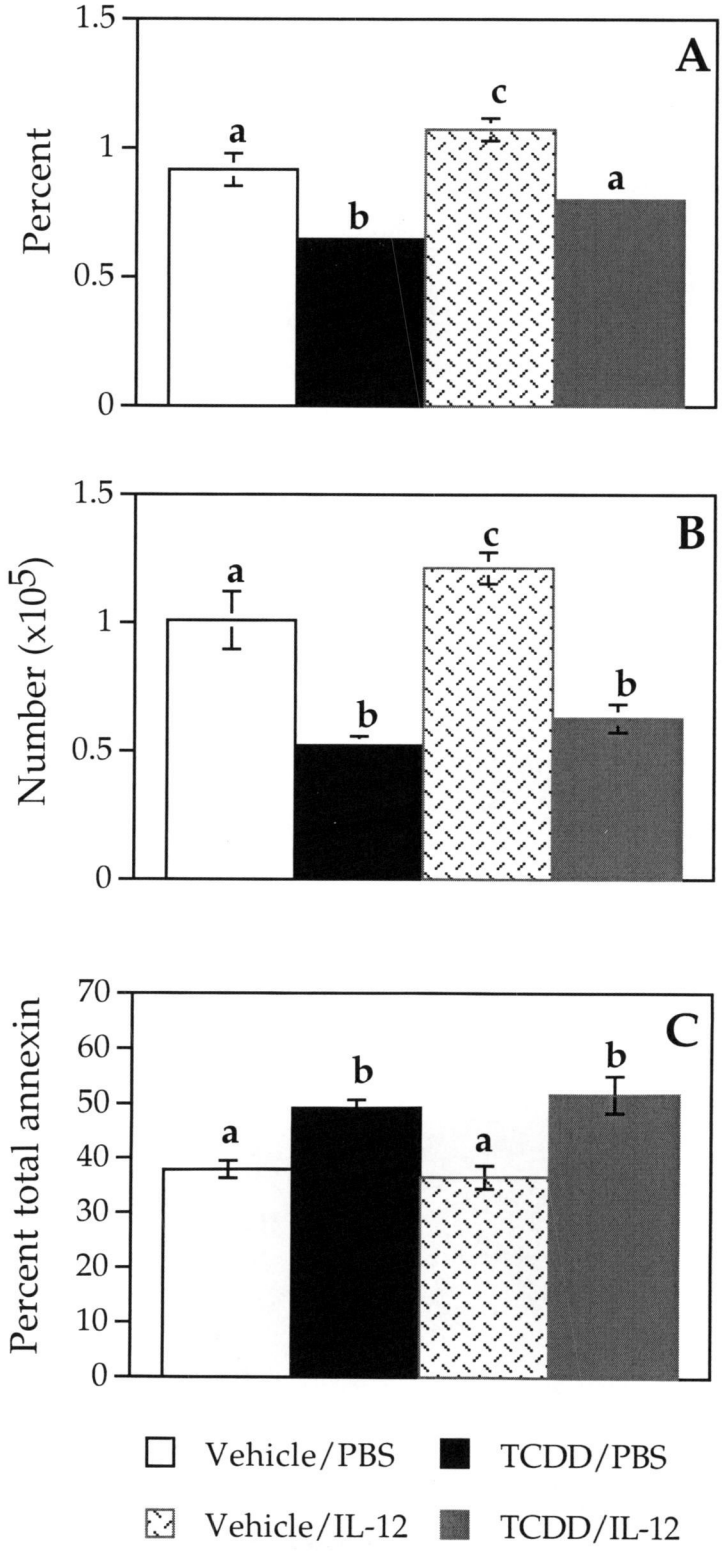
5.3.2 Potential mechanisms of TCDD-induced OVA-specific CD4+ T cell deletion

5.3.2.1 The effect of early IL-12 administration in adoptively-transferred mice on the percent, number, and annexin staining of OVA-specific CD4+ T cells

It has been reported that administration of a neutralizing IL-12 antibody induces T cell apoptosis in models of immune tolerance (Marth et al., 1996; Marth et al., 1999). IL-12 production from APC was significantly suppressed in TCDD-treated mice (DM Shepherd, manuscript in preparation). Thus, it is possible that the TCDD-induced decrease of OVA-specific T cells may be mediated by the decrease in IL-12. As shown in Figure 5.8, on day 5 after OVA/CFA injection, IL-12 treatment increased the percent (Fig 5.8A) and number (Fig 5.8B) of OVA-specific CD4+ T cells in adoptively-transferred mice. In TCDD-treated mice, IL-12 administration increased the percentage of OVA-specific CD4+ T cells, but did not increase the number of these cells. Furthermore, analysis of PS expression revealed that IL-12 treatment did not affect the percent of OVA-specific CD4+ T cells staining positive for annexin (Fig 5.8C). These results suggest that decreased IL-12 may not be responsible for mediating the deletion of OVA-specific CD4+ T cells in TCDD-treated mice.

Figure 5.8: The effect of IL-12 treatment on the deletion of OVA-specific CD4+ T cells from adoptively-transferred mice. Vehicle- or TCDD (15 μg/kg)-treated adoptively-transferred mice were treated with recombinant mouse IL-12 (0.5 μg/injection) on days -1, 0 and +1 relative to injection of 2 mg OVA in CFA. The percent (A) and number (B) of OVA-specific CD4+ T cells were determined as described in Figure 5.4. The percent of OVA-specific CD4+ T cells staining positive for annexin v (C) was determined as described in Figure 5.7. Data represent mean ± SEM of five mice per group per timepoint. Significant differences between the groups are represented by different letters.

Figure 5.8


A
Percent
1.5
1
0.5
0
a
b
c
a
B
Number (x10^5)
1.5
1
0.5
0
a
b
c
b
C
Percent total annexin
70
60
50
40
30
20
10
0
a
b
a
b
Vehicle/PBS
TCDD/PBS
Vehicle/IL-12
TCDD/IL-12

5.3.2.2 The effect of TCDD on Fas expression on the OVA-specific CD4+ T cells

In order to determine if an increase in Fas expression is a mechanism of TCDD-induced T cell deletion, we measured Fas expression on the OVA-specific CD4+ T cells. As mentioned earlier, Fas/FasL interaction plays a critical role in peripheral T cell deletion, AICD, and down regulation of immune responses. As shown in Figure 5.9, Fas was upregulated on the OVA-specific CD4+ T cells as early as one day after immunization with OVA/CFA, and continued to rise through day 4. In contrast, only small changes in Fas expression were observed in the bystander CD4+ T cells, suggesting that these cells do not become activated. Interestingly, TCDD significantly decreased Fas expression on the OVA-specific CD4+ T cells starting at day 3. The TCDD-induced decrease was evident through day 10 (data not shown). TCDD did not affect Fas expression on the bystander CD4+ T cells. These results suggest that a TCDD-induced increase in Fas expression may not mediate the deletion of OVA-specific CD4+ T cells in TCDD-treated mice.

5.3.2.3 The effect of TCDD on OX40 expression

Another potential mechanism by which TCDD could cause T cell deletion is through the inhibition of survival signals delivered to the T cell. Ligation of OX40, a member of the TNFR family that is primarily expressed on activated CD4+ T cells, leads to increased proliferation, survival, and function of CD4+ T cells (Gramaglia et al., 1998; Weinberg et al., 1998; Maxwell et al., 2000; Weinberg et al., 2000). We measured OX40 expression on the OVA-specific and bystander CD4+ T cells by flow cytometry. As shown in Figure 5.10, the amount of OX40 expressed was very low. The median channel fluorescence of OX40 on OVA-specific T cells was elevated above non-OVA-specific T cells on days one and two after OVA/CFA injection. There was no effect of TCDD on the expression (Fig 5.10) or percent (data not shown) of CD4+ T cells positive for OX40 on

Figure 5.9: The effect of TCDD on Fas expression on OVA-specific CD4+ T cells from adoptively-transferred mice. The expression of Fas was determined by flow cytometry on the OVA-specific (circles) and non-OVA-specific (triangles) CD4+ T cells at various times after OVA/CFA injection from mice treated with vehicle (open symbols) or TCDD (closed symbols). Data represent mean ± SEM of three mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.10: The effect of TCDD on OX40 expression on OVA-specific CD4+ T cells from adoptively-transferred mice. The expression of OX40 was determined by flow cytometry on the OVA-specific (circles) and non-OVA-specific (triangles) CD4+ T cells at various times after OVA/CFA injection from mice treated with vehicle (open symbols) or TCDD (closed symbols). Data represent mean ± SEM of three mice per group per timepoint.

Figure 5.9

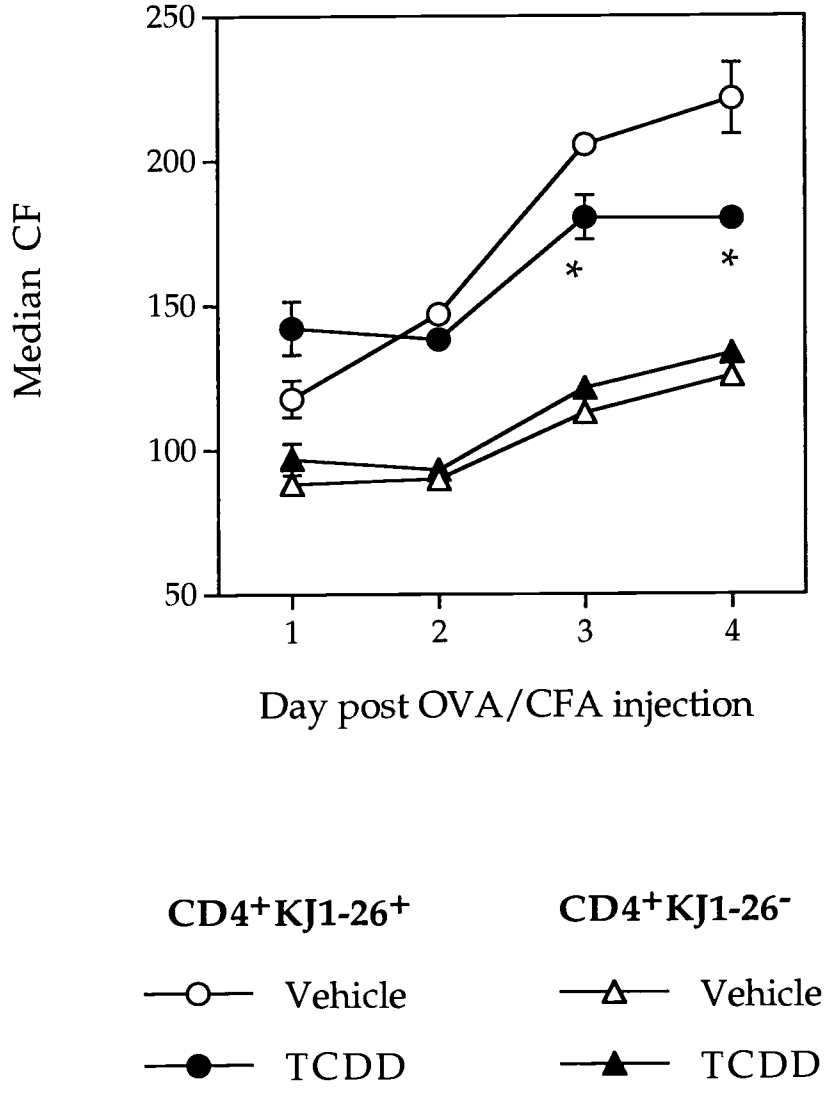
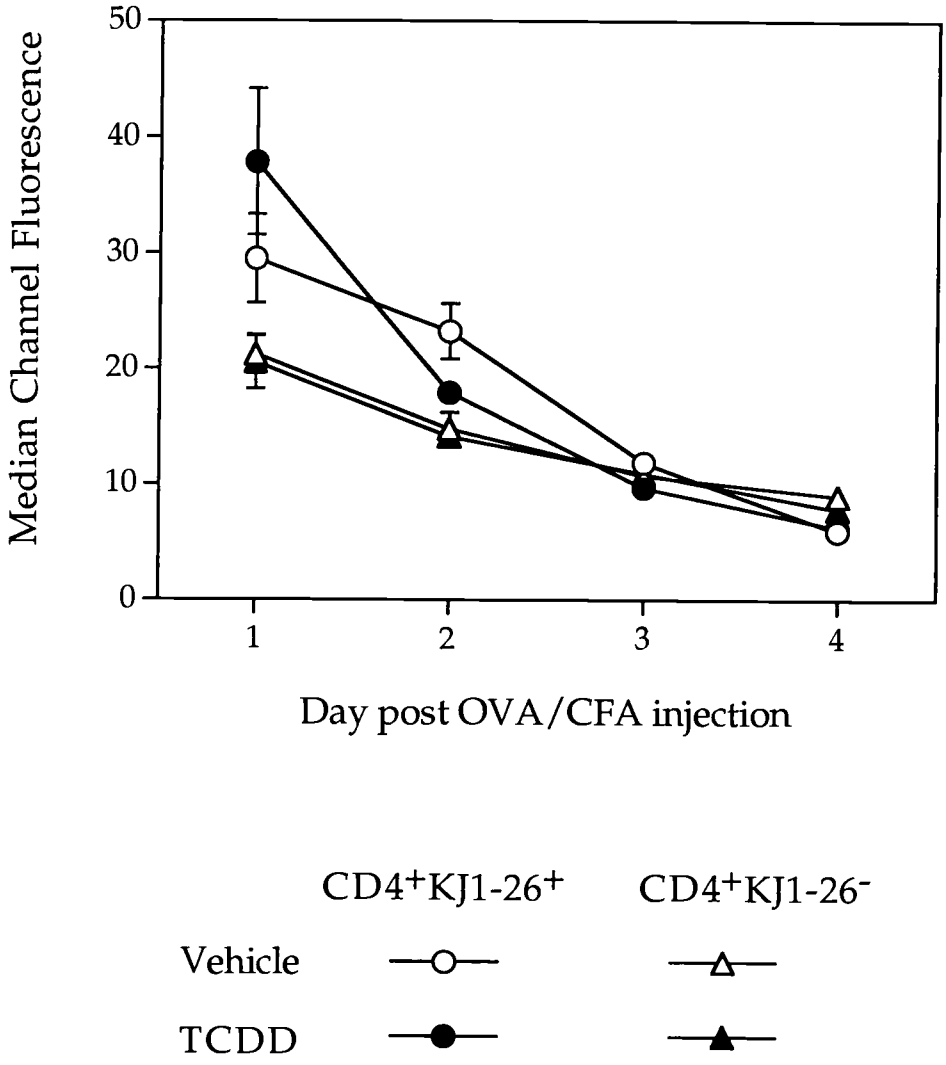

250
200
150
100
50
Median CF
1
2
3
4
Day post OVA/CFA injection
*
*
CD4+KJ1-26+
Vehicle
TCDD
CD4+KJ1-26-
Vehicle
TCDD

Figure 5.10


Median Channel Fluorescence
50
40
30
20
10
0
1
2
3
4
Day post OVA/CFA injection
CD4+KJ1-26+
CD4+KJ1-26-
Vehicle
TCDD

any day tested. In a separate experiment, TCDD exposure did not affect OX40 expression on day 5 after immunization (data not shown).

5.3.2.4 The effect of Anti-OX40 treatment on the survival and proliferation of the OVA-specific CD4+ T cells from TCDD-treated adoptively-transferred mice

In order to address the possibility that TCDD could be interfering with OX40 signaling, we treated adoptively-transferred mice with an agonistic anti-OX40 antibody and measured the effect of this treatment on the survival of the OVA-specific CD4+ T cells. As shown in Figure 5.11, administration of anti-OX40 did not increase total spleen cell number on days 1-5 from adoptively-transferred mice treated with OVA/CFA. TCDD exposure caused a decrease in total spleen cell number in both control and anti-OX40-treated mice. Thus, it appears that anti-OX40 treatment does not cause an increase in overall spleen cell number and does not overcome the decrease induced by TCDD.

As shown in Figure 5.12A and 5.12B, the OVA-specific T cells reached maximal expansion on day 3 after immunization with OVA/CFA, and declined by day 5. As expected, TCDD did not affect the expansion of these cells on day 3, but enhanced their deletion on day 5 (albeit the deletion was not as impressive as it had been in other experiments). Anti-OX40 treatment caused a slight increase in the number of OVA-specific CD4+ T cells on day 3 in vehicle-treated mice, but had no effect in TCDD-treated mice. However, on day 5 after immunization, anti-OX40 treatment significantly increased the percent and number of OVA-specific cells in both vehicle- and TCDD-treated mice as compared to ratIgG-treated control mice. In fact, the number of T cells present in the spleen at day 5 was identical to the number present at day 3. Thus, these data suggest that activated antigen-specific T cells from both vehicle- and TCDD-treated mice can respond to anti-OX40 treatment which results in an increase in the percent and number of OVA-specific CD4+ T cells.

Figure 5.11: The effect of anti-OX40 treatment on total spleen cell number from adoptively-transferred mice treated with vehicle or TCDD. The number of spleen cells from vehicle- (open symbol) and TCDD- (filled symbol) treated mice injected with either rat IgG (circles) or anti-OX40 (triangles) on days 0 and +3 relative to OVA/CFA were enumerated as described in Materials and Methods. Data represent mean ± SEM of four mice per group per timepoint. * Denotes significant difference ($p \leq 0.05$) from vehicle-treated group.

Figure 5.12: Administration of anti-OX40 increases the proliferation of the activated OVA-specific CD4+ T cells in vehicle- and TCDD-treated mice. The percent (A) and number (B) of the OVA-specific $CD4^+$ T cells were measured in vehicle- (open symbols) and TCDD- (filled symbols) treated mice injected with either rat IgG (red symbols) or anti-OX40 (blue symbols). Data represent mean ± SEM of four mice per group per timepoint. CFSE staining (C) was measured by flow cytometry in vehicle- (open histograms) and TCDD- (filled histograms) treated mice injected with either rat IgG (red histograms) or anti-OX40 (blue histograms). One representative histogram of four is shown for each group at each timepoint. The percent of OVA-specific $CD4^+$ T cells that have undergone at least 7 divisions is indicated on each histogram for day 3 and day 5. The percent of OVA-specific CD4+ T cells staining positive for annexin v (D) was determined by flow cytometry. * Indicates significant difference ($p \leq 0.05$) from vehicle-treated group. # Indicates significant difference ($p \leq 0.05$) from rat IgG-treated control group.

Figure 5.11

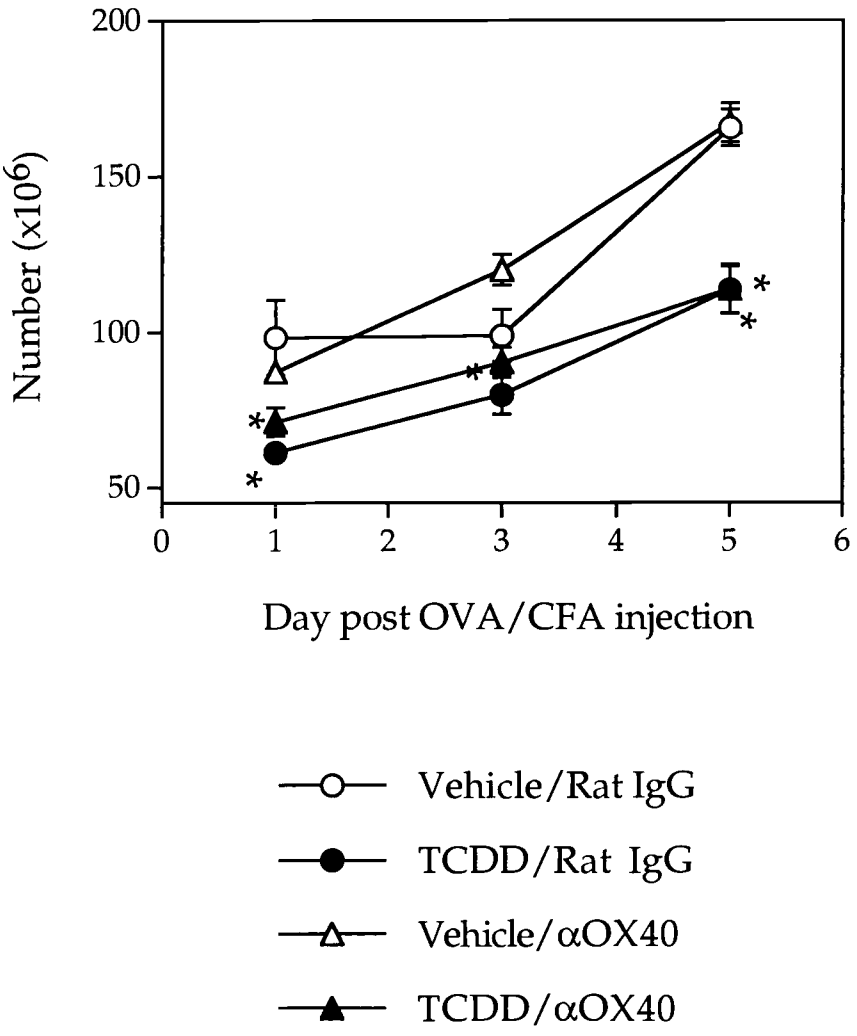
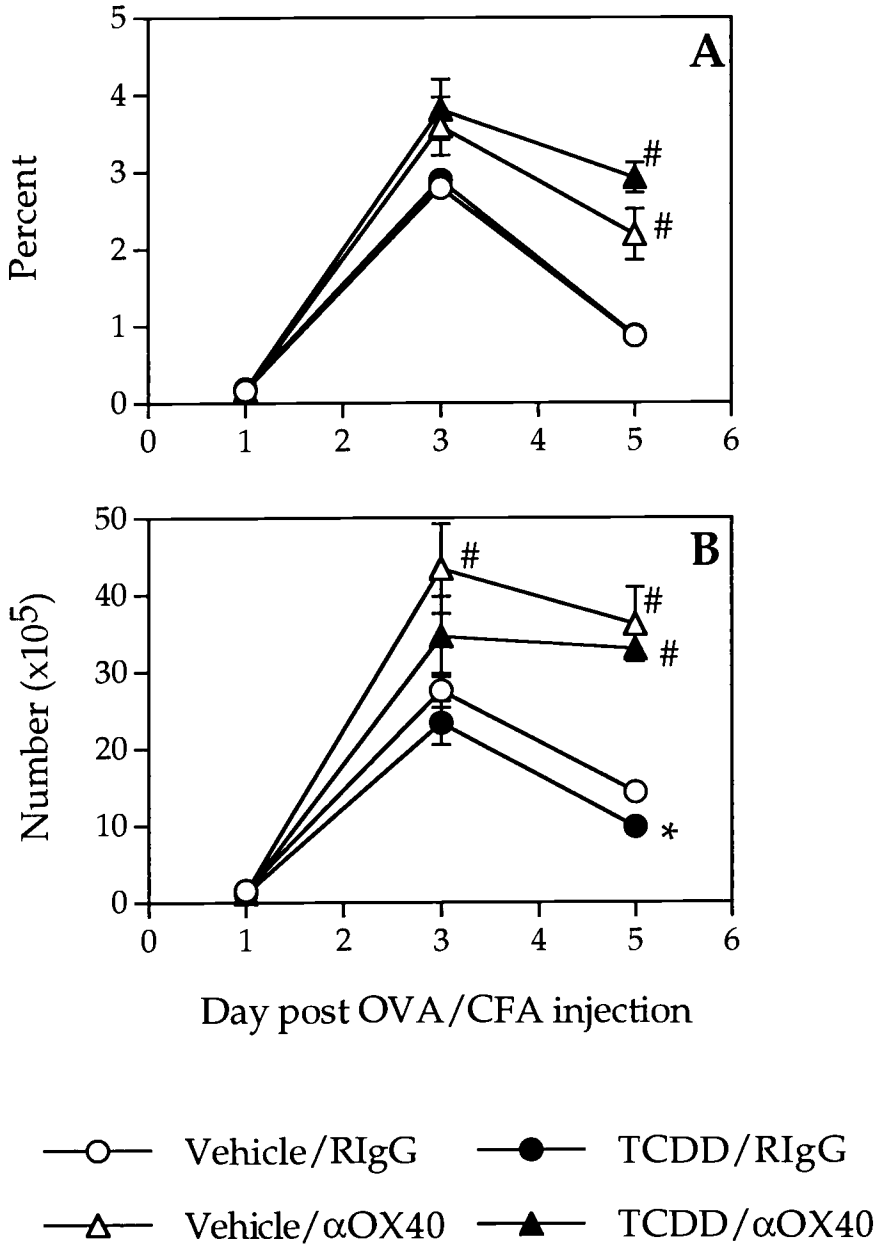

200
150
100
50
Number (x10^6)
0
1
2
3
4
5
6
Day post OVA/CFA injection
Vehicle/Rat IgG
TCDD/Rat IgG
Vehicle/αOX40
TCDD/αOX40

Figure 5.12


A
B
Percent
Number (x10^5)
Day post OVA/CFA injection
#
*
Vehicle/RIgG
TCDD/RIgG
Vehicle/αOX40
TCDD/αOX40

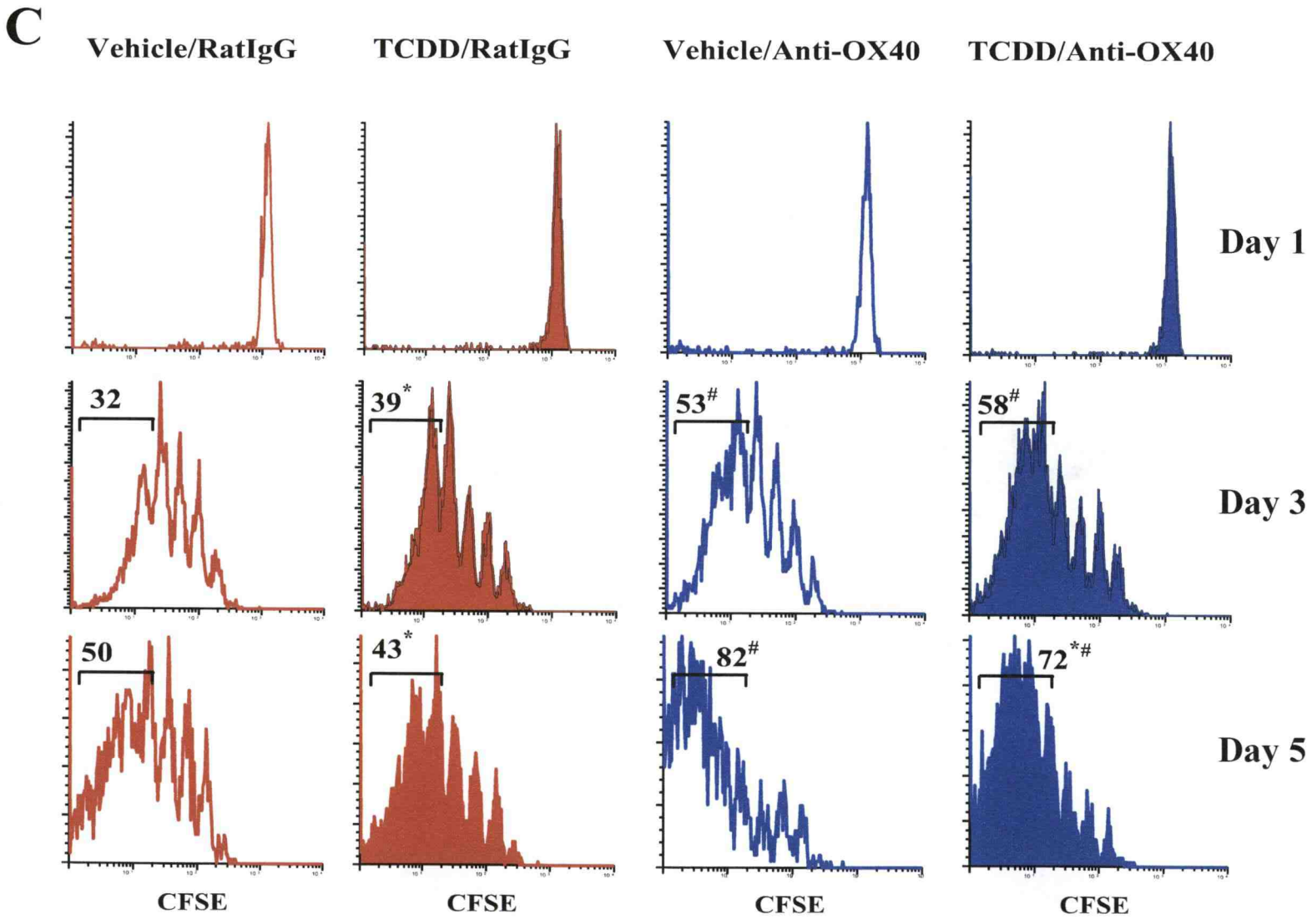
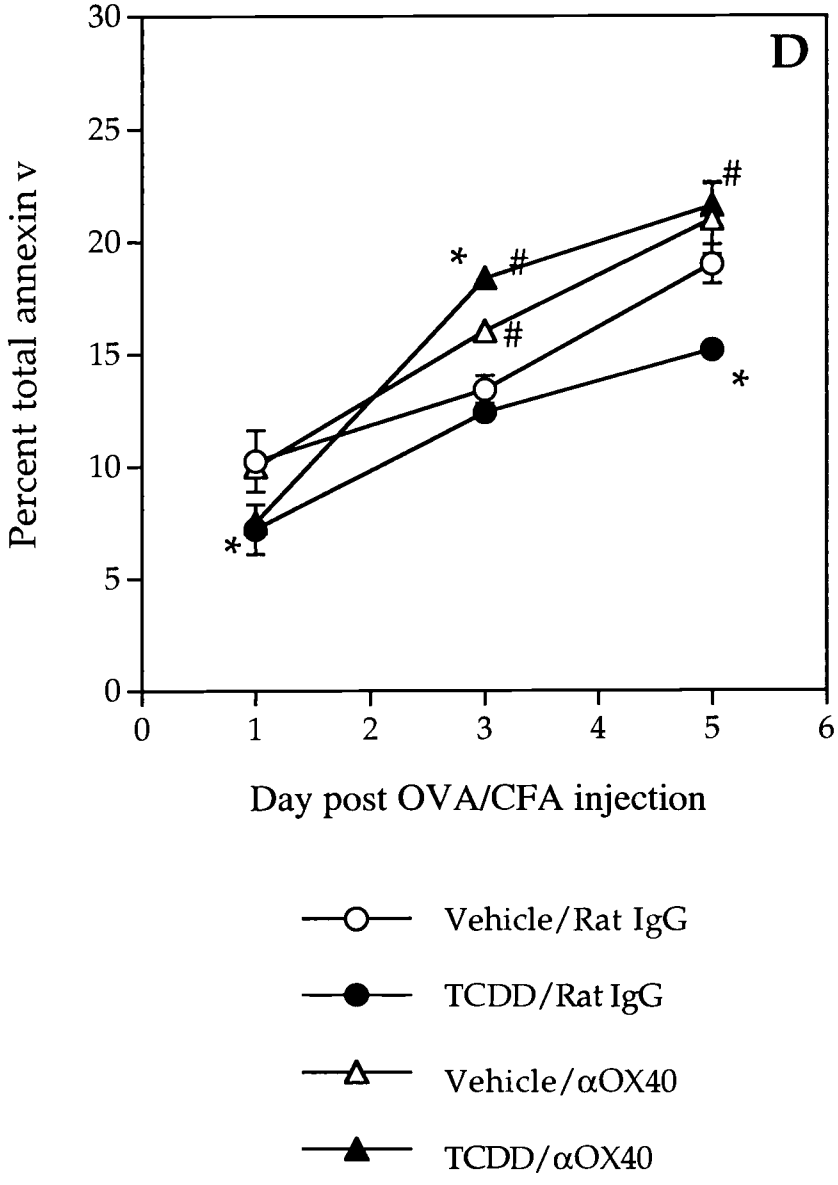


Figure 5.12 (continued)

Figure 5.12 (continued)



A possible mechanism by which anti-OX40 increases the percent and number of the OVA-specific CD4+ T cells could involve enhanced proliferation of these cells. In order to determine if anti-OX40 increased the proliferation of the OVA-specific CD4+ T cells, we adoptively-transferred CFSE-labeled DO11.10 spleen cells into Balb/c recipients. The CFSE is equally divided among daughter cells, thereby decreasing the fluorescence intensity of the stain by half with each cell division. As shown in Figure 5.12C, the OVA-specific CD4+ T cells had not begun to proliferate one day after OVA/CFA injection. By day 3, however, a significant portion of the cells in ratIgG-treated control mice had undergone at least seven rounds of division. Interestingly, TCDD treatment enhanced the early proliferation of the transgenic T cells as measured by an increased percentage of cell which had undergone at least seven cell division cycles. In contrast, exposure to TCDD significantly decreased the proliferation of the T cells remaining on day 5 as compared to vehicle controls. Anti-OX40 treatment significantly increased the proliferation of OVA-specific CD4+ T cells from both vehicle- and TCDD-treated mice on days 3 and 5 after treatment with OVA/CFA. Interestingly, TCDD treatment also had a significant effect on inhibiting proliferation in anti-OX40-treated mice on day 5 after immunization.

Another potential mechanism by which anti-OX40 could increase the percent and number of the OVA-specific CD4+ T cells could involve a decrease in apoptosis of these T cells. Unfortunately, as shown in Figure 5.12D, in ratIgG control mice, exposure to TCDD unexpectedly decreased the percent of OVA-specific cells staining positive for annexin v. This was the only experiment out of seven in which TCDD induced a significant decrease in annexin v staining. Anti-OX40 treatment enhanced the percent of the cells positive for annexin v on days 3 and 5, however, on day 5, only TCDD/anti-OX40-treated mice were significantly higher than TCDD/ratIgG-treated mice. Thus, these data suggest that anti-OX40 increases the percent and number of OVA-specific CD4+ T cells in

adoptively-transferred mice by increasing proliferation of the cells and not by decreasing apoptosis.

5.3.2.5 The effect of anti-OX40 on cytokine production from 24 hour spleen cell culture supernatents

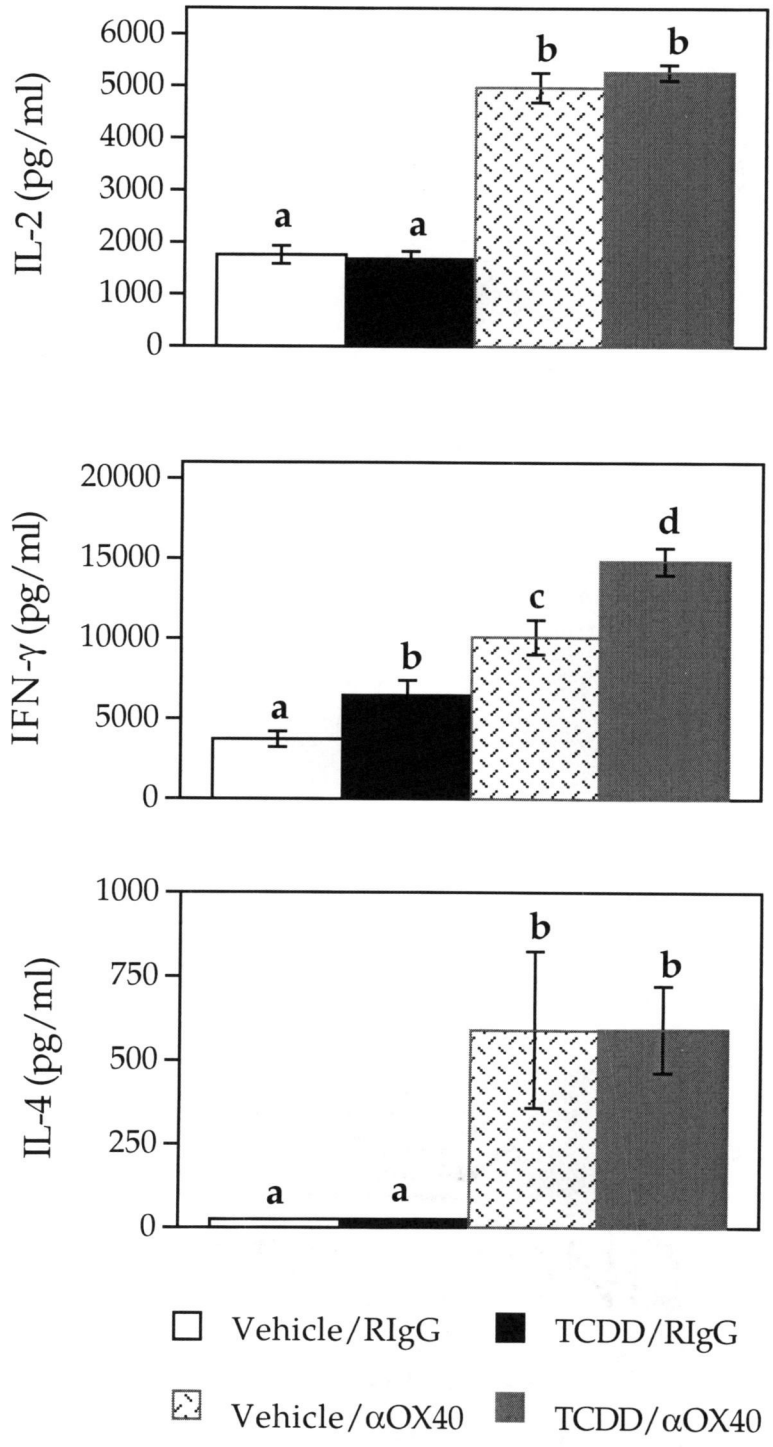
Since anti-OX40 treatment had a significant effect on the proliferation of the OVA-specific CD4+ T cells, we also wanted to determine whether anti-OX40 treatment would also augment cytokine production. It has been shown that anti-OX40 treatment can cause an increase in cytokine production from activated T cells (Ohshima et al., 1998; Kopf et al., 1999; Murata et al., 2000). We measured cytokine production in supernatents from total spleen cell cultures incubated with 10μM ovalbumin for 24 hours. As shown in Figure 5.13, anti-OX40 treatment significantly increased the production of IL-2, IFN-γ, and IL-4 from vehicle- and TCDD-treated mice on day 5 as compared to ratIgG-treated controls. Similar results were also obtained when cytokine production was measured on days 1 and 3 after immunization and TCDD did not suppress the production of IL-2 from anti-OX40 treated mice at either timepoint (data not shown). Thus it appeared that anti-OX40 treatment increases cytokine production from spleen cell cultures. We also attempted to determine cytokine production from OVA-specific CD4+ T cells restimulated for 6 hours with 10μM OVA in vitro on days 1 and 3 after immunization by intracellular staining. Unfortunately, we did not detect any staining above background levels (data not shown).

5.3.2.6 The effect of anti-OX40 treatment on OVA-specific antibody production

OX40 ligation has been shown to enhance antibody production from B cells. Although the obligatory role for this interaction is questionable, it does appear that OX40-OX40L interaction can augment germinal center formation and drive B cell differentiation (Stuber et al., 1995; Stuber and

Figure 5.13: The effect of anti-OX40 treatment on cytokine production from ex vivo restimulated spleen cells cultures. Spleen cells were harvested from vehicle- or TCDD-treated mice injected with rat IgG or anti-OX40 on day 5 after OVA/CFA immunization and restimulated ex vivo with 10 μM OVA for 24 hours. The levels of IL-2, IFN-γ, and IL-4 were measured by cytokine-specific ELISA as described in Materials and Methods. Data represent the mean ± SEM of four mice per group. Significant differences between the groups are represented by different letters.

Figure 5.13


IL-2 (pg/ml)
6000
5000
4000
3000
2000
1000
0
a
a
b
b
IFN-γ (pg/ml)
20000
15000
10000
5000
0
a
b
c
d
IL-4 (pg/ml)
1000
750
500
250
0
a
a
b
b
Vehicle/RIgG
TCDD/RIgG
Vehicle/αOX40
TCDD/αOX40

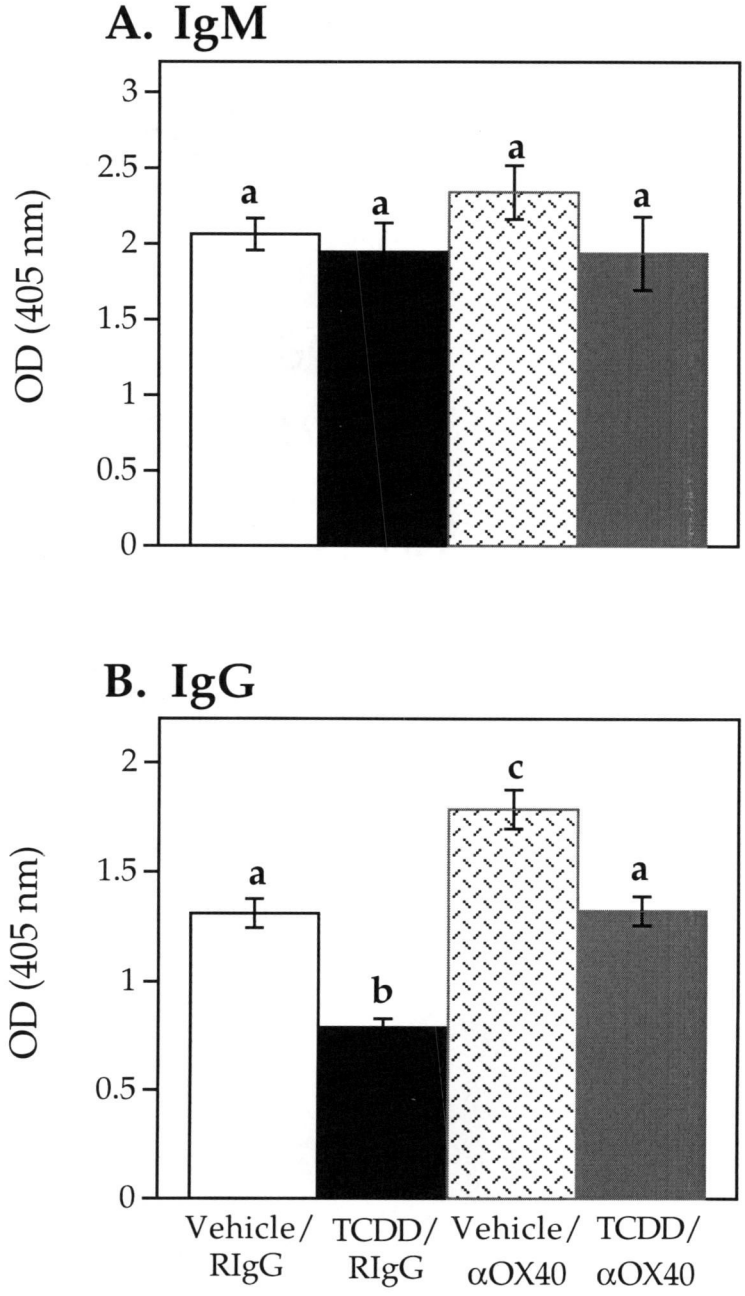
Strober, 1996; Brocker et al., 1999). Since the antibody response to ovalbumin is suppressed in TCDD-treated adoptively-transferred mice and these mice have decreased numbers of OVA-specific T cells, we wanted to determine if the deletion of T cells was involved in the suppression of the antibody response. We measured the levels of OVA-specific IgM and total IgG in the plasma from adoptively-transferred mice treated with vehicle or TCDD and ratIgG or anti-OX40 on day 5 after immunization with OVA/CFA. As shown in Figure 5.14A, IgM levels were not affected by either TCDD or anti-OX40 treatment. In contrast, TCDD treatment significantly decreased the level of total IgG in ratIgG control mice (Fig 5.14B). As expected, anti-OX40 treatment elevated the level of IgG in the plasma in both vehicle- and TCDD-treated mice. However, a significant suppression in the production of IgG was still evident in TCDD/anti-OX40-treated mice as compared to vehicle/anti-OX40-treated mice. Similar data were obtained on day 10 after OVA/CFA injection (data not shown). Overall, these data suggest that anti-OX40 may have significant effects on the proliferation of OVA-specific CD4+ T cells, but it can not overcome the TCDD-induced suppression of the antibody response. Therefore, it appears that the decrease in activated antigen-specific T cells induced by TCDD does not solely mediate the suppression of the overall immune response.

5.4 Discussion

Previous studies in our laboratory have demonstrated that TCDD suppresses the antibody response to ovalbumin (OVA) in adoptively-transferred mice (Shepherd et al., 2000). Surprisingly, TCDD had very little effect on the initial activation of OVA-specific CD4+ T cells on days 1-3 as measured by alterations of activation markers and expansion. In contrast, TCDD caused a significant decrease in the percent and number of

Figure 5.14: Anti-OX40 treatment does not fully restore the anti-OVA IgG production from TCDD-treated mice. The plasma levels of IgM (A) and total IgG (B) were measured on day 5 by ELISA from vehicle- and TCDD-treated mice injected with either rat IgG or anti-OX40. Data represent the mean ± SEM of four mice per group at the 1:100 dilution of plasma. Significant differences between the groups are represented by different letters.

Figure 5.14


A. IgM
OD (405 nm)
3
2.5
2
1.5
1
0.5
0
a
a
a
a
B. IgG
OD (405 nm)
2
1.5
1
0.5
0
a
b
c
a
Vehicle/ RIgG
TCDD/ RIgG
Vehicle/ αOX40
TCDD/ αOX40

OVA-specific CD4+ T cells on days 4-7 after immunization with OVA/CFA. In the present studies, we evaluated whether apoptosis was a mechanism by which TCDD decreased OVA-specific CD4+ T cells and whether the reduced number of OVA-specific CD4+ T cells was responsible for suppressing the antibody response.

When spleen cells were stained with antibodies to identify the OVA-specific CD4+ T cells and analyzed for apoptosis using annexin v and 7-AAD, an increased percent of apoptotic OVA-specific CD4+ T cells was observed in TCDD-treated mice on days 3 and 5 after immunization. Thus, these results support the hypothesis that TCDD decreases OVA-specific CD4+ T cells by increased apoptosis. Furthermore, TCDD also increased the percent of dead OVA-specific CD4+ T cells on days 5 and 7 after OVA/CFA. It is worth noting that cells which stain positive for both annexin v and 7-AAD represent cells that are either in the late phases of apoptosis (apoptotic cells which have lost their membrane integrity) or that have undergone necrosis. Therefore, cells that are designated as "dead" could result from an apoptotic event.

Another interesting finding in our studies was that TCDD did not affect the percent of non-OVA-specific CD4+ T cells staining positive for annexin v. Thus, our data suggest that TCDD only affects activated CD4+ T cells. This interpretation is consistent with a recent report that showed TCDD increased apoptosis of activated, but not resting, T cells from mice treated with anti-CD3 and CFA (Pryputniewicz et al., 1998).

There are many potential mechanisms by which TCDD could increase death of OVA-specific CD4+ T cells. For example, insufficient cytokine production has been shown to result in deletion of T cells by apoptosis. Recent studies in our laboratory have shown that exposure to TCDD suppresses IL-2 and IL-10 production from in vitro restimulated OVA-specific CD4+ T cells on days 1 and 3 after immunization, respectively (Shepherd et al., 2000). However, the modest degree and duration of suppression of the T cell-derived cytokines in TCDD-treated

mice did not appear to be proportional to the magnitude of the decrease of OVA-specific CD4+ T cells observed in these mice.

In contrast to the moderate suppression of T cell-derived cytokines, IL-12 production by APC was significantly suppressed in TCDD-treated mice at 16 hours, 3 and 7 days after immunization (Shepherd, DM, Dearstyne, EA, and Kerkvliet, NI, manuscript in preparation). IL-12 is an inflammatory cytokine that is critically important in the differentiation of CD4+ T cells into type 1 helper (Th1) cells, which produce IFN-γ, IL-2, and TNF α (Lane et al., 1998). These Th1-derived cytokines are important in immunoglobulin (Ig) class switching of antibodies in B cells from the IgM isotype to downstream isotypes such as IgG2a (Finkelman et al., 1990). In addition, a few studies have suggested a link between IL-12 production and T cell survival. For example, blocking IL-12 production with a neutralizing antibody (anti-IL-12) increased apoptosis of T cells in models of oral tolerance or superantigen activation (Marth et al., 1996). In addition, anti-IL-12 was also shown to increase apoptosis of T cells from wildtype, but not Fas deficient lpr mice, suggesting that IL-12 can block Fas-mediated apoptosis (Marth et al., 1999).

Based on these studies we afterulated that IL-12 can enhance T cell survival, presumably via inhibition of apoptosis. Therefore, we examined the effect of administration of exogenous IL-12 on the reduction of OVA-specific T cells in TCDD-treated adoptively-transferred mice. However, treatment with IL-12 did not reverse the TCDD-induced decrease of OVA-specific cells nor did it alter the TCDD-induced increase in annexin staining. A possible explanation for the lack of effect of IL-12 on the OVA-specific T cells is that the dose of IL-12 administered to the mice was not effective. However, this interpretation seems unlikely since IgG2a production was restored in TCDD-treated adoptively-transferred mice given recombinant IL-12 (Shepherd, DM, Dearstyne, EA, and Kerkvliet, NI, manuscript in preparation). Thus, these results suggest the decreased IL-12

production may not be a mechanism by which TCDD causes T cell deletion. Interestingly, these results are consistent with a recent report which showed that administration of IL-12 to adoptively-transferred mice at the same time as a tolerizing dose of OVA peptide (300 μg iv) restored Th1 cell differentiation but not proliferation of the OVA-specific T cells (Van Parijs et al., 1997b).

In TCDD-treated adoptively-transferred mice, OVA-specific CD4+ T cells undergo normal expansion, which is followed by a rapid depletion from the spleen. The kinetics of the expansion and depletion are consistent with agents that induce peripheral tolerance, such as superantigens (McCormack et al., 1993; McCormack et al., 1994; Vella et al., 1997) or tolerizing doses of antigen (Kearney et al., 1994). In superantigen models, the deletion of antigen-reactive T cells is mediated by Fas/FasL interaction (Ettinger et al., 1995). One potential way that TCDD could affect the Fas pathway is by upregulation of Fas or FasL expression (Kamath et al., 1999). However, when we examined Fas expression on OVA-specific CD4+ T cells on days 3-7 after antigen administration, exposure to TCDD decreased Fas expression. Thus, it seems unlikely that TCDD decreased activated antigen-specific T cells by upregulation of Fas. Unfortunately, we were not able to detect any message for FasL in OVA-specific CD4+ T cells. Thus, the impact of TCDD on FasL remains unknown. In addition, it was recently reported that FasL expression in nonlymphoid tissues, such as the liver and intestine, may be responsible for killing superantigen-activated T cells (Bonfoco et al., 1998). Therefore, further studies need to be done to address the role that Fas/FasL interaction may have on the TCDD-induced deletion of the OVA-specific CD4+ T cells.

An alternative mechanism by which TCDD could cause deletion of OVA-specific CD4+ T cells is through the inhibition of survival signals delivered to the T cells. Several members of the TNFR superfamily, such as 4-1BB, TNFRII, and OX40, provide potent survival signals to T cells (Ware et al., 1996). OX40 is expressed primarily on recently activated CD4+

T cells (Weinberg, 1998; Weinberg et al., 1998; Maxwell et al., 2000). In addition, OX40+ T cells have been identified in areas of inflammation, autoimmune disease, and sites of tumor infiltration (Weinberg, 1998; Weinberg et al., 2000). Engagement of OX40 on the T cell has been shown to decrease apoptosis and increase both proliferation and cytokine production from T cells, leading to enhanced T cell survival and immune responses (Gramaglia et al., 1998; Ohshima et al., 1998; Weinberg et al., 1998; Lane, 2000; Maxwell et al., 2000).

When we examined OX40 expression on OVA-specific CD4+ T cells, we only detected very low amounts of OX40. However, in spite of the low level of expression, OX40 was elevated on days 1 and 2 as compared to the level present on naive CD4+ T cells. The low level of OX40 expression could reflect a lack of activation of the OVA-specific CD4+ T cells in this experiment. However, several other activation markers, such as CD44, CD62L, and CD95, were altered, suggesting that the OVA-specific T cells were indeed activated. Another possible explanation for the low level of OX40 expression detected on the OVA-specific T cells could be that the fusion protein did not bind to OX40. However, this was also unlikely since ConA stimulated DO11.10 blasts showed high levels of OX40 expression. Alternatively, the low level of staining detected in our experiment could potentially be due to a high degree of background staining that is observed in the spleens of mice (RA Prell, personal communication). Further study will be necessary to determine if TCDD alters OX40 expression.

As an alternative way to evaluate if OX40/OX40L interaction was interrupted in TCDD-treated mice, we treated adoptively-transferred mice with agonistic anti-OX40. Maxwell et al., (2000) recently showed that anti-OX40 treatment enhanced the survival and memory formation of OVA-specific CD4+ T cells in adoptively-transferred mice. In our studies, treatment with anti-OX40 significantly increased the percent and number of OVA-specific T cells on day 5 after immunization in both vehicle- and TCDD-treated mice. In addition, anti-OX40 increased the production of

cytokines (IL-2, IFN-γ, and IL-4) on all days examined. Unexpectedly, however, anti-OX40 treatment did not decrease apoptosis of CD4+ T cells as measured by annexin staining on the OVA-specific cells on days 3 and 5. These results suggest that treatment with anti-OX40 increased the population of the OVA-specific cells by increasing proliferation rather than decreasing apoptosis.

When we examined proliferation of the OVA-specific CD4+ T cells using CFSE-labeled DO11.10 cells adoptively-transferred into Balb/c mice, anti-OX40 treatment increased the proliferative capacity of OVA-specific cells at both 3 and 5 days after immunization, as measured by an increase in the percent of T cells that had undergone at least seven rounds of division. These results correlate with the increase in cell number observed on day 5 and provide evidence that OX40 ligation increased the proliferation of OVA-specific CD4+ T cells. One interesting finding in our studies using CFSE was that exposure to TCDD alone increased the proliferative capacity of the OVA-specific CD4+ T cells on day 3 after immunization. These data are consistent with the results of Neumann et al (1993), in which TCDD dose-dependently increased the proliferation of lymph node T cells 24 hours after anti-CD3 injection. In addition, recent studies conducted in our laboratory have shown that DC removed from TCDD-treated mice increased the in vitro proliferation of DO11.10 T cells (BA Vorderstrasse and NI Kerkvliet, manuscript in preparation). These data suggest that TCDD may enhance the initial activation of T cells, which could make them more susceptible to apoptosis, possibly via activation-induced cell death.

The effect of anti-OX40 treatment on OVA-specific antibody production in TCDD-treated mice was also investigated. OX40/OX40L interaction has been implicated in driving optimal germinal center formation and antibody production from B cells (Stuber et al., 1995; Stuber and Strober, 1996; Brocker et al., 1999). Interestingly, on day 5 after immunization, anti-OX40 did not have any effect on plasma IgM levels,

however, when total IgG levels were evaluated, anti-OX40 treatment significantly enhanced the production of IgG in both vehicle- and TCDD-treated mice. Nonetheless, there remained a significant suppression of plasma IgG levels in TCDD/anti-OX40-treated mice as compared to vehicle/anti-OX40-treated mice. Thus, it appears that while anti-OX40 can increase antibody production in adoptively-transferred mice, it does not completely restore antibody production in TCDD-treated mice. Therefore, the TCDD-induced decrease in antibody response could result from an effect on some other parameter of CD4+ T cell function that we have not measured, such as CD40L expression, or from a direct effect on B cells.

In conclusion, the results of these studies using the DO11.10 OVA-specific adoptive transfer model support our hypothesis that TCDD causes a decrease in activated OVA-specific cells by a mechanism that involves increased cell death (by apoptosis and/or necrosis). Although the exact mechanism by which TCDD decreases the activated antigen-specific CD4+ T cells remains to be fully elucidated, the mechanism may involve decreased survival signals delivered to the T cell. Provision of an exogenous survival signal was able to completely reverse the TCDD-induced decrease of OVA-specific CD4+ T cells and was able to enhance cytokine production. However, despite the presence of large numbers of apparently functional T cells, anti-OX40 treatment did not overcome the TCDD-induced decrease in antibody production. Therefore, our results suggest that the decrease of OVA-specific CD4+ T cells does not solely mediate the TCDD-induced suppression of the anti-OVA response.

Chapter 6

Summary and Conclusions

The studies presented in this dissertation addressed the hypothesis that exposure to TCDD decreases T lymphocyte number by a mechanism that involves augmented cell death. In addition, we also wanted to determine whether the TCDD-induced decrease in T cells was responsible for the suppression of the immune response. In order to test our hypothesis, we evaluated T cell death in three different in vivo models in which TCDD suppresses T cell numbers and/or function.

The results obtained in the DO11.10 OVA-specific adoptive-transfer model support the hypothesis that TCDD causes a decrease in activated OVA-specific CD4+ T cells by a mechanism that involves increased cell death, as measured by annexin v and 7-AAD. Likewise, in anti-CD3-treated mice, TCDD-induced T cell deletion was correlated with an increase in the percent of T cells staining positive for annexin v and 7-AAD. In contrast, increased T cell death did not appear to be a mechanism by which TCDD causes the decrease in the number of T cells in mice injected with P815 allogeneic tumor cells. Thus, there are alternative mechanisms involved in the decrease of T cells in TCDD- and P815-treated mice. For instance, the TCDD-induced decrease in CD8+ T cells may be caused by a lack of activation and expansion of the cells, as opposed to increased death. However, due to the inability to specifically assess the CD4+ T cells responding to P815 allogeneic tumor cells, it remains a possibility that TCDD increases cell death of these cells.

Although our data indicate that TCDD augments T cell death, the role of apoptosis in the decrease of T cells is unclear. In the DO11.10 adoptive-transfer model, the TCDD-induced increase in cell death was a result of an increase in the percent of both apoptotic and dead T cells, thus

suggesting that apoptosis is involved in the decrease of OVA-specific CD4+ T cells. In contrast, in mice treated with anti-CD3, the TCDD-induced increase in T cell death was due to an increase in the percent of dead T cells, which would indicate that apoptosis is not mediating the decrease observed in T cells. However, upon further examination of potential mechanisms of TCDD-induced T cell deletion, we found that Fas/FasL interaction may be involved in the TCDD-induced decrease in anti-CD3-activated T cells, thus providing suggestive evidence that apoptosis may actually be involved.

While the exact mechanism by which TCDD increases cell death remains to be elucidated, it is possible that TCDD may promote a death signal or prohibit a survival signal being delivered to the T cells. In anti-CD3-treated mice, the TCDD-induced deletion of T cells may be mediated by the Fas pathway. It remains to be determine if the Fas pathway is involved in the TCDD-induced decrease of OVA-specific CD4+ T cells. Alternatively, in the DO11.10 adoptive-transfer model, the provision of a survival signal reversed the TCDD-induced decrease in OVA-specific CD4+ T cells, suggesting that TCDD may inhibit the delivery of survival signals to activated T cells. Taken together, these results indicate that TCDD may augment T cell death by multiple mechanisms.

In conclusion, these data suggest that the TCDD-induced decrease in T cells is mediated in part by increased cell death. This conclusion is supported by the fact that either inhibiting a death pathway or providing a survival signal appears to reverse the TCDD-induced decrease in T cell numbers. However, the TCDD-induced decrease in T cells does not appear to be solely responsible for the suppression of the immune response in TCDD-treated animals, since large numbers of apparently functional T cells were not able to overcome the TCDD-induced suppression of the OVA-specific antibody response. Therefore, the exact mechanism by which TCDD induces immune suppression remains to be elucidated.

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