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Abstract approved: Redacted for privacy

Nancy I. Kerkvliet

T cell activation requires a signal delivered through the T cell receptor (TCR) in combination with a costimulatory signal, both of which are delivered by antigen presenting cell (APCs). Complete T cell activation results in proliferation, differentiation and acquisition of effector function (e.g. cytokine production or lytic activity). Previous studies have shown that exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes a suppression of specific effector functions associated with $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. In this thesis, the hypothesis is tested that exposure to TCDD alters T cell activation which leads to decreased T cell function. Three in vivo models of T cell activation that differ in their requirement for APC-mediated signals were used to study TCDD-induced alterations on T cell function. The first model utilized anti-CD3 (145-2C11), a monoclonal antibody that binds to the CD3 epsilon portion of the murine TCR and activates T cells independent of major histocompatibility complex (MHC) proteins on APCs. Anti-CD3-induced activation induces a transient release of several cytokines and transient T cell proliferation. In model two, staphylococcal enterotoxin A (SEA), a bacterial superantigen, binds to MHC class II proteins in an unprocessed form and specifically activates T cells bearing the Vβ3 chain of the TCR was used. Injection of SEA causes the clonal expansion and subsequent peripheral deletion of $V\beta3^+$ T cells. Thirdly, T

cell activation following injection of allogeneic P815 tumor cells was used as a model in which activation is completely dependent on APCs. Exposure to TCDD did not significantly affect anti-CD3-induced cytokine production or SEA-induced clonal expansion or deletion of $V\beta3^+$ T cells. However, TCDD significantly increased anti-CD3-induced depletion of $CD4^+$ but not $CD8^+$ lymph node cells. In contrast, exposure to TCDD decreased cytokine production and the generation of effector cytotoxic T cells (CTL_E) in P815-challenged mice. These results suggested that the APC is important for TCDD-induced immunotoxic effects. Subsequent studies showed that the expression of CD86 (B7-2), a critical costimulatory molecule expressed on the surface of APCs, was decreased following TCDD exposure. Furthermore, providing exogenous CD86 significantly augmented the CTL response in TCDD-treated mice. Similarly, administration of exogenous IL-2 increased the CTL response in TCDD-treated mice. Taken together these results suggest that the APC rather than the T cell may represents the primary target of TCDD and are consistent with the induction of anergy in T cells by TCDD due to defective costimulation by APCs.

Investigation of the Mechanism of 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Immunotoxicity in C57Bl/6 Mice: Effects on T Cell Activation

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Rodney A. Prell

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APPROVED:

Redacted for privacy

Major Professor, representing Toxicology

Redacted for privacy

Chair of Program of Toxicology

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Dean of Graduate School

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

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Rodney A. Prell, Author

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

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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.	Technical support; critical review of the experimental design, data, and manuscripts; guidance; patience.
Oughton, Julie A.	Technical support; critical review of data and manuscript.
Steppan, Linda B.	Technical support.
Vella, Anthony T.	Technical support; critical review of experimental design, data, and manuscript.

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CHAPTER I

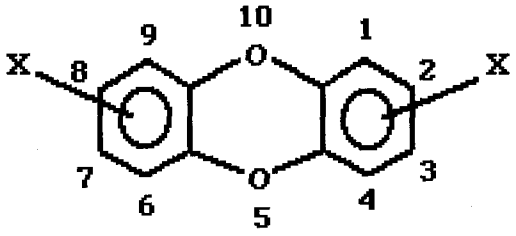
INTRODUCTION

The research described herein have addressed the mechanism(s) involved in the immune suppression caused by exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin). The primary focus of the studies was on the potential effects of TCDD on T cell activation. This introductory chapter is organized into the following sections: 1) a general summary of halogenated aromatic hydrocarbons (HAH) toxicity, 2) the immunotoxicity of TCDD, and 3) a brief description of the three models used to investigate TCDD-induced alterations in *in vivo* T cell function.

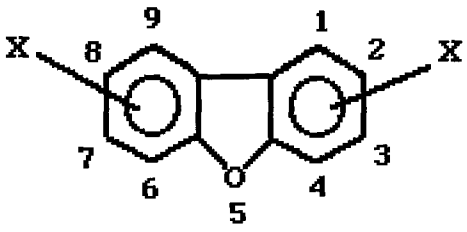
HALOGENATED AROMATIC HYDROCARBONS

Overview

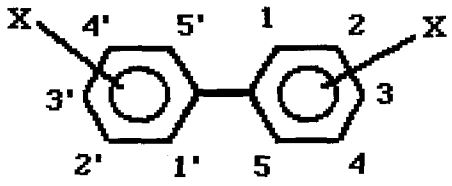
Halogenated aromatic hydrocarbons (HAH) are a family of structurally related chemicals which include the biphenyls, dibenzofurans, azobenzenes, azoxybenzenes, diphenyl ethers, naphthalenes, and dibenzo-*p*-dioxins (figure I-1). The multiple ring structure of this family of chemicals allows for several possible halogenation sites which results in the formation of many different isomers. For example, there are 209 possible isomers of polychlorinated biphenyls (PCBs), 135 polychlorinated dibenzofurans (PCDFs), 209 polychlorinated azo- and azoxybenzenes, 75 polychlorinated naphthalenes,



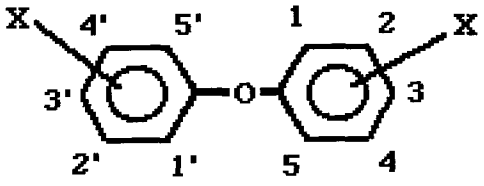
Dibenzodioxins



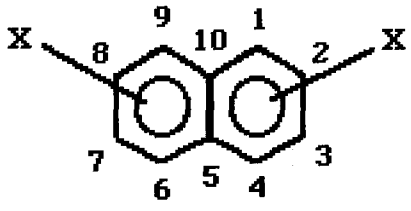
Dibenzofurans



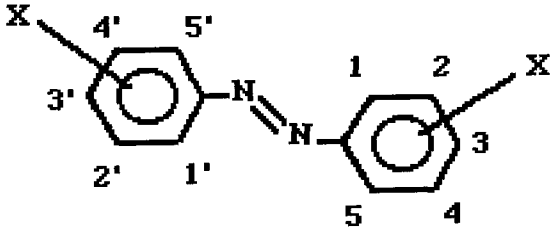
Biphenyls



Diphenyl Ethers



Naphthalenes



Azobenzenes

Figure I-1. Structures of various halogenated aromatic hydrocarbons

and 75 polychlorinated dibenzo-*p*-dioxins (PCDDs). More than 5000 different combinations are possible for HAH containing a mixture of chlorine and bromine substitutions (De Vito and Birnbaum, 1994).

The United States started commercial production of PCBs in 1929. Their stable physical-chemical properties, which include low vapor pressure, thermal stability, and little or no flammability (Lang, 1992; Pohjanvirta and Tuomisto, 1994; Webster and Commoner, 1994), led to their widespread use in hydraulic fluids, transformers, plasticizers, immersion oils, fire retardants, and many other industrial processes (Safe, 1990). These same properties led to the halt in their production roughly five decades later after certain health and environmental problems were found to be associated with HAH. During the 48 years of production, approximately 1.5 million metric tons of PCBs were produced worldwide, of which 650,000 metric tons were produced in the United States (De Voogt and Brinkman, 1989).

In contrast to PCBs, PCDDs and PCDFs were never intentionally manufactured. Instead, these compounds are unwanted by-products of numerous industrial processes including the production or burning of polyvinyl chloride (PVC) plastics and certain herbicides such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Tiernan et al., 1985; Webster and Commoner, 1994), incineration of medical and municipal waste (Olie et al., 1977; Vogg and Stieglitz, 1986; Lindner et al., 1990), combustion of leaded gasoline (Fielder et al., 1990; Marklund et al., 1990), and the chlorine bleaching processes (Amendola et al. 1989).

Environmental Distribution and Fate

HAH have been dispersed in the environment as a result of herbicide use, industrial accidents, fires, and negligence. Due to their chemical stability HAH persist and accumulate in the environment .

The discovery of HAH in pristine areas increased public awareness to the fact that atmospheric distribution was a major contributor to environmental contamination of HAH (Czuczwa et al. 1984; Stalling et al. 1983). Many HAH, especially the highly substituted congeners, are apparently stable enough to travel long distances in the atmosphere, leading to their deposition in the terrestrial and aquatic compartments in the environment (Zook and Rappe 1994). The highly substituted species more readily adsorb to particulates in the atmosphere which protects them from photodegradation (Rappe et al., 1989; Zook and Rappe, 1994). Biological transformation and degradation of PCDDs/PCDFs are thought to be relatively slow processes. The half-life of TCDD in soil has been estimated to be approximately 10 years (Vanden Heuvel and Lucier, 1993).

The primary route of exposure of terrestrial animals, including humans, to HAH is through the consumption of HAH-contaminated meat, fish and vegetation; minor routes include inhalation and dermal exposure (Startin, 1994). Since HAHs are lipophilic, they tend to bioaccumulate in the fatty tissue where they are sequestered and remain highly resistant to metabolic breakdown. The relative resistance to metabolic breakdown is evidenced by whole body half-life times. The half life for TCDD ranges from 10-15 days in mice, 12-31 days in rats, and 10-12 days in the hamster. In comparison, the whole body half life of TCDD in humans has been estimated to be between 7-14 years (Olson, 1994).

Identification of the Aryl Hydrocarbon Receptor

Nebert and co-workers (Nebert and Gielen, 1972; Nebert et al., 1972) first observed that certain strains of mice, but not others, increased aryl hydrocarbon hydroxylase (AHH) activity in response to treatment with 3-methylcholanthrene (3-MC). Further studies revealed that exposure to other structurally related aromatic hydrocarbons, such as PCBs and PCDDs, induced high levels of AHH and ethoxyresorufin-O-deethylase (EROD) activity as a result of increased levels of cytochrome P450 1A1 (CYP1A1). In

fact, TCDD was found to be approximately 30,000 times more potent than 3-MC in inducing CYP1A1 enzyme activity (Poland et al., 1982). Genetic crosses and back crosses between C57Bl/6 (B6) and DBA/2 (D2) mice demonstrated that the responsiveness to 3-MC, as measured by induction of CYP1A1, segregated as a dominant trait that was controlled by a single autosomal gene. This gene has been designated the aryl hydrocarbon (*Ah*) locus and encodes a protein known as the Ah or dioxin receptor (Green, 1973; Thomas and Hutton, 1973). Poland et al. (1976) were the first to identify and characterize the Ah receptor (AhR) which signified a major advancement in understanding the toxicities associated with TCDD and dioxin-like chemicals.

Subsequent genetic studies revealed that B6 mice are homozygous for the Ah^b allele and are the prototypic responsive strain to AhR ligands, whereas D2 mice are homozygous for the Ah^d allele and are the prototypic nonresponsive strain. Although considered nonresponsive to 3-MC, D2 mice show similar CYP1A1 induction to that of B6 mice if exposed to approximately 10-fold higher doses of TCDD. The difference in inducibility has been attributed to the fact that the Ah receptor in D2 mice has a lower binding affinity (~ 6 fold) for TCDD as compared to B6 mice (Okey et al., 1989; Poland et al., 1994). Sequence analysis has revealed that the Ah^b allele encodes an 89-kDa protein of 805 amino acids with an estimated equilibrium dissociation constant (K_d) for TCDD in the range of 10^{-10}-10^{-12} M (Hankinson, 1995). In comparison, the Ah^d allele encodes a protein with an additional 43 amino acids at the carboxy terminus and also differs from the Ah^b allele at four other amino acids (Chang et al., 1993). Recent data suggest that the reduced ligand binding affinity of the Ah^d receptor is a result of the polymorphism at amino acid 375. The high affinity receptor contains a valine at this position whereas the low affinity receptor contains an alanine. The location of this polymorphism has been demonstrated to be within the ligand-binding domain (Poland et al., 1994).

Mechanism of Action: Aromatic Hydrocarbon Receptor Model

Most, if not all, of the toxicity of TCDD and structurally related HAH appears to result from interaction with the AhR (Lucier et al., 1993). In its unbound form, the AhR exists in the cytosol complexed with a dimer of 90 kDa heat shock proteins (Hsp90) and a putative third protein of about 46 kDa (Hsp 50) (Perdew, 1992). These associated proteins may help maintain the AhR in a ligand-binding conformation and/or prevent nuclear translocation (Pongratz, et al, 1992; Whitelaw et al, 1994). Since TCDD is highly lipophilic, it is able to freely diffuse through the cell membrane. Once in the cytosol, TCDD can bind to the AhR causing a conformational change that results in the dissociation of Hsp90 and Hsp50. The ligand-bound AhR then binds to a second protein called the aryl hydrocarbon nuclear translocator (ARNT) which forms an activated DNA-binding complex (reviewed in Hankinson, 1995; Schmidt and Bradfield, 1996). During the dimerization between the ligand-bound AhR and ARNT, a translocation from the cytoplasm to the nucleus occurs, permitting the activated complex to interact with consensus DNA sequences known as xenobiotic response elements (XRE) or dioxin response elements (DRE). These response elements have been identified in the enhancer region of a number of genes (Lai et al., 1996) and contain a core sequence 5'-GCGTG-3' which is required for recognition by the activated complex.

Cloning of the AhR and ARNT proteins revealed that both subunits were members of the basic helix-loop-helix (bHLH) superfamily of transcription factors. Other members of this family include *Drosophila melanogaster* proteins Per and Sim, and the mammalian myogenic proteins myc, max, myo D and the newly identified hypoxia-inducible factor 1α (HIF-1α). All members of this family contain a homologous domain termed the PAS domain, for Per-ARNT-Sim and are involved in sequence-specific transcriptional regulation. The bHLH and PAS domains contain subdomains that are involved in protein dimerization and DNA binding (Hankinson, 1995).

Binding of activated AhR-ARNT complexes to DREs has been shown to alter gene transcription (figure I-2). Several genes involved in xenobiotic metabolism, including cytochrome P4501A1, NAD(P)H menadione oxidoreductase, UDP glucuronosyltransferase, glutathione transferase Ya, and aldehyde dehydrogenase have been reported to be transcriptionally regulated through an AhR-dependent mechanism (Israel and Whitlock, 1984; Fischer et al., 1989; Nebert, 1989; Nebert et al., 1993). It is hypothesized that other pleiotropic effects of TCDD such as immunotoxicity, teratogenicity, and antiestrogenicity are also a consequence of alterating the transcription of genes containing DREs. Consistent with this idea, TCDD has been shown to affect the transcription of several growth factors or cytokines including transforming growth factor (TGF)-α and TGF-β, and IL-1β (Sutter et al., 1991; Gaido et al., 1992). TCDD has also been shown to modulate the expression and/or ligand binding of several growth factor receptors. For instance, TCDD downregulates estrogen receptor expression (Wang et al., 1993) and also inhibits insulin-like growth factor (Liu et al., 1992), epidermal growth factor, and TGF-induced (Fernandez and Safe, 1992) cell growth in human breast cancer cells. Therefore, it is possible that TCDD alters cellular homeostasis by directly interfering with normal signalling pathways involved in growth and differentiation.

Other mechanisms have also been proposed that may account for TCDD-induced alterations in gene transcription (Okino and Whitlock, 1995; Safe, 1995). For example, the activated AhR complex may alter chromatin structure, allowing other transcription factors to bind more easily. Alternatively, DNA binding may disrupt or displace endogenous transcription factors required for transcription. Consistent with this mechanism, Krishnan et al (1994) reported that the activated AhR heterodimeric complex directly disrupted an estrogen receptor/Sp1 complex by binding to an imperfect DRE sequence located between the ER and Sp1 genomic binding sites. Similarly, Zacharewski et al (1994) have shown that a DRE sequence plays a role in the inhibition of 17β-estradiol-induced pS2 expression.

Figure I-2. The Ah receptor model.

A general schematic of the Ah receptor model is shown. The ligand passively diffuses through the cell membrane and binds to the Ah receptor. Ligand binding causes the release of the previously associated heat shock proteins. The ligand-bound AhR complex then binds to the AhR nuclear translocator protein (ARNT). The ligand-bound heterodimeric complex then translocates to the nucleus where it binds to specific DNA sequences known as dioxin response elements (DREs) regulating transcriptional events. Numerous biological events such as induction of CYP 1A1 protein have been shown to occur through an AhR-mediated mechanism. Regulation of other DRE-containing genes that encode for growth factors, cytokines, and/or receptors may play a role in the teratogenic, carcinogenic, antiestrogenic and immunotoxic effects of HAH.

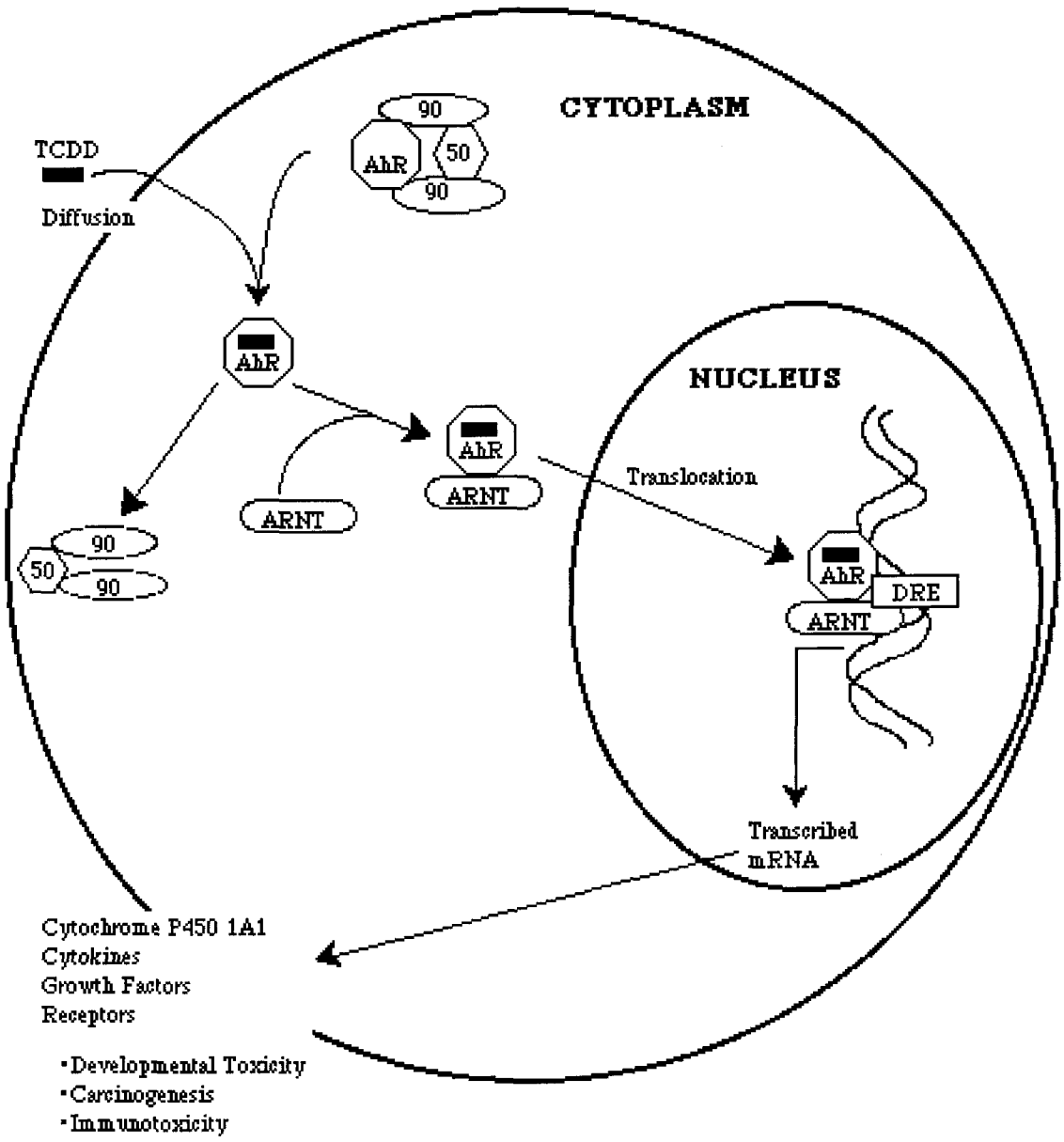

CYTOPLASM
TCDD
Diffusion
90
AhR
50
90
AhR
ARNT
AhR
ARNT
NUCLEUS
Translocation
90
50
90
AhR
DRE
ARNT
Transcribed
mRNA
Cytochrome P450 1A1
Cytokines
Growth Factors
Receptors
•Developmental Toxicity
•Carcinogenesis
•Immunotoxicity

Figure I-2.

However, the connection between modulation of specific transcriptional events caused by exposure to TCDD and the subsequent toxic effects remains to be determined.

DRE-Independent Mechanisms of Action: Role of Phosphorylation

Not all of the observed effects of TCDD exposure can be explained by the AhR model in which an activated complex composed of the ligand-bound AhR and ARNT translocates to the nucleus, binds to DREs and actively alters transcription. For example, Puga et al. (1992) reported that TCDD caused a rise in intracellular Ca^{2+} as early as 2 minutes after in vitro treatment of cultured Hepa-1 cells, and this phenomenon also occurred in a Hepa-1 cell line which is known to be deficient in ARNT. TCDD exposure also increased the level of mRNA for junB, jun-D and c-fos within 2 hours in C4 cells. Since ARNT is required for binding of the activated AhR complex with DREs and subsequent transcriptional activation, these data suggest that ARNT is not required for the induction of fos or jun mRNA expression by TCDD in this cell line. These same studies showed that TCDD exposure increased c-ras GTP-binding activity within 15 minutes (Puga et al., 1992). Based on the speed of occurrence, these effects are unlikely to be mediated through AhR-dependent transcriptional activation and protein synthesis. Therefore it appears that a separate DRE-independent pathway may be responsible for these events.

In order to help interpret these otherwise paradoxical phenomena, Matsumura (1994) recently described a hypothetical model in which the action of ligand binding to the AhR activates a kinase cascade that does not require AhR/DRE interactions and subsequent *de novo* protein synthesis (figure I-3). According to this model, a protein kinase associated with the AhR, presumably within HSP90 and/or HSP50 becomes activated following TCDD binding to the AhR and alters protein phosphorylation. Consistent with this idea, numerous reports indicate that TCDD exposure results in altered phosphorylation patterns in various cells. For instance, TCDD-induced alterations in phosphorylation have been reported in hepatocytes (Matsumura et al., 1984), B lymphocytes (Clark et al., 1991a),

mouse thymocytes (Bombick and Matsumura, 1987), adipocytes (Enan and Matsumura, 1993), and pancreatic cells (Ebner et al., 1993). Furthermore, Enan and Matsumura (1995) showed that addition of TCDD to a cell-free system containing a purified AhR or to an isolated HSP90 complex preparation containing the AhR caused an increase in protein tyrosine kinase activity. Activation occurred in the presence of actinomycin D and cycloheximide, indicating that *de novo* protein synthesis was not required. Finally, Enan and Matsumura (1996) identified the oncoprotein c-src as the integral component of the Ah receptor complex that transduced a signal through a protein phosphorylation pathway following ligand binding to the AhR. Taken together, these data strongly support the existence of an alternative pathway, distinct from the AhR-ARNT-DRE-dependent model, by which dioxin-like compounds can alter cellular responses. However, direct evidence for a kinase-associated pathway in mediating the toxic effects of TCDD has yet to be determined.

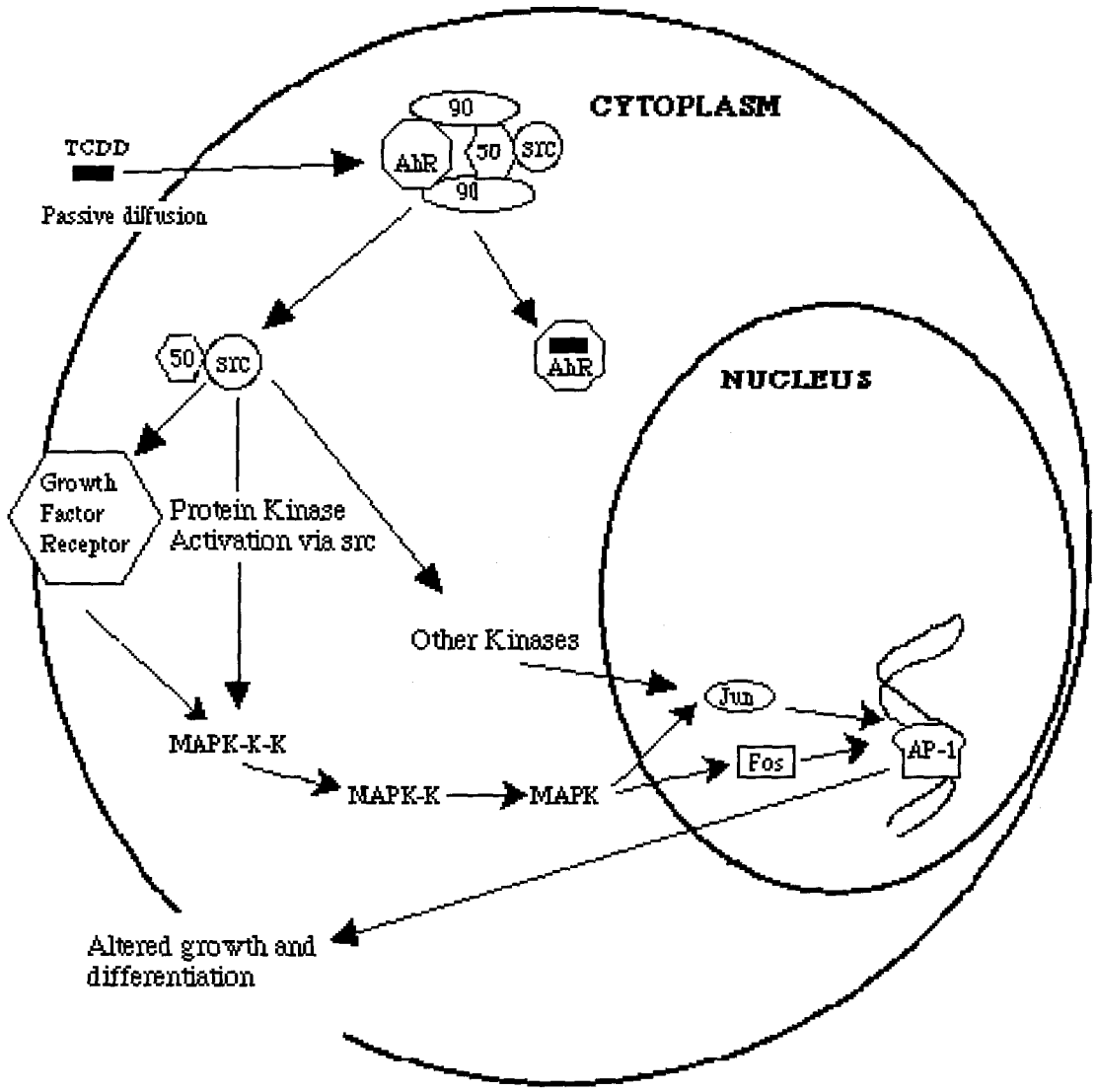
TOXIC EFFECTS OF HAHS

The toxicity of HAH differs widely between different congeners. Structure-activity relationship studies have shown that the most toxic congeners are the more highly substituted HAH. However, the arrangement of the halogen atoms on the aromatic rings also plays a role in determining toxic potency. For the dioxins and furans, halogenation at the lateral position (positions 2,3,7,8) increases the toxicity of these compounds whereas halogen substitution at the nonlateral positions (1,4,6,9) decreases their toxicity (Poland and Knutson, 1982). Similarly, biphenyls which contain halogen molecules in at least two adjacent lateral positions (3,3',4,4',5, or 5') are more toxic than those containing halogens adjacent to the biphenyl bridge (figure I-1). Comparison of the toxic HAH reveals that they are all planar or can assume a planar configuration, which is critical for binding with high affinity to the AhR. Addition of halogens at any of the nonlateral positions decreases

Figure I-3. DRE-independent mechanism of HAH-induced toxicity.

A schematic of the hypothetical DRE-independent "protein phosphorylation pathway" is shown. Ligand passively diffuses through plasma membrane and binds to the Ah receptor complex consisting of heat shock proteins 90 and 50 and the protein kinase SRC. After ligand binding, the AhR dissociates from the complex releasing the SRC and activating its protein kinase activity. The activated SRC can then activate other intracellular kinases such as the mitogen activated protein (MAP)-kinase by inappropriately triggering growth factor receptor activation. Alternatively, the activated SRC could directly activate the MAP-kinase cascade or other pathways that eventually lead to activation of transcription factors such as AP-1, that are involved in cellular growth and differentiation.

Figure I-3.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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

receptor binding by altering size or coplanarity (Poland and Knutson, 1982; Vanden Heuvel and Lucier, 1993). Of the HAH, TCDD is used as the prototype congener to investigate HAH-induced toxicities because it has the highest toxic potency of this family of compounds (DeVito and Birnbaum, 1994).

Metabolism is also very important in determining the toxicity of HAH. Two of the major factors that influence the rate of HAH metabolism are the degree of ring substitution and the ring substitution pattern (Safe, 1992). Early studies demonstrated that lower-halogenated congeners are preferentially metabolized into polar metabolites that can be excreted as glucuronides (Poiger and Schlatter, 1979; Sundstrom et al., 1976). However, other more highly-substituted congeners appear to be biotransformed at a fairly slow rate. In addition, many HAH are highly lipophilic and tend to accumulate in the fatty tissue where they are sequestered and remain highly resistant to metabolic breakdown. Thus, several HAH tend to have a long whole body half life which can influence their overall toxicity.

A number of studies in accidentally- or occupationally-exposed humans have been conducted to examine the toxic effects of HAH in humans (reviewed in Kimbrough 1987; Dickson and Buzik, 1993). For instance, Cook et al. (1986) evaluated the mortality patterns for the time period between 1940 and 1979 of 2189 men with potential occupational exposure to PCDDs. Particular attention was directed to TCDD and mortality due to cancers of the liver, stomach and nasopharyngeal tissue as well as soft-tissue sarcomas, non-Hodgkins lymphoma, and Hodgkins disease. The cause-specific mortality for the cohort study was compared to that of white males in the United States. No TCDD-related increases in mortality due to these cancers were found. In contrast, Fingerhut et al. (1991) reported that mortality from all cancers was significantly increased in 5172 industrial workers exposed to chemicals contaminated with TCDD when compared to the control cohort population. Interestingly, in a subcohort in which workers had 1 year or more exposure period and 20 years or more of latency, a 46% increase in all cancers was

found. However, no increase in mortality due to soft tissue sarcomas, Hodgkins disease or non-Hodgkins lymphoma could be detected. Furthermore, no dose-response relationship with TCDD and specific malignancies could be established in either study.

Numerous other clinical symptoms have been reported following exposure to HAH and include weight loss, headache, hyperkeratosis and sexual dysfunction. However, the only lesion that has been directly attributed to HAH exposure is chloracne. This disease resembles juvenile acne and can be distinguished only based on exposure history, age of onset and other factors (Kimbrough, 1984). Chloracne involves hyperkeratosis of the hair follicle and hyperplasia of the interfollicular epidermis resulting in the formation of cystic acne, and can be experimentally induced by high-dose oral or dermal exposure in monkeys, hairless mice and rabbits. Chloracne has been used as a marker of accidental exposure in humans, especially in chemical workers.

Studies in laboratory animals have shown that exposure to HAH results in numerous toxic effects including a wasting syndrome, thymic atrophy, immune suppression, hepatotoxicity, reproductive toxicity, teratogenicity, and carcinogenicity. Several biochemical alterations involving enzyme induction or suppression, hormone imbalances, and membrane receptor expression have also been reported following exposure to HAH (reviewed in Poland and Knutson, 1982; DeVito and Birnbaum, 1994; Pohjanvirta and Tuomisto, 1994). However, it is important to note that the toxic effects of TCDD and related chemicals vary between animal species. Even within the same species, the toxicological effects can vary, depending on age, sex, and strain of the animal. It is evident that TCDD and other dioxin-like chemicals are toxic to many organ systems. However, despite the considerable knowledge regarding TCDD-induced toxicity, the exact biochemical and/or cellular targets remain to be elucidated.

THE IMMUNE SYSTEM: AN OVERVIEW

The immune system is responsible for the protection of an organism against infections by viruses, parasites, bacteria, and other foreign bodies. It is also involved in the detection of altered "self" tissue and is critical in the prevention of cancer.

The immune system can be divided into two major arms: 1) the innate or non-specific system, and 2) the acquired or adaptive system. The innate immune system represents the first line of defense of the host and is composed of preformed serum proteins such as complement, as well as a number of effector cells (natural killer cells, eosinophils, neutrophils and monocyte/macrophages). The specificity and response time of the innate immune system do not change with repeated exposure to antigen.

The acquired immune system can be further subdivided into two effector arms, the humoral and cellular immune responses which are mediated by B lymphocytes and T lymphocytes, respectively. B cells are responsible for two major functions including the production of antibodies and presentation of antigen to T cells (Benjamini and Leskowitz, 1988). Antibodies bind to antigenic determinants with high specificity and directly inactivate the antigen or mark them for inactivation by other components of the immune system (Benjamini and Leskowitz, 1988). In general, antibody production by B cells requires the help of T cells. T lymphocytes are divided into two major subtypes by the differential expression of the CD4 and CD8 glycoproteins. $CD4^+$ T cells are collectively known as T helper (T_H) cells. The function of activated T_H cells is to provide help, in the form of soluble mediators (cytokines) and/or surface associated molecules, to other immune cells. In comparison, $CD8^+$ T cells are recognized as suppressor T cells (T_S) as well as cytotoxic T lymphocytes (CTL). The primary function of an activated $CD8^+$ T lymphocyte is to actively seek out and lyse altered cells, such as virus-infected or cancer cells. A subset of the activated T and B lymphocytes differentiates into memory cells which persist long after the antigen has been eliminated and is the basis of immunological

memory. Therefore, in contrast to an innate immune response, an acquired immune response ensures a more rapid and effective response upon subsequent exposure to the same antigen .

IMMUNOTOXICITY OF HAHS

Laboratory animal studies

Data from laboratory animal studies suggest that the immune system is one of the most sensitive targets of TCDD and other AhR ligands. This evidence is derived from numerous studies in which adverse effects on the immune system occurred at doses much lower than those required to cause other pathological changes. Although mice have been the preferred animal species to study the potential immunotoxic effects of TCDD and related HAHs, many other species including rats, rabbits, guinea pigs, monkeys, and marmosets have also been employed and shown to be sensitive to HAH-induced immunotoxicity (Kerkvliet and Burleson, 1994).

Most, if not all, of the immunotoxic effects of TCDD and related HAHs appear to be mediated through an Ah receptor-dependent mechanism. This conclusion is based on the following lines of evidence: 1) approximately 10-fold lower doses of TCDD are required to suppress the response to sheep red blood cells (SRBCs) in C57Bl/6 mice (high affinity AhR) as compared to DBA/2 mice (low affinity AhR; Vecchi et al., 1983), 2) the immunosuppressive potency of different PCDD, PCDF, and PCB congeners is positively correlated with their ability to bind the AhR (Silkworth and Grabstein, 1982; Kerkvliet et al.,1985), and 3) the sensitivity of C57Bl/6 mice congenic at the Ah locus segregates with the expression of the high affinity AhR (Ah^{bb}). Congenic mice expressing the low affinity AhR (Ah^{dd}) are significantly less sensitive to TCDD-induced suppression following challenge with SRBC (Kerkvliet et al., 1990a; Silkworth et al, 1993), TNP-LPS (Kerkvliet et al, 1990a), or allogeneic tumor cells (Kerkvliet et al, 1990b).

Thymic atrophy has been observed following exposure to TCDD in all animal species studied and is characterized by cellular depletion in the thymic cortex (Poland and Knutson, 1982; Vos et al., 1991). When animals are exposed prenatally, during the time when the thymus is critical for the development of T cells, TCDD-induced thymic atrophy is accompanied by immune suppression (Vos and Moore, 1974). In contrast, thymic atrophy in adult animals exposed to TCDD does not correlate with immune suppression (Vos et al., 1978). In fact, removal of the thymus in adult animals does not affect TCDD-induced immunotoxicity (Tucker et al., 1986; Kerkvliet and Brauner, 1987). The mechanism of TCDD-induced thymic involution remains unclear, but a number of potential mechanisms have been proposed including Ca^{2+}-dependent (McConkey et al, 1988) or Fas-dependent (Rhile et al., 1996) apoptosis of thymocytes, inhibition of terminal deoxynucleotidyl transferase activity in bone marrow and thymus cells (Fine et al., 1990), and decreased ability of thymic epithelial cells to support thymocyte proliferation (Greenlee et al. 1985). Therefore, the age at which the animal is exposed to TCDD is a critical factor in determining the role that TCDD-induced thymic atrophy plays in immune suppression.

Exposure to TCDD decreases host resistance to bacterial disease. Numerous studies have demonstrated that exposure to TCDD decreases host resistance to the gram negative bacteria *Salmonella* (Thigpen et al., 1975; Vos et al., 1978; Hinsdill et al., 1980). Thigpen et al (1975) reported that treatment of mice with TCDD at a dose of 1 μg/kg once a week for 4 weeks (total dose, 4 μg/kg) increased mortality following *Salmonella* infection but did not result in any clinical or pathological changes. Various results have been observed for the immunotoxic effect of TCDD in the host resistance of mice infected with *Listeria monocytogenes*. Hinsdill et al. (1980) demonstrated increased mortality of *L. monocytogene*-infected Swiss Webster mice fed a diet containing 50 ppb TCDD. In contrast, no increase in the number of viable *Listeria* organisms were detected in the spleen of Swiss mice gavaged with 50 μg TCDD/kg once a week for 4 weeks (Vos et al., 1978). Likewise, House et al. (1990) reported that a single oral dose of 10 μg TCDD/kg did not

increase mortality in mice challenged with *Listeria* seven days later. These conflicting results may reflect differences in study designs including dose, route of administration, single versus multiple administration, strain or age of the animals. However, these data suggest that under certain conditions, exposure to TCDD enhances susceptibility to *Listeria* infection.

In addition to inhibiting antibacterial responses, exposure to TCDD decreases host resistance to parasitic and viral disease. A single dose of 5 μg TCDD/kg increased the duration and magnitude of peak parasitemia in B6C3F1 mice challenged with the nonlethal strain of malaria, *Plasmodium yoelii* 17 XNL (Tucker et al., 1986). Enhanced mortality due to herpes simplex type II was observed in mice previously dosed with TCDD ip once a week for 4 weeks with doses ranging from 0.04-4.0 μg/kg (total dose range 0.16-16.0 μg/kg; Clark et al., 1983). Similarly, House et al. (1990) reported increased mortality of mice to *influenza/A/Taiwan/1/64 (H2N2)* after a single dose of TCDD as low as 0.1 μg/kg. Interestingly, a recent study by Burleson et al. (1996) demonstrated that exposure to a single dose of 0.01 μg/kg TCDD increased mortality in mice challenged intranasally with *influenza A/Hong Kong/8/68 (H3N2) virus.* No enhancement of mortality was noted at TCDD doses of 0.001μg/kg or 0.005 μg/kg. To date, this is the most sensitive immunosuppressive effect reported.

Decreased host resistance to infectious diseases may reflect suppressed immune responses in animals exposed to TCDD. Several studies have shown that acute in vivo exposure to TCDD and related HAH significantly suppresses humoral immune responses (reviewed in Vos and Luster, 1989; Kerkvliet, 1994). In fact, the antibody response to SRBCs is one of the most sensitive measures of TCDD-induced immune suppression with the ID_{50} value in the 0.65-0.70 μg/kg range (Vecchi et al., 1980; Kerkvliet et al., 1985). Interestingly, the antibody response to the T helper cell independent antigen trinitrophenol (TNP)-LPS was shown to be approximately 10 fold less sensitive to the immunosuppressive effects of TCDD than was the antibody response to SRBC, a T cell

dependent response (House et al., 1990; Kerkvliet et al., 1990a). These results have been interpreted to suggest that the extreme sensitivity of the in vivo anti-SRBC response to TCDD may depend on the T cell and/or antigen presenting cell components of the response.

Relatively few reports exist that examined the effects of exposure to TCDD on effector functions associated with $CD4^+$ T helper cells. Lundberg et al. (1990) reported that thymocytes from mice exposed to a dose of 50 μg TCDD/kg body weight were less able to support an in vitro anti-SRBC response. In addition, Tomar and Kerkvliet (1991) reported that exposure of SRBC-primed mice to a dose of 5 μg/kg TCDD suppressed the in vivo generation of T helper cells specific for trinitrophenol-benzenesulfonate conjugated SRBC. However, Clark et al. (1983) reported that in response to mitogen stimulation, T cells from TCDD-treated mice produced normal levels of interleukin (IL)-2. Therefore, the toxic effects of TCDD on T helper cell function remain unclear.

In contrast, several studies have shown that TCDD and related HAH suppress $CD8^+$ CTL responses in C57Bl/6 mice challenged with allogeneic P815 tumor cells (Clark et al., 1981; Clark et al., 1983; Kerkvliet and Baecher-Steppan 1988 a,b; Kerkvliet et al., 1990b; DeKrey et al., 1993; Hanson and Smialowicz, 1994; DeKrey and Kerkvliet, 1995; Kerkvliet et al., 1996). The suppression of splenic CTL activity has been shown to be dose-dependent and mediated via an AhR-dependent mechanism (Clark et al., 1983; Kerkvliet et al., 1990b; DeKrey and Kerkvliet, 1995). Clark et al. (1981) demonstrated that in vivo CTL activity in C57Bl/6 mice to allogeneic tumor cells was inhibited by as little as 0.4 μg/kg TCDD and that the generation of CTL activity in vitro could be suppressed by doses as low as 0.004 μg/kg. It was reported that the frequency of CTL precursors (CTL_p) was not reduced and suggested that the suppressed CTL activity represented a defect in the development of CTL_p into CTL as a result of increased suppressor T cell activity. This study has generated controversy, as other laboratories have been unable to duplicate the immunosuppressive effects of TCDD at such low exposure levels (Kerkvliet et al., 1990; Hanson and Smialowicz, 1994). Interestingly, however, a recent study by Kerkvliet et al.

(1996) indicated that the activation and/or clonal expansion of CTL_P into CTL effectors (CTL_E) is inhibited by much higher doses of TCDD, which is consistent with the initial interpretation by Clark et al. (1981). In addition, Kerkvliet et al. (1996) showed that the TCDD-induced suppression of CTL activity correlated with a profound suppression in IL-2, interferon (IFN)-gamma, and tumor necrosis factor (TNF) production in P815-challenged mice.

Overall, these studies provide evidence that exposure to TCDD suppresses both the humoral and cell mediated immune responses within the adaptive immune system. This is especially apparent in host resistance models where the enhanced susceptibility may reflect several small decreases in a number of different immune parameters that by themselves are not significant, but when combined may cause a significant immunosuppression. However, the mechanism(s) and immunological target(s) responsible for the immune suppression remain to be defined.

The effects of TCDD on the innate immune system are less apparent. House et al. (1990) reported that splenic spontaneous natural killer (NK) cell activity was not affected after intraperitoneal (ip) injection of TCDD at doses ranging from 0.1 to 10 μg/kg. Similarly, Mantovani et al. (1980) reported no effect on NK cell activity following a single dose of TCDD of 30 μg/kg. In contrast, Funseth and Ilbäck (1992) reported an increase in spontaneous NK activity on days 28 and 120 in the spleen and blood of male A/J mice after an initial ip dose of 5 μg/kg followed by 3 weekly doses of 1.42 μg/kg (total dose 9.26 μg/kg). However, in this study control mice had extremely low NK activity (2%) and an increase to only 6% in TCDD-treated mice was reported as significant. Interestingly, Yang et al. (1994) found that doses of 3 and 10 μg/kg TCDD did not alter spontaneous pulmonary NK activity but significantly suppressed influenza virus-augmented NK activity in rats. Thus, the effects of TCDD exposure on NK activity are inconsistent but may reflect differences in animal species or strains used or exposure regimes.

A limited number of studies have addressed the effects of TCDD on other components within the innate immune system. Ackerman et al. (1989) demonstrated that following an acute dose of 5 μg/kg TCDD, neutrophil-mediated tumor cytotoxicity was suppressed but degranulation or production of reactive oxygen was not affected. Macrophage functions, as assessed by phagocytosis, oxidative reactions, and cytolytic activity, were also not affected by exposure to TCDD at doses as high as 30 μg/kg (Mantovani et al., 1980; Vos et al., 1978). White et al. (1986) reported that complement levels were suppressed in mice following acute or subchronic exposure to a total dose of 14 μg/kg TCDD. Therefore, it appears that the innate immune system is more resistant to TCDD-mediated toxicity than is the acquired immune system (e.g. cell-mediated and humoral immunity).

Human evidence

The potential immunotoxic effects of TCDD and related HAHs in humans has been the subject of several studies involving long-term occupational exposure (reviewed in Nicholson and Landrigan, 1994). For instance, Tonn et al. (1996) recently reported on immunological parameters of industrial workers 13-15 years after the last known exposure to TCDD. The level of TCDD in blood fat of the 11 individuals ranged from 43-874 pg/g, which was above that reported for the general population of Germany (approximately 4 pg/g). At the time of the study 5 subjects still suffered from chloracne. When the mitogenic responses of T and B cells were investigated, no TCDD effects were observed (Tonn et al., 1996). Interestingly, a decreased T helper cell function, as indicated by a reduced response to in vitro alloantigen and proliferative response to IL-2, was observed in the TCDD-exposed group (Tonn et al., 1996). Because the frequency and absolute numbers of T helper cells were not different between groups, these data suggested that the immunosuppressive effects more likely reflected a decreased functionality of individual T helper cells rather than a reduction in the T helper cell subset. However, the observed

changes in the in vitro assays may not correlate with increases in diseases such as certain cancers or infections (Webb et al., 1989). Also these conclusions were based on only 11 individuals; therefore, the results should be interpreted with caution.

Other human studies which report on the potential immunosuppressive effects of TCDD and related HAHs are a result of accidental exposure. Such was the case when an explosion occurred in 1976 at the ICMESA plant near Seveso, Italy, in which at least 1.3 kg of 2,3,7,8-tetrachlorodibenzo-p-dioxin contaminated a populated area of approximately 2.8 km^2. Initial studies of 44 children exposed to TCDD as a result of the explosion found no abnormalities in the proliferative responses to T and B cell mitogens, serum immunoglobulin titers, or circulating levels of complement (Mocarelli et al,. 1986). In contrast, a second study conducted on a different cohort of children six years after the explosion revealed a significant increase in complement protein levels, which correlated with an increased incidence of chloracne. However, no specific health problems were associated with increased TCDD levels in these children (Tognoni and Bonaccorsi, 1982). More recently, Mocarelli et al. (1991) analyzed 30 serum samples for TCDD measurements that were taken from individuals within 6 months after the Seveso explosion and linked them with the patients' medical history from 1976 through 1985. These samples represented 10 residents who had developed severe chloracne, 10 residents who lived in the most heavily contaminated area but did not develop chloracne, and 10 residents from a noncontaminated site as the controls. Lipid-adjusted TCDD concentrations ranged from non-detectable in controls to 56,000 parts per trillion in a subject who lived in the most severely contaminated site. No differences were detected in complete blood cell counts, differentials, nor in T or B lymphocyte blastogenic responses. The authors concluded that these data strongly support the hypothesis that there are minimal adverse health effects, with the exception of chloracne, in humans exposed to the levels of TCDD observed

following the Seveso accident. However, it should be noted that these parameters are also not affected in laboratory animals even when adaptive immune responses are profoundly suppressed.

Unlike the Seveso accident, residents in the Times Beach/Quail Run area of Missouri were exposed to TCDD-contaminated dust and soil as a result of spraying TCDD-tainted oil on roadways as a means of dust control. Although the contamination occurred in 1971, epidemiologic studies were not initiated until 1983 (Burrell et al., 1992) and results were often conflicting. For example, Hoffman et al. (1986) reported a decrease in cell-mediated immunity and T cell numbers (T3, CD4, and T11), while two other studies failed to detect any alterations in T cell subsets or immune function (Stehr et al., 1986; Evens, et al., 1988). In agreement with these later studies, Webb et al. (1989) reported on immunological parameters from 41 individuals with known adipose levels of TCDD. Individuals were separated into groups based on adipose tissue levels: 1) less than 20 ppt (n=16), 2) between 20 and 60 ppt (n=13), and 3) greater than 60 ppt (n=12). After controlling for sex and age, increased TCDD levels were correlated with increased percentage of T3, T4, T8 and T11 positive T cells. In addition, the absolute numbers of T8 positive cells and total IgG were increased. However, the proliferative responses to several mitogens were unaltered. Similarly, no increase in anergy to seven different antigens (diphtheria, tetanus, streptococcus, trichophyton, proteus, candida, and tuberculin) or association with adverse health effects was observed.

Accidental exposure to PBBs, PCBs and PCDFs have also provided opportunities to study potential human health problems associated with exposure to HAH. For example, a commercial preparation of PBB was accidentally used in preparing a feed supplement for cows and poultry (Fries et al., 1986). The contaminated feed was widely distributed throughout Michigan in 1973 and 1974. Ingestion of PBB-contaminated beef, dairy products, poultry and eggs resulted in human exposure. An original report by Bekesi et al. (1983) showed that 18 of 45 Michigan dairy farmers sampled had a significant decrease in

the total number of T cells as well as suppressed T and B cell mitogenic response when compared to dairy farmers from Wisconsin. However, a subsequent study revealed a persistent increase in natural killer (NK) cells and elevated levels of IgG, IgA, and IgM in PBB-exposed farmers (Bekesi et al., 1985). Because NK cells represent one of the first lines of defense against tumor formation, it is possible that PBB could potentially increase tumor resistance by increasing the number of NK cells. Interestingly, an epidemiologic study by Brown and Jones (1981) reported a lower than expected mortality from all cancers in 2500 industrial workers exposed to PBB.

Finally, in two separate incidents, one in Japan in 1968 and the other in Taiwan in 1971, thousands of people were exposed to PCBs and PCDFs through consumption of contaminated cooking oil. Significant decreases in the total T cells, active T cells and T helper cells were measured one year after exposure. However, when remeasured three years later, a partial recovery of the T helper cells and an increase in $CD8^+$ cells was detected (Lü and Wu, 1985). Furthermore, a decrease in delayed-type skin hypersensitivity to tuberculin and streptococcal enzymes was detected. Interestingly, in vitro mitogenesis assays using PWM, Con A, or PHA showed enhanced stimulation indices.

In summary, it is apparent that the data on the potential immunotoxic effects of HAHs in humans are inconsistent and often conflicting. Many of the endpoints used to examine potential HAH-induced effects (e.g., serum antibody titers, phenotypic subset analysis, and mitogenic responsiveness) show a wide range of normal values which decreases the sensitivity and makes it extremely difficult to detect small changes. Another potential problem may be attributed to the fact that epidemiologic studies rely on the human population which is outbred and genetically dissimilar. This variability could play a significant role when one considers the importance of the AhR in HAH-induced toxicity. Furthermore, very few studies have correlated known body burdens with the assays used to represent immune function, since a majority of the studies are conducted on subjects in

which the exact tissue level of HAH is unknown. This could influence the reported outcome by inclusion of individuals with little or no actual exposure. Finally, problems such as the occurrence of long-term effects (e.g., cancer and other rare disease endpoints) with long latency periods may not be detectable at this time and will require follow-up studies.

In vitro immunotoxic effects of HAH

One of the difficulties encountered by scientists studying the mechanism of TCDD-induced immune suppression is the apparent lack of consistent in vitro effects. Some laboratories have been able to demonstrate TCDD effects on B lymphocytes following in vitro exposure (Tucker et al., 1986; Dooley and Holsapple, 1988; Luster et al., 1988). For example, Tucker et al. (1986) showed that TCDD directly suppressed the anti-SRBC response in vitro. Also, Luster et al (1988) observed that while B cell viability and antigen-induced proliferation were unaltered, B cell differentiation and antibody production were suppressed by in vitro exposure to TCDD. However, in contrast to in vivo antibody responses, TCDD-induced suppression of in vitro antibody responses appears to involve both AhR-dependent (Tucker et al., 1986) and AhR-independent (Holsapple et al., 1986; Davis and Safe, 1991) mechanisms.

In contrast, T lymphocyte function appears to be unaltered by in vitro TCDD exposure (Kerkvliet and Baecher-Steppan, 1988b; Lundberg et al., 1990; DeKrey and Kerkvliet, 1995). Recently, Lawrence et al (1996) demonstrated that T cells (T cell clones and isolated splenic T cells) contain both the AhR and ARNT which translocate to the nucleus upon in vitro TCDD exposure. However, the activated complex was unable to bind a consensus DRE sequence. The authors interpreted these results to suggest that T cells may not be the direct target of TCDD and may explain the lack of in vitro effects of TCDD.

The question remains as to why it has been so difficult to demonstrate consistent in vitro immunotoxic effects of TCDD and other related HAHs. A few explanations are possible: 1) In vitro culture systems are set up at optimal growth and survival conditions for the cells of interest. Because of this, the conditions within the in vitro system may simply overcome any potential HAH-induced immunotoxic effect. This idea is supported in a study by Morris et al. (1991) in which the effect of TCDD on the in vitro anti-SRBC response was absolutely dependent on the serum used in the culture system. They showed that only 3 of 23 lots of serum were able to support a TCDD-induced dose-dependent suppression of the anti-SRBC response. In fact, the anti-SRBC response was shown to be enhanced by over 15 fold by TCDD if performed in serum free culture conditions. 2) Because an effective immune response in vivo depends on the interaction of numerous cells types (i.e., antigen presenting cells, T cells, B cells, etc.) it is possible that in vitro cultures do not contain the target cells. For example, dendritic cells are the most potent antigen presenting cells in the immune system and are thought to be responsible for initiating an immune response in vivo (Moser et al., 1995). However, normal mechanical disruption of lymphoid organs such as the spleen and lymph nodes for in vitro culture does not dislodge the dendritic cells from the organ capsule. The end result is that most of the dendritic cells, which may be the target cells, are be missing from the culture system. 3) The immunotoxic effects of TCDD and related HAHs may be indirect. In vitro cultures try to mimic entire organs or systems within a well-defined and controlled environment. However, by doing so, the cells of interest are displaced from any potential outside influences of other organ systems. Therefore, many investigators have had to rely on in vivo animal models to study the immunotoxic mechanism of TCDD and related HAHs.

HYPOTHESIS

The hypothesis tested in this dissertation is that the immunotoxicity of TCDD is induced by alterations in T cell activation involving either the antigen presenting cell or the T cell. This hypothesis is based on the fact that T cell activation is controlled by the T cell receptor (TCR) in combination with additional signals from cytokines or accessory molecules delivered by the APC. T cell activation is a highly complex process that starts with the recognition of antigen by the TCR/CD3 complex. Antigen recognition triggers an array of intracellular signal transduction molecules that are pivotal for cellular activation (Cantrell, 1996). The end result of complete T cell activation is proliferation, differentiation and acquisition of effector function (e.g. cytokine production or lytic activity). T cell effector functions following different methods of activation were used to investigate the potential mechanisms of TCDD-induced immune suppression.

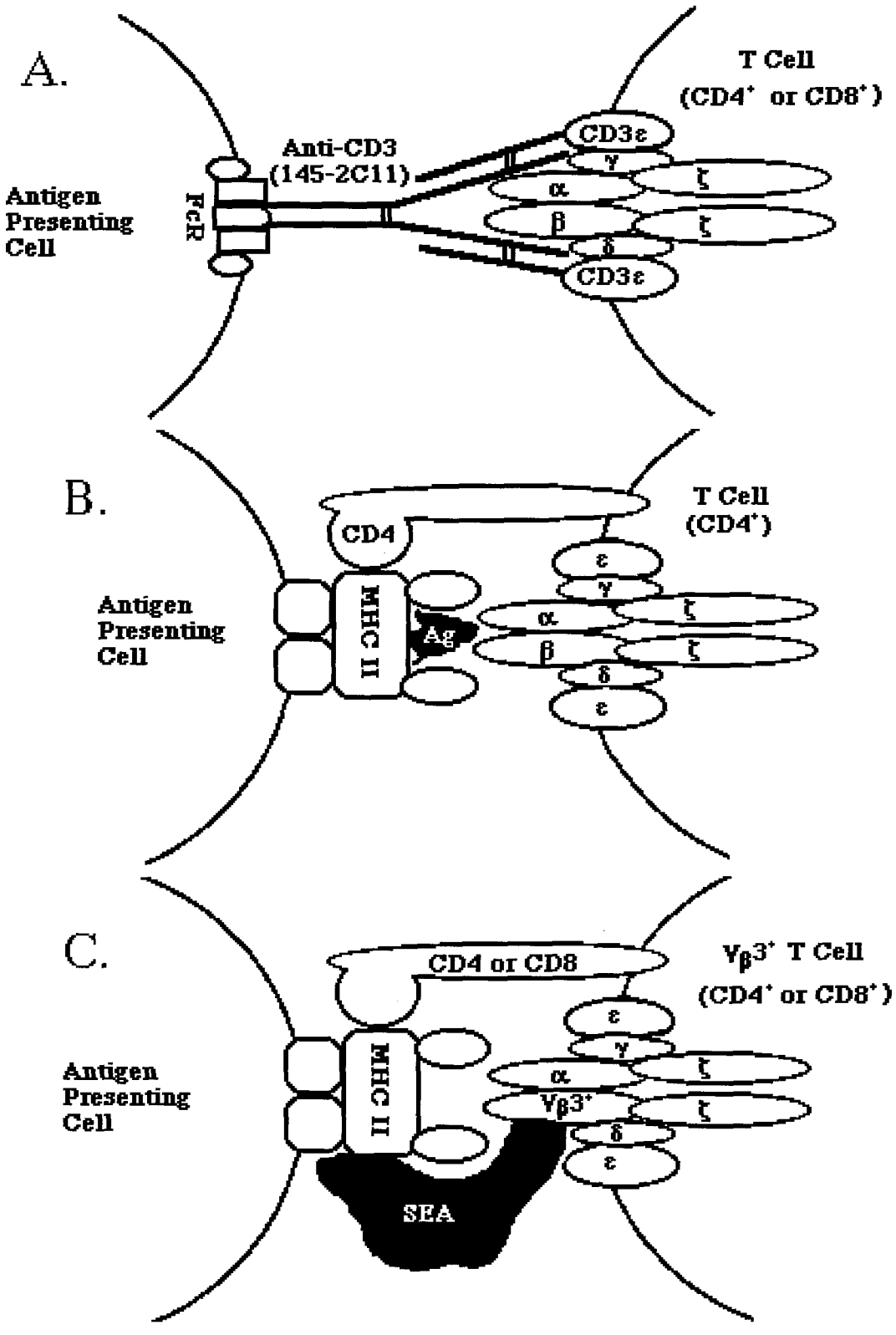
Three in vivo models were employed to investigate the potential effects of TCDD exposure on T cell activation. These models were chosen because of the differences in the initial interaction between the antigen and the T cell receptor complex. In addition, all three models differ in their requirement for costimulatory signals that are provided by antigen presenting cells. In the first approach, anti-CD3 monoclonal antibodies were injected into mice to directly crosslink the CD3 complex of the TCR and activate all T lymphocytes. Anti-CD3-induced T cell activation results in massive cytokine release that is readily detected in vivo. The function of the APC in the anti-CD3 model is to anchor the monoclonal antibody via the Fc receptor (figure I-4A). In contrast, injection of allogeneic P815 tumor cells closely resembles the initiation of an immune response following exposure to a conventional antigen since T cell activation and acquisition of effector function is completely dependent on APCs. P815 antigen must be processed into antigenic peptide, then presented to $CD4^+$ T helper cells in the context of MHC class II proteins (figure I-4B). The activated $CD4^+$ T cells provide help to the $CD8^+$ precursor cytotoxic T

cells (CTL_P) which then proliferate and differentiate into allospecific effector cytotoxic T cells (CTL_E). The population of CTL_E is readily detectable and allows easy quantitation of $CD8^+$ T cell function. Finally, superantigens activate T cells via binding to specific Vβ chains of the T cell receptor. All superantigens discovered thus far require presentation by MHC II proteins; however, they are presented in an unprocessed form. Thus, presentation of superantigen is dependent on APCs, but in such a way that the APCs main function is to provide an anchoring site for the superantigen (figure I-4C). For example, injection of the superantigen staphylococcal enterotoxin A (SEA) specifically activates $V\beta3^+$ T cells. SEA-induced activation causes the clonal expansion and subsequent deletion of $V\beta3^+$ T lymphocytes. Since 5% of T lymphocytes express Vβ3 in naive B10BR/SgSnJ mice, this model allows one to track the response of antigen-specific T cells in vivo. Together, these models allowed us to compare and contrast the effects of TCDD on in vivo T cell function following different methods of activation and to gain insight into the mechanism of TCDD-induced immune suppression.

Figure I-4. Initial interactions between antigen presenting cells and T cells with three different in vivo models of T cell activation.

Model of the interactions between APCs and T cells with three different antigens. A) Anti-CD3 monoclonal antibodies are bound by the APC via the Fc receptor. This anchors the antibody so that the variable end can crosslink the CD3 portion of the TCR and activate the T cell. B) A processed antigenic peptide from P815 tumor cells is presented to the CD4+ TCR in the context of MHC class II proteins. Binding of the CD4 molecule with the outside region of the MHC class II protein helps stabilize and strengthen the interaction. C) SEA requires presentation by MHC class II proteins. SEA binds to and activates $CD4^+$ and $CD8^+$ T lymphocytes that express the Vβ3 chain of the T cell receptor.

Figure I-4.


A.
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CHAPTER II

EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN ON ANTI CD3-INDUCED CHANGES IN T CELL SUBSETS AND CYTOKINE PRODUCTION

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ABSTRACT

The influence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure on the cytokine-dependent toxicity syndrome induced by the injection of 145-2C11 (anti-CD3), a hamster monoclonal antibody to the CD3 epsilon portion of the murine T cell receptor, was studied. This syndrome has been attributed to the transient release of several cytokines including TNF-α, IFN-γ, IL-2, IL-3, IL-6, and GM-CSF. Exposure of C57Bl/6 mice to TCDD (15 μg/kg) 2 days prior to anti-CD3 injection exacerbated anti-CD3-induced toxicity as evidenced by significantly enhanced and prolonged body weight loss and lymphoid tissue atrophy. Unexpectedly, TCDD exposure did not alter plasma levels of TNF or IL-2 at any time after anti-CD3 injection. However, plasma IFN-γ was significantly reduced at 24 hours and plasma IL-6 levels were elevated 48 hours after anti-CD3 injection in TCDD-treated mice. In addition, TCDD exposure resulted in elevated levels of plasma GM-CSF at 24 and 48 hours. Since the body weight of TCDD-treated mice diverged from vehicle-treated mice at 48 hr, it suggests that the increased IL-6 and GM-CSF may have contributed to the prolonged loss of body weight. The ability of spleen cells from vehicle- and TCDD-treated mice to produce cytokines was evaluated *in vitro* at various times after anti-CD3 injection. TCDD treatment resulted in reduced IL-2 and GM-CSF production at 90 minutes but increased GM-CSF production at 48 hours post anti-CD3 injection. In contrast, TCDD exposure did not influence cytokine production by spleen cells from mice injected with a control IgG and activated *in vitro* with anti-CD3. Flow cytometric analysis showed that the percentage of $CD4^+$ cells in the draining lymph nodes from TCDD-treated mice was reduced 48-144 hours post anti-CD3 injection. In contrast, the percentage of $CD8^+$ cells was not affected by TCDD exposure. A high fraction of lymph node cells (LNC) from TCDD-treated animals showed decreased forward angle light scatter and increased 90° light scatter following anti-CD3 injection, which is a pattern characteristic of cells undergoing apoptosis. In contrast, few LNC from vehicle-treated animals showed this light scatter

profile. These data suggest that TCDD may be targeting T helper cells during activation resulting in activation-driven cell death (apoptosis) rather than differentiation.

INTRODUCTION

Halogenated aromatic hydrocarbons (HAHs) are a class of structurally related compounds that are widespread environmental contaminants. There has been increased public concern over HAHs due to their toxicity and accumulation in the environment. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent congener of the HAHs and is often used to study the mechanisms of HAH induced toxicity. Many of the toxic effects caused by TCDD and related HAHs have been shown to be mediated through specific binding to the cytosolic aromatic hydrocarbon receptor (AhR) (Poland and Knutson, 1982; Nebert, 1989; Landers and Bunce, 1991).

One of the characteristic toxic responses induced by high doses of TCDD in many animal species is a wasting disease characterized by body weight loss and thymic involution (Poland and Knutson, 1982). At lower doses of TCDD, immune suppression is evidenced by decreased resistance to infections and suppressed immune function (Vos and Luster, 1989). TCDD has been shown to alter multiple immunological parameters including suppression of the T cell-dependent antibody response to sheep red blood cells (Davis and Safe, 1988; Kerkvliet and Brauner, 1990) and inhibition of cytotoxic T-lymphocyte activity (Clark *et al.*, 1981; Kerkvliet *et al.*, 1990). In addition, TCDD inhibits T-helper cell function (Tomar and Kerkvliet, 1991) and B cell differentiation (Tucker *et al.*, 1986). Although TCDD-induced immune suppression has been widely studied, the underlying biochemical mechanisms by which it occurs remain unidentified (reviewed by Kerkvliet and Burleson, 1994).

Activation of T cells by anti-CD3, a monoclonal antibody (mAb) specific for the CD3 epsilon portion of the murine T cell receptor, mimics antigen-induced T cell activation (Leo *et al.*, 1987) and provides a unique *in vivo* tool for studying the events leading to T

cell activation and proliferation (Pereira *et al.*, 1988). However, injection of high doses of anti-CD3 mAb results in an acute toxicity syndrome characterized in mice by piloerection, hypothermia, hypoglycemia, diarrhea, necrosis and edema of lymphoid organs, and body weight loss (Ferran *et al.*, 1990b). These effects have been associated with the transient release of several cytokines, including interleukin 2 (IL-2), IL-3, IL-6, tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) (Ferran *et al.*, 1990a; Matthys *et al.*, 1993). TNF-α appears to be one of the major cytokines involved in anti-CD3 induced toxicity since administration of mAb to TNF-α reverses many of the acute toxicity symptoms (Alegre *et al.*, 1990; Ferran *et al.*, 1991b).

Our laboratory has used the *in vivo* anti-CD3 model to investigate the effects of TCDD on early events (0-24 hr) in T cell activation (Neumann *et al.*, 1993). In these studies, TCDD exposure unexpectedly increased T cell activation as measured by the percentage of $CD4^+$ and $CD8^+$ T cells in the S/G_2M phase of the cell cycle. At later timepoints (48 and 72 hr), treatment with TCDD appeared to increase the acute toxicity of anti-CD3, as indicated by increased body weight loss. Like anti-CD3-induced toxicity, TNF has been suggested to play an important role in TCDD toxicity (Clark and Taylor, 1994), and anti-TNF antibody treatment reduces the acute toxicity of TCDD (Taylor *et al.*, 1992). Interestingly, like TCDD, TNF has been shown to enhance T cell activation by anti-CD3 (Yokota *et al.*, 1988). Therefore, we hypothesized that TCDD exposure alters TNF production in response to anti-CD3 treatment, leading to increased toxicity and alterations in T cell activation. In the studies described here, the effects of TCDD exposure on anti-CD3-induced toxicity, cytokine production, and lymphocyte phenotypes were examined.

EXPERIMENTAL PROCEDURES

Animals

Male C57Bl/6 mice (6-8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in front of a laminar flow unit. Animals were given standard rodent chow and tap water *ad libitum*. Sera from monthly bleeding of sentinel mice were analyzed by Charles River Biotechnical Services, Inc. (Wilmington, MA) for the presence of antibodies to murine hepatitis virus. The colony remained virus-free throughout the studies.

Antibodies

The hamster anti-murine CD3 mAb, 145-2C11, (a generous gift from Dr. J. A. Bluestone, Univ. of Chicago, Chicago, IL) was obtained from culture supernatants, purified by gel filtration and diluted in phosphate buffered saline (PBS) to 1 mg/ml. The antibody preparation was tested for the presence of lipopolysaccharide by Microbiology Reference Laboratory (Cincinnati, OH) using the limulus amebocyte lysate assay and found to contain 1.29 EU/ml (0.129 ng/ml). Phycoerythrin (PE)-labeled antibodies to CD4 or CD8 cell surface molecules were purchased from Becton-Dickinson (Mountainview, CA) and Pharmingen (San Diego, CA) respectively. PE-rat IgG_{2a} (Pharmingen) was used as the isotypic control antibody for both CD4 and CD8 staining.

Animal treatment

TCDD, purchased as a certified reference standard (> 98% purity; Cambridge Isotope Laboratories, Inc., Woburn, MA), was dissolved in anisole and diluted in peanut oil. Mice were treated with vehicle or TCDD at 15 μg/kg body weight by gavage 2 days prior to anti-CD3 injection. Anti-CD3 mAb or a non-activating control hamster IgG (Ham IgG; Organon Teknika Corporation, Durham, NC) was injected at 25 μg/rear foot pad.

Body weights were recorded daily throughout the studies. Animals were killed at various times after anti-CD3 injection by CO_2 asphyxiation. All animals injected with Ham IgG were killed 48 hours later (96 hrs after TCDD treatment). Blood was collected via heart puncture using syringes prefilled with 12 units sodium heparin. Plasma was separated by centrifugation and stored at -70°C. In some experiments spleens and lymph nodes were removed and processed for cytokine production or flow cytometric analysis.

Spleen cell cultures

Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Spleen cells were washed once and resuspended to $2x10^7$ cells/ml in Ultraculture® media (BioWhittaker) supplemented with 2 mM HEPES, 2 mM L-glutamine, 1.5 mM sodium pyruvate, 1 mM penicillin-streptomycin and 50 μM 2-mercaptoethanol (Sigma, St Louis, MO). Spleen cells from anti-CD3 injected mice were cultured in duplicate wells without additional stimulation in 24-well plates whereas cultures from Ham IgG injected mice contained 0.05 μg/ml anti-CD3 for *in vitro* stimulation. Plates were incubated at 37°C in an atmosphere of 5% CO_2 for 6 hours. Supernatants were collected and stored at -70°C.

Cytokine analysis

IL-2, IL-6, IFN-γ and GM-CSF levels in plasma and culture supernatants were measured using enzyme-linked immunosorbant assays (ELISA). Antibody pairs, both capture and detection, were purchased from Pharmingen. Recombinant IL-6 (Genzyme, Cambridge, MA), IL-2, IFN-γ, and GM-CSF (Pharmingen) were used as standards. Assays were performed as recommended by the manufacturer (Pharmingen) and fluorescence at 405 nm was measured using a microplate reader (Biotek Corp., Winooski, VT).

TNF activity was measured using a murine L929 fibroblast bioassay as described by Flick and Gifford (1984). Recombinant TNF-α standard was purchased from Genzyme. Plates were read at 595 nm.

Flow cytometric analysis

Popliteal, inguinal, axillary and superficial dorsal axillary nodes were removed and pooled at various times after anti-CD3 injection. Single cell suspensions of lymph node cells (LNC) were prepared as described for the spleen. LNC ($1x10^6$) were incubated on ice in round bottom 96-well plates (Flow Laboratories, Inc., McLean, VA) in PBS containing 1.0% bovine serum albumin and 0.1% sodium azide. Non-specific binding of antibodies via the Fc receptor was blocked with excess rat IgG prior to staining with PE-conjugated anti-CD4 or anti-CD8. Immediately after staining, cells were passed through a 40 μm nylon mesh to remove cell clumps and analyzed on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). Fluorescent, forward angle light scatter (FALS), and 90° light scatter (LI90) measurements were collected on 20,000 cells by listmode acquisition and analyzed using Cyclops software (Cytomation, Inc., Fort Collins, CO). Dead cells were excluded from analysis on the basis of FALS and propidium iodide (PI) staining. The proportion of LNC expressing a particular phenotype was determined by integration of the log amplified fluorescent signal.

Statistics

ANOVA modeling was performed to determine significant changes in body weight and cytokine levels over time. The Student's t test was used to compare treatment effects. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of TCDD on anti-CD3 induced body weight loss.

Body weight loss was monitored as a gross measure of overt toxicity. As shown in figure II-1A, mice injected with Ham IgG did not lose body weight even if they were treated with TCDD. In contrast, within 24 hr following injection of anti-CD3, body weights were significantly decreased, with a maximum weight loss of 15% on day 3-4 (figure II-1B). In vehicle-treated mice, body weight was regained by day 6. Co-exposure to TCDD and anti-CD3 resulted in significantly greater body weight loss, and recovery was delayed until day 9. In addition, one TCDD-treated animal died on day 8, whereas no mortality occurred in the vehicle-treated group. In a separate study, co-exposure of female mice to TCDD and anti-CD3 also resulted in enhanced and prolonged weight loss when compared to treatment with anti-CD3 alone (data not shown). However, male mice appeared to be more sensitive to the effect of TCDD on anti-CD3-induced weight loss and were thus used in all subsequent studies.

Effect of TCDD on spleen weight and cellularity.

As shown in figure II-2, spleen weight, normalized to body weight, and spleen cellularity in Ham IgG-injected mice were not altered by TCDD treatment (presented as 0 hr post anti-CD3). Following anti-CD3 injection, spleen weight increased over time in vehicle-treated mice. In TCDD-treated animals, spleen weight increased up to 48 hr and then decreased at 144 hr. Spleen weight in TCDD-treated mice was significantly lower than vehicle-treated mice at 24, 48 and 144 hours.

In contrast to spleen weight, spleen cell number decreased in both vehicle-and TCDD-treated animals at 24 hours following anti-CD3 injection, suggesting that the early increase in spleen weight may have been due to edema. When vehicle- and TCDD-treated

Figure II-1. Effect of TCDD on body weight of mice injected with Ham IgG (A) or anti-CD3 (B).

Mice were treated with vehicle (open circle) or 15 μg/kg TCDD (closed circle) 2 days prior to antibody injection and body weight was recorded daily for 14 days. Each point represents mean ± SEM of 6 mice per treatment group per timepoint. * indicates $p <$ 0.05 between treatment groups.

Figure II-2. Effect of TCDD on spleen weight to body weight ratio (A) and number of spleen cells (B).

Mice were treated with vehicle (open bar) or 15 μg/kg TCDD (solid bar) 2 days prior to Ham IgG (represented as time 0) or anti-CD3 administration and killed at various times following antibody injection. Mice injected with Ham IgG were killed at 48 hours. Each bar represents the mean ± SEM of 4 mice per treatment group per timepoint. * indicates $p < 0.05$ between treatment groups.

Figure II-1.

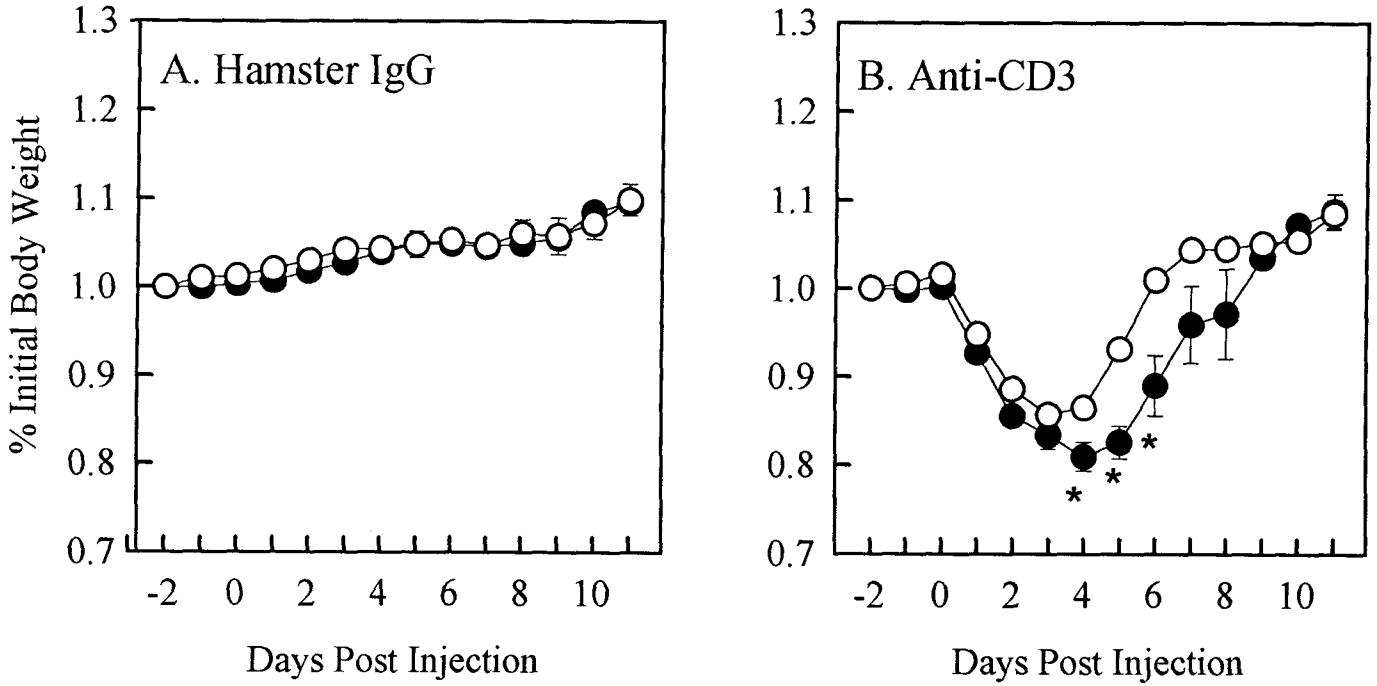
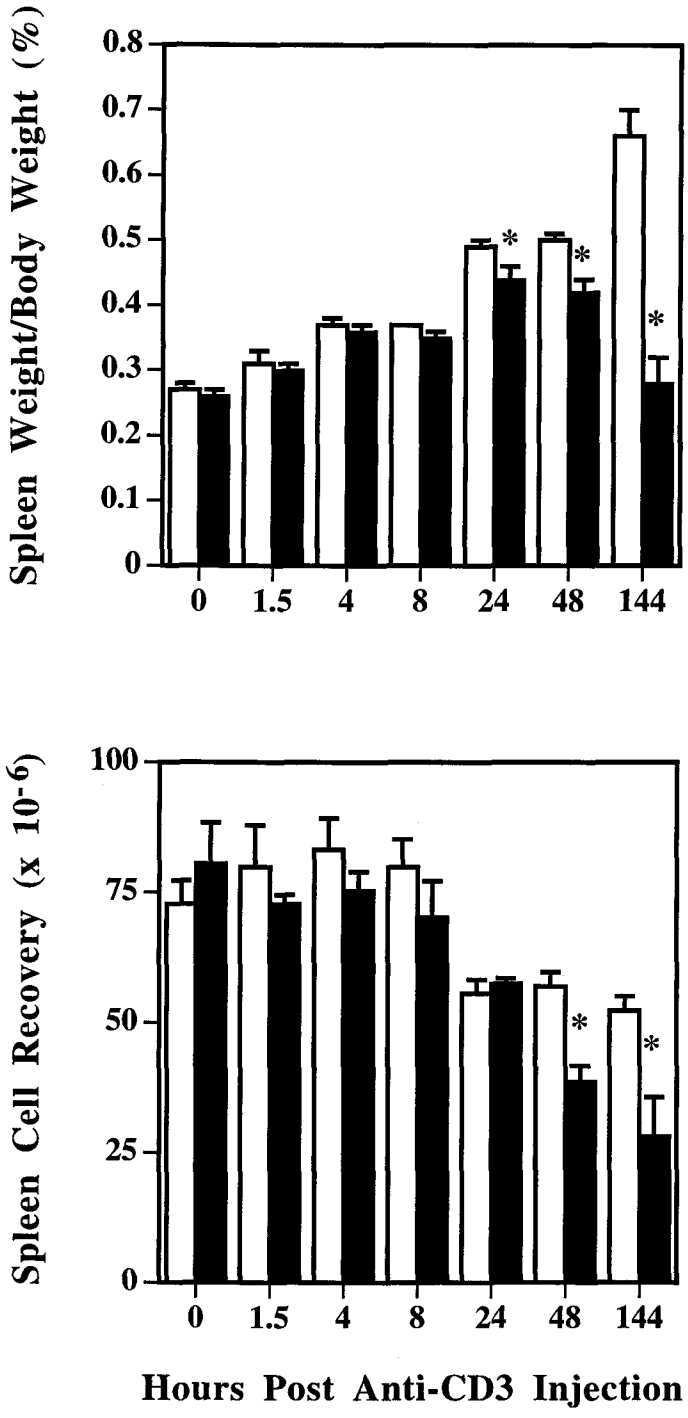

A. Hamster IgG
B. Anti-CD3
% Initial Body Weight
1.3
1.2
1.1
1.0
0.9
0.8
0.7
-2
0
2
4
6
8
10
Days Post Injection
*

Figure II-2.


Spleen Weight/Body Weight (%)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1.5 4 8 24 48 144
Spleen Cell Recovery (x 10-6)
100
75
50
25
0
0 1.5 4 8 24 48 144
Hours Post Anti-CD3 Injection

groups were compared, spleen cell number was significantly decreased in TCDD-treated mice at 48 and 144 hours following anti-CD3.

Effect of TCDD on plasma cytokine levels.

To investigate the effect of TCDD on anti-CD3-induced cytokine production, plasma cytokine levels were measured at various times following anti-CD3 injection. As shown in figure III-3, vehicle-treated animals responded to anti-CD3 injection with a rapid increase in plasma IL-2, TNF, IFN-γ and IL-6 levels, in agreement with previous reports (Ferran *et al.*, 1990b; Matthys *et al.*, 1993). TCDD treatment did not significantly alter the magnitude or kinetics of plasma cytokine levels with the exception of two points. Plasma IFN-γ levels were significantly suppressed by 2-fold at 24 hours and IL-6 levels were elevated by about 3-fold ($p = 0.07$) at 48 hours in TCDD-treated animals. In an independent study, plasma levels of IFN-γ, IL-6 and GM-CSF were measured at 24, 48, and 72 hours after anti-CD3 injection. TCDD-treated animals again showed significant suppression of IFN-γ at 24 hours as well as significant elevation of IL-6 at 48 hours (data not shown). In the same experiment, TCDD-exposed mice had elevated plasma levels of GM-CSF (61-63 pg/ml) at 24 and 48 hours after anti-CD3 injection whereas levels in vehicle-treated mice were below the limit of detection (31 pg/ml). Plasma cytokine levels were below detection limit in both vehicle- and TCDD-treated Ham IgG-injected mice (represented as 0 hr on figure II-3).

Effect of TCDD on cytokine production by cultured spleen cells.

As shown in Table II-1, when cells from Ham IgG-injected mice were stimulated with anti-CD3 *in vitro*, cytokine production was not altered by prior *in vivo* exposure to TCDD. Likewise, the production of TNF, IFN-γ, and IL-6 by cultured spleen cells from anti-CD3 injected mice was not altered by TCDD exposure (figure II-4). However, when

Figure II-3. Effect of TCDD on anti-CD3-induced cytokine levels in plasma.

Mice were treated with vehicle (open bars) or 15 μg/kg TCDD (solid bars) 2 days prior to Ham IgG (represented as time 0) or anti-CD3 injection. Mice were killed at various times after antibody injection and plasma cytokine levels were measured as described in Materials and Methods. Mice injected with Ham IgG were killed 48 hours post administration. The lower limits of detection for each cytokine was as follows: IL-2 (31 pg/ml), IL-6 (31 pg/ml), IFN-γ (100 pg/ml) and TNF-α (0.5 pg/ml). Values below detection limit were derived from the regression curve and used for calculating group means. Each bar represents the mean ± SEM of 4 mice per treatment group per timepoint. * indicates $p < 0.05$ between treatment groups. † indicates $p = 0.07$

Figure II-3.

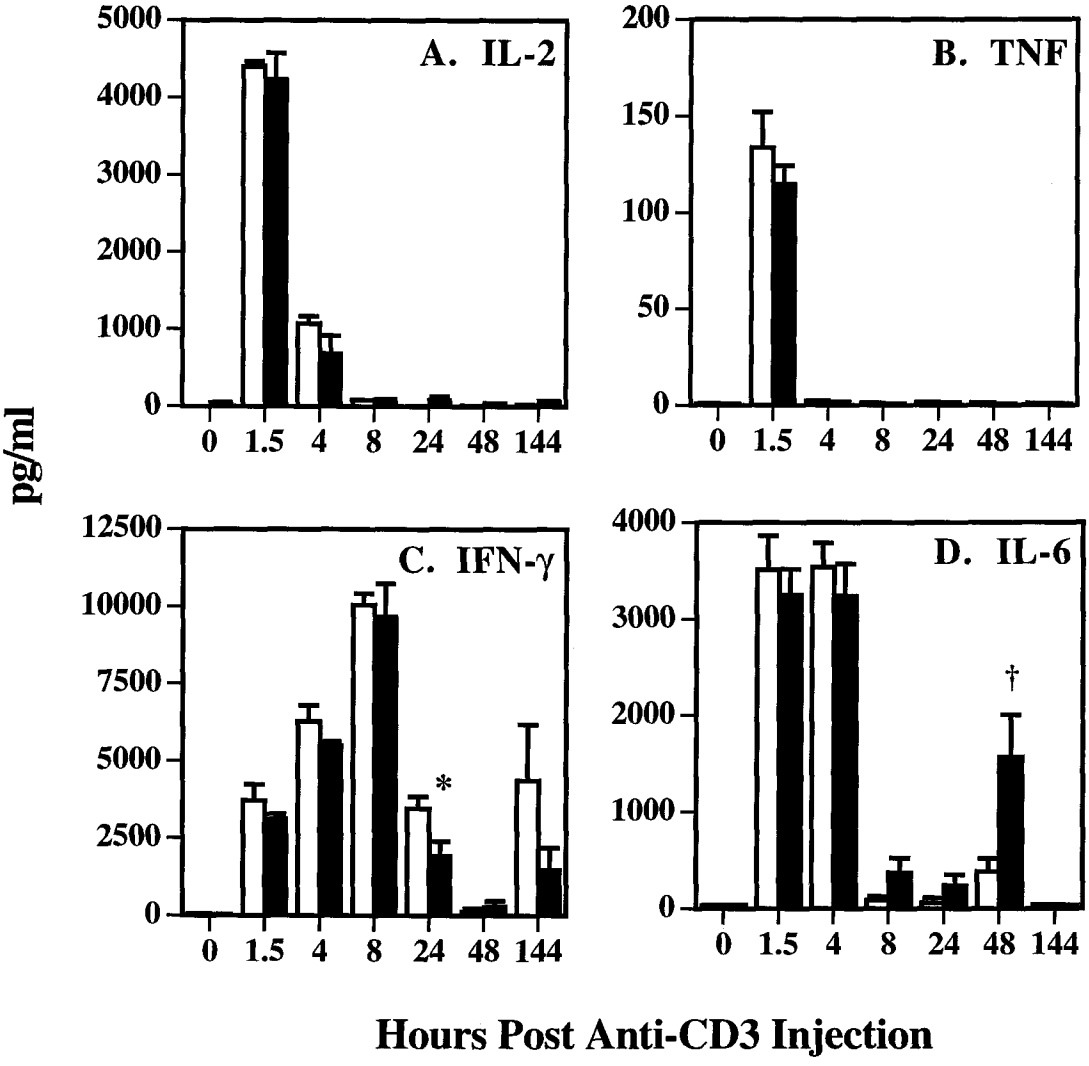

A. IL-2
B. TNF
C. IFN-γ
D. IL-6
pg/ml
5000
4000
3000
2000
1000
0
200
150
100
50
12500
10000
7500
5000
2500
0 1.5 4 8 24 48 144
*
†
Hours Post Anti-CD3 Injection

Table I. Cytokine Production By Spleen Cells Stimulated With Anti-CD3 *In Vitro* Is Unaffected By *In Vivo* TCDD Exposure.*,†

	Cytokine Levels (pg/ml)‡	
Cytokine	Vehicle	TCDD
IFN-γ	4218 ± 894	5434 ± 483
IL-2	808 ± 129	806 ± 54
IL-6	210 ± 32	236 ± 28
TNF-α	28 ± 2	29 ± 1
GM-CSF	85 ± 9	62 ± 7

* Vehicle or TCDD (15 μg/kg) was given by gavage 2 days prior to the injection of hamster IgG. Mice were killed 48 hr later. Spleen cells were incubated for 6 hr with 0.05 μg/ml anti-CD3.

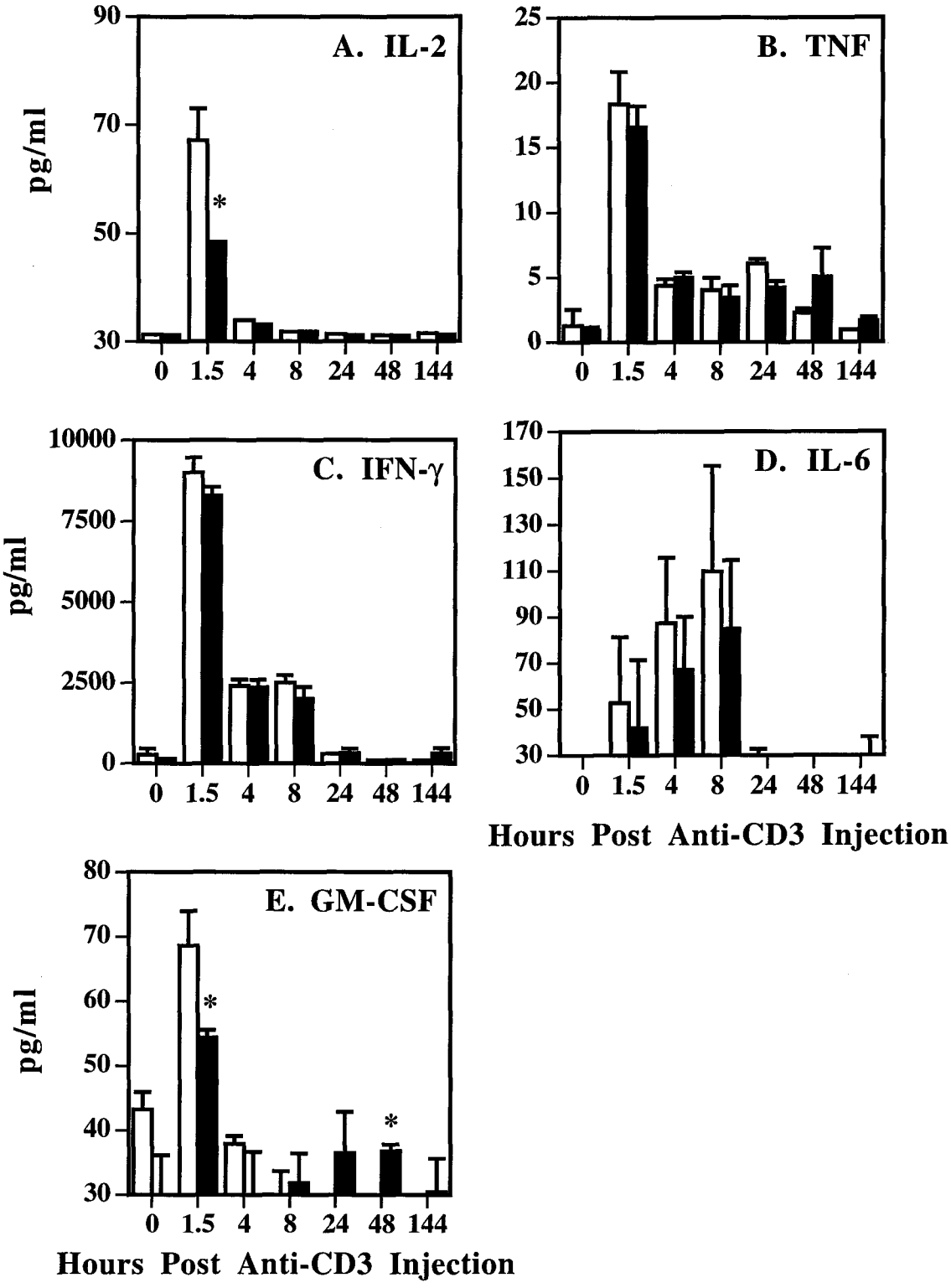
† Data represent mean ± SEM of 4 mice/treatment.

‡ No statistically significant changes were seen ($p < 0.05$).

Figure II-4. Effect of TCDD on cytokine profiles in spleen cell cultures from animals injected with anti-CD3.

Mice were treated with vehicle (open bar) or 15 μg/kg TCDD (solid bar) 2 days prior to Ham IgG (represented as time 0) or anti-CD3 injection. Spleens were removed at various times following anti-CD3 injection. Spleens were removed from Ham IgG injected mice 48 hours post administration. Spleen cells (1×10^6) were cultured for 6 hr and cytokine levels in culture supernatants were measured as described under Materials and Methods. The lower limits of detection for each cytokine was as follows: IL-2 (31 pg/ml), IL-6 (31 pg/ml), GM-CSF (31 pg/ml), IFN-γ (100 pg/ml) and TNF-α (0.5 pg/ml). Values below detection limit were derived from the regression curve and used for calculating group means. Each bar represents mean ± SEM of 4 mice per treatment group per timepoint. * indicates $p < 0.05$ between treatment groups.

Figure II-4.


A. IL-2
B. TNF
C. IFN-γ
D. IL-6
E. GM-CSF
pg/ml
0 1.5 4 8 24 48 144
Hours Post Anti-CD3 Injection

cells were taken from mice 90 minutes after anti-CD3 injection, the production of IL-2 and GM-CSF was significantly lower in cells from TCDD-treated animals. In contrast, GM-CSF levels were significantly higher in spleen cell cultures from TCDD-treated mice when cells were removed 48 hours after anti-CD3 injection.

Effect of TCDD on light scatter and phenotypes of LNC.

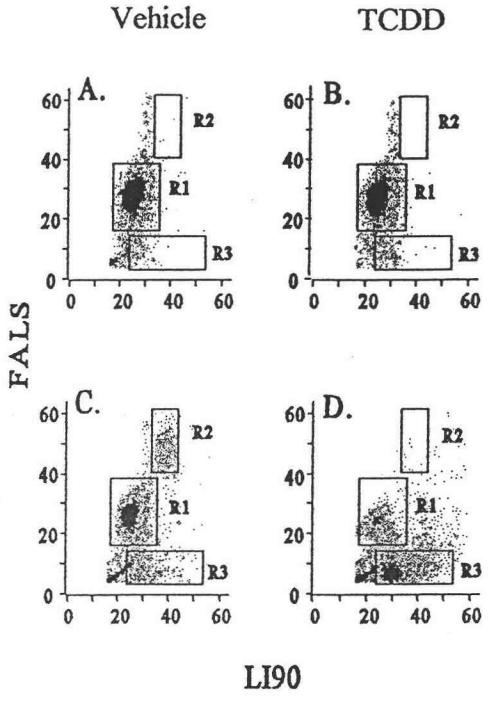
LNC were removed at various times after anti-CD3 injection and processed for flow cytometric analysis. Total LNC recovery from Ham IgG-injected mice was not affected by TCDD exposure (data not shown). In contrast, LNC recovery was significantly reduced in TCDD-treated mice by 2-fold at 72 hours and 4-fold at 144 hours after anti-CD3 administration. As shown in figure II-5, viable (PI negative) LNC from Ham IgG-injected mice represented a relatively homogeneous population of cells based on their FALS and LI90 measurements, with 88% of the cells in region 1. TCDD treatment of Ham IgG injected mice did not significantly alter the light scatter profile (86% of the cells in region 1). In contrast, LNC from anti-CD3-injected mice showed an altered light scatter profile, characterized by an additional population of cells with increased FALS and LI90 (region 2) and an increased percentage of cells with decreased FALS and increased LI90 (region 3). These changes were most severe at 144 hrs. Exposure to TCDD exacerbated the anti-CD3-induced changes in light scatter characteristics and there was a clear shift in the majority of cells from TCDD-treated mice from region 1 to region 3. In addition, the population of cells in region 2 was not evident in TCDD-treated mice. These changes induced by TCDD progressed in severity from 48 through 144 hr (representative histograms of 144 hr data are shown in figure II-5).

The influence of TCDD treatment on the frequency of viable $CD4^+$ and $CD8^+$ T cells was examined at 24, 48, 72, and 144 hours after anti-CD3 injection by analyzing the cells in regions 1 and 2. As shown in Table II-2, when compared to Ham IgG-treated

Figure II-5. Effect of TCDD on lymph node cell (LNC) forward angle (FALS) and 90° (LI90) light scatter measurements of PI^- cells.

Mice were treated with vehicle or 15 μg/kg TCDD 2 days prior to injection with Ham IgG or anti-CD3. Light scatter measurements were analyzed 48 hours after Ham IgG injection and 144 hours after anti-CD3 injection by flow cytometric analysis. The histograms shown are representative of each treatment group. Values indicate the percentage of LNC in each region.

Figure II-5.



% of LNC in each region (R)

	R1	R2	R3
A.	88	0.9	4.4
B.	86	0.9	5.1
C.	52	8.9	15
D.	20	1.0	47

Table II. Phenotypic analysis of T cells in the draining lymph nodes of mice injected with 25 μg anti-CD3 antibody in the rear footpads: Influence of exposure to TCDD*.

Time (h) Post	%CD4		%CD8	
Anti-CD3	Vehicle	TCDD	Vehicle	TCDD
0 §	31.4 ± 1.8	33.1 ± 0.9	23.5 ± 1.8	24.2 ± 0.4
24	20.7 ± 0.8 †	21.8 ± 0.9 †	8.2 ± 0.3 †	9.8 ± 0.9 †
48	20.6 ± 1.4 †	13.7 ± 0.8 †‡	24.2 ± 1.2	28.2 ± 1.8
72	12.5 ± 0.9 †	9.3 ± 0.7 †‡	20.2 ± 1.6	22.2 ± 1.2
144	15.0 ± 0.9 †	5.6 ± 0.5 †‡	6.0 ± 1.5 †	3.1 ± 0.4 †

* Data represent mean ± SEM of 4 mice/treatment except for TCDD-treated mice at 144 hr where n = 2 pools of 2 mice due to poor cell recovery.
† Significantly different than Ham IgG control for respective treatment group, $p < 0.05$.
‡ Significantly different from anti-CD3 + vehicle at same timepoint, $p < 0.05$.
§ Mice were killed 48 hrs after Ham IgG injection (96 hrs after TCDD treatment).

mice, injection of anti-CD3 alone resulted in a significant decrease in the percentage of $CD4^+$ cells at all timepoints examined. In contrast, the percentage of $CD8^+$ cells was transiently decreased at 24 hr, followed by recovery at 48 hr, and then a second decrease at 144 hr. TCDD treatment did not alter the anti-CD3-induced changes in the frequency of $CD8^+$ cells but intensified the decrease in $CD4^+$ cells at all times after 24 hr. In contrast, TCDD treatment did not affect T cell subsets in Ham IgG-injected mice.

DISCUSSION

The immune system as a target for TCDD toxicity has been recognized for a long time (reviewed by Kerkvliet and Burleson, 1994). Because T cells play a critical role in determining the type and magnitude of an immune response, it is important to understand the mechanisms by which TCDD affects T cell function. Previous studies in our laboratory have used anti-CD3 antibody injected into the rear foot pads of mice as an *in vivo* model to study early T cell responses in the draining lymph nodes and spleen. We have shown that cyclosporin A (CsA) and TCDD, both potent immunosuppressive chemicals, produce divergent effects on T cells following anti-CD3 stimulation. While CsA suppressed T cell proliferative responses (Neumann *et al.*, 1992), TCDD unexpectedly enhanced T cell responsiveness (Neumann *et al.*, 1993). Cells from TCDD-treated mice showed an increased percentage of both $CD4^+$ and $CD8^+$ T cells that entered S phase, and, when cultured *in vitro* with or without exogenous IL-2, incorporated more ^{3}H-TdR. No effect of TCDD on IL-2 receptor expression was found. Interestingly, TCDD-treated mice had greater body weight loss induced by anti-CD3 treatment, suggesting that cytokine production may be altered in the TCDD-treated mice. In the present studies, the effects of TCDD exposure on anti-CD3-induced toxicity, cytokine production, and T cell phenotypes were examined in an effort to further characterize the interaction between TCDD and anti-CD3.

When body weight was monitored over a 14 day period following the injection of a total of 50 μg anti-CD3, TCDD-treated mice lost more body weight and took longer to regain the lost weight compared to vehicle-treated mice. Although TCDD is known to cause cachexia and body weight loss in all animal species when administered at or near the LD50 (Poland & Knutson, 1982), the increased weight loss was not directly related to overt TCDD toxicity since the same dose of TCDD did not alter body weight in mice treated with the nonspecific Ham IgG. Also, the 15 μg/kg dose of TCDD used in these experiments is about 10-fold below the LD50 (132 μg/kg) of TCDD for C57Bl/6 mice (Neal *et al.*, 1982).

TNF has been implicated as a major contributor to the physical symptoms in response to anti-CD3. Administration of antibodies to TNF or high doses of methylprednisolone attenuates many of the acute physical symptoms (Alegre *et al.*, 1991; Ferran *et al.*, 1994) and Finck *et al.* (1992) reported that TNF-deficient NZW mice are resistant to anti-CD3-induced toxicity. TNF has also been shown to play a key role in the acute toxicity following exposure to high doses of TCDD (Taylor *et al.*,1992). In addition, the hyperinflammatory response to sheep red blood cells (Moos *et al.*, 1994) and hypersensitivity to endotoxin (Clark *et al.*, 1991) in TCDD-treated mice appear to be mediated through altered TNF production and/or activity. We were therefore surprised to find that TCDD did not alter plasma TNF levels or the kinetics of TNF production in response to anti-CD3 treatment. However, a recent study by Ferran *et al.* (1994) reported that T cells activated with anti-CD3 express a membrane-associated form of TNF within 1 hour of stimulation. There have been reports on the relevance of membrane-associated TNF in tumor cell lysis and as an autocrine or paracrine T cell growth factor (Lopez-Cepero *et al.*, 1994; Kriegler *et al.*, 1988; Vassali, 1992). Since membrane-associated TNF would not be detected in plasma, the possibility remains that TCDD influences anti-CD3 toxicity by increasing membrane-associated TNF. Alternatively, TCDD exposure could increase the responsiveness of T cells to TNF. However, when LNC from vehicle- or TCDD-

treated mice were stimulated *in vitro* with a combination of anti-CD3 and rTNF-α, no differences in the proliferative response was observed (unpublished data).

Although TNF has been reported to be a major contributor to anti-CD3 toxicity, other cytokines have been implicated as well. Ferran *et al.* (1991a) examined strain differences in response to anti-CD3 toxicity and reported that the most severe physical reaction was seen in BALB/c mice which correlated with the highest levels of IFN-γ. CBA/J mice had the longest recovery period which correlated with the highest levels of IL-3/GM-CSF. In our studies TCDD exposure resulted in significantly decreased IFN-γ levels at 24 hours and significantly increased IL-6 levels at 48 hours. In addition, TCDD exposure resulted in elevated levels of plasma GM-CSF at 24 and 48 hours. Since the body weight of TCDD-treated mice diverged from vehicle-treated mice at 48 hr, it suggests that the increased IL-6 and GM-CSF may have contributed to the prolonged loss of body weight. Interestingly, Matthys *et al.* (1993) recently reported that blocking IFN-γ availability in anti-CD3-treated animals led to increased IL-6 production. Thus, in TCDD-treated mice, the elevated IL-6 at 48 hours may be causally related to the decreased IFN-γ at 24 hours.

The effect of TCDD on cytokine production in spleen cell cultures was also investigated. However, TCDD exposure did not alter cytokine production when cells were activated with anti-CD3 *in vitro* or when spleen cells were obtained from mice at various times after anti-CD3 injection with two exceptions. Small but significant decreases in IL-2 and GM-CSF levels were noted 90 min after anti-CD3 injection. Also, at later timepoints, GM-CSF was detected in cultures from TCDD-treated mice but not vehicle-treated mice. These later timepoints correlate with elevated GM-CSF levels found in plasma of TCDD-treated mice.

T cell depletion from peripheral lymphoid tissues following anti-CD3 injection has been reported to be a consequence of T cell destruction (Hirsch *et al.* 1988). In our studies, anti-CD3 administration resulted in time-dependent changes in the percentage of

$CD4^+$ and $CD8^+$ cells in the draining lymph nodes. Depletion of $CD8^+$ cells at 24 hours was followed by recovery to normal frequency by 48 hr as previously reported (Neumann *et al.*, 1992). Interestingly, $CD8^+$ cells were highly depleted again at 144 hr. The frequency of $CD4^+$ cells was decreased at all times following anti-CD3 injection with the maximum decrease observed at 72 hr.

Exposure to TCDD did not alter the pattern of anti-CD3-induced changes in the frequency of $CD8^+$ cells. In contrast, TCDD exposure exacerbated the anti-CD3-induced loss of $CD4^+$ cells, starting at 48 hours and continuing to 144 hours post-injection. These changes were driven by anti-CD3 since TCDD did not affect T cell frequencies in Ham IgG-injected mice. These results suggest that TCDD may preferentially target T-helper cells undergoing activation. TCDD has been shown to alter specific events involved in T cell activation such as increasing cytosolic Ca^{2+} levels in rat thymocytes (McConkey *et al.* 1988) and $pp60^{c\text{-}src}$ tyrosine kinase activity in mouse thymocytes (Bombick and Matsumura, 1987). By providing an inappropriate signal to the $CD4^+$ cells, TCDD could alter the signal transduction pathway resulting in activation-driven cell death rather than differentiation. Superoptimal activation has been shown to induce apoptosis in CTL clones (Ucker *et al.*, 1992). In support of this possibility, we identified by flow cytometric analysis a population of LNC from mice co-exposed to TCDD and anti-CD3 that has been characterized by Dive *et al.* (1992) as cells undergoing apoptosis (i.e., decreased FALS and increased LI90). It has been suggested that the decreased FALS reflects cell shrinkage and increased LI90 reflects condensation of chromatin and fragmentation of nuclei (Swat *et al.*, 1991). On the other hand, Ferran *et al.* (1990b) reported necrosis and edema in lymphoid organs 24 and 48 hours after anti-CD3 injection. In our study, histological examination of lymph nodes from mice treated with anti-CD3 at 48 and 72 hrs revealed extensive necrosis. In lymph nodes from TCDD-treated mice, necrosis was more severe at 72 hours as compared to vehicle-treated mice (data not shown). Thus, further studies are necessary to clarify the mechanisms of TCDD-induced depletion of $CD4^+$ LNC in the anti-CD3 model.

In conclusion, we have shown that prior treatment with TCDD enhances the acute physical toxicity associated with anti-CD3 injection and promotes the loss of $CD4^+$ T cells in the lymph nodes. However, surprisingly few changes in cytokine production were observed in TCDD-treated mice, except for a small decrease in plasma levels of IFN-γ and small increases in plasma IL-6 and GM-CSF. Whether these or other cytokine changes are directly related to the effects of TCDD on body weight loss and $CD4^+$ cell depletion in anti-CD3 treated mice remain to be determined.

CHAPTER III

INVOLVEMENT OF ALTERED B7 EXPRESSION IN DIOXIN IMMUNOTOXICITY: B7 TRANSFECTION RESTORES THE CTL BUT NOT THE ALLOANTIBODY RESPONSE TO THE P815 MASTOCYTOMA

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a highly toxic environmental contaminant, suppresses both cytotoxic T lymphocyte (CTL) and cytotoxic alloantibody production in C57Bl/6 mice challenged with allogeneic P815 tumor cells. Recent evidence suggests that TCDD interferes with the initial activation of $CD4^+$ T helper cells, possibly through an indirect mechanism. In this study, we examined the effect of TCDD on the expression of the important costimulatory molecules, B7-1 and B7-2, in P815 allograft immunity. Expression of B7-2, but not B7-1, was upregulated on splenic $B220^+$ and Mac-1^+ cells in P815-challenged mice. Exposure to TCDD significantly decreased the expression of B7-2 on $B220^+$ and Mac-1^+ cells in P815-challenged mice. Providing exogenous B7-mediated costimulation, in the form of B7-transfected P815 tumor cells, induced CTL activity in TCDD-treated mice by a mechanism that was independent of $CD4^+$ T cells. In contrast, B7-transfected P815 cells did not restore the cytotoxic alloantibody response in TCDD-treated mice. These results are consistent with a model in which MHC Class II^-, B7-transfected P815 tumor cells can directly activate $CD8^+$ CTL precursors but cannot directly stimulate $CD4^+$ T helper cells required for B cell activation. These results also demonstrate that CTL precursors in TCDD-treated mice are functional and able to differentiate into effector CTL provided they receive adequate costimulation via B7 and suggest that defective costimulation, through reduced B7-2 expression, may play a role in TCDD-induced immunotoxicity. In support of this hypothesis, we show that blocking B7-2/CD28 interactions, and to a lesser degree B7-1/CD28 interactions, suppressed the alloimmune responses to P815 tumor cells, which further indicates that B7-2 represents the dominant B7 molecule involved in the generation of an immune response to allogeneic P815 tumor cells.

INTRODUCTION

It is generally accepted that antigen presenting cells (APCs) provide at least two signals to T lymphocytes that are required for complete T cell activation and acquisition of effector function (Allison, 1994; Boussiotis et al., 1994; Chen et al., 1993; Harlan et al., 1995; Guerder et al., 1995). The first signal, which confers antigen specificity, is mediated through the TCR following engagement with antigenic peptide presented in association with the MHC complex. The second signal, termed costimulation, is neither antigen-specific nor MHC-restricted. These costimulatory signals can be delivered by cytokines, via binding their specific receptor, or through the cognate interaction of costimulatory molecules expressed on the surface of APCs with their counter-receptors on the T cells. Of the known APC-associated costimulatory molecules, the family of surface proteins known as B7 appear to be the most critical for delivery of costimulatory signals (Guinan et al., 1994; Johnson and Jenkins, 1992; Schwartz, 1992). To date, two B7 molecules, B7-1 (BB-1, CD80) and B7-2 (B70, CD86), have been described (Freeman et al., 1991; Freedman et al., 1991; Azuma et al., 1993; Hathcock et al., 1993; Freeman et al., 1993a; Freeman et al., 1993b; Freeman et al., 1993c). B7-1 and B7-2 are differentially expressed on the surface of "professional" APCs which include dendritic cells, macrophages, and activated B cells. B7-2, but not B7-1, is constitutively expressed at low levels on unstimulated dendritic cells and monocytes; however, both molecules are rapidly upregulated following activation (Inaba et al., 1994; Engel et al., 1994; Mondino and Jenkins, 1994). Neither molecule is expressed on resting B cells; however, *in vitro* studies indicate that B7-2 is upregulated earlier and to higher levels than B7-1 following activation (Hathcock et al., 1994; Lenschow et al., 1994). Despite their differential expression, both B7-1 and B7-2 interact with two counter-receptors, CD28 and CTLA-4 (Linsley and Ledbetter, 1993), on T cells. B7/CD28 interactions upregulate the production of several cytokines essential for T cell activation and clonal expansion, including interleukin (IL)-2, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) through enhanced

transcription and/or message stabilization (Lindsten et al., 1989; Walter et al., 1994). Delivery of the TCR-mediated signal in the absence of sufficient costimulation has been shown to induce a state of clonal anergy (Schwartz, 1990; Guerder et al., 1994; Chen and Nabavi, 1994), or, under certain circumstances, apoptosis (Van Parijs et al., 1996). The importance of the B7/CD28 costimulatory pathway to immune function has been demonstrated in several *in vivo* studies. For example, blocking B7/CD28 interactions using soluble CTLA-4Ig, a CD28 antagonist, has been shown to inhibit many T cell-dependent immune responses including xenograft and allograft rejection and antibody production to sheep red blood cells (reviewed in June et al., 1994; Bluestone, 1995).

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous and persistent environmental contaminant that produces a wide spectrum of toxic effects which are species- and tissue-specific (Poland and Knutson, 1982). These effects, which include teratogenicity, carcinogenicity, hepatotoxicity, cachexia, thymic involution, and immunosuppression, are mediated through specific binding of TCDD to the cytosolic aromatic hydrocarbon receptor (AhR), a ligand-activated transcription factor (Landers and Bunce, 1991; Whitlock, 1993; Nebert et al., 1993). AhR-mediated alterations in transcription of genes encoding growth factors and cytokines have been hypothesized to play a role in many of the tissue-specific effects (Greenlee et al., 1985; Abbott et al., 1992; Gaido and Maness, 1994). Studies in laboratory animals indicate that the immune system is one of the most sensitive targets of TCDD-induced toxicity. Exposure of mice to very low doses of TCDD results in decreased resistance to bacterial and viral infections, as well as suppression of specific effector functions associated with T helper cells, cytotoxic T cells, and B cells (Kerkvliet and Burleson, 1994; Kerkvliet, 1994). Although TCDD-induced immunotoxicity has been widely studied, the cellular targets and biochemical mechanism(s) remain poorly understood.

Our laboratory has utilized an allogeneic tumor model to study the immunotoxic effects of TCDD and other AhR ligands (Kerkvliet and Baecher-Steppan, 1988; DeKrey et

al., 1995; Kerkvliet et al., 1990). Using this model, we and others have shown that TCDD exposure dose-dependently suppresses CTL activity in C57Bl/6 mice challenged with P815 tumor cells via an AhR-dependent mechanism (DeKrey et al., 1995; Kerkvliet et al., 1990; Clark et al., 1983). Recent data from our laboratory indicate that the activation and/or clonal expansion of CTL precursors (CTL_P) into CTL effectors (CTL_E) is inhibited by TCDD. Suppression of CTL activity was shown to correlate with a significant reduction in the production of IL-2, IFN-γ and TNF-α in P815-challenged mice (Kerkvliet et al., 1996). Recent studies have also demonstrated that the cytotoxic alloantibody response to P815 tumor cells was suppressed in TCDD-treated mice even though IL-4 and IL-6 production was unaffected (Kerkvliet et al., 1996). Since B7-mediated costimulation is necessary for optimal cytokine production, CTL_E generation, and antibody production to T cell-dependent antigens, we sought to investigate the possible role of B7-1 and B7-2 expression in TCDD-induced immunosuppression in mice challenged with P815 tumor cells.

In this study, we show that exposure to TCDD results in decreased expression of B7-2 on splenic B cells and macrophages in response to P815 tumor cell challenge. Additionally, we report that injection of either B7-2 or B7-1 transfected P815 tumor cells restores the CTL but not the alloantibody response in TCDD-treated mice. These results suggest that inadequate costimulation represents one mechanism by which TCDD induces immune suppression.

MATERIALS AND METHODS

Animals

Six week old DBA/2 (H-2^d) and C57Bl/6 (H-2^b) and C3H/HeJ (H-2^k) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in front of a laminar flow unit. C57Bl/6 mice were used in experimental studies at 8-12 weeks of age.

P815 mastocytoma cells

Wild-type P815 tumor cells, derived from a methylcholanthrene-induced mastocytoma of DBA/2 origin, were maintained *in vivo* as an ascites tumor by weekly *i.p.* transfer in DBA/2 mice. B7-1-transfected P815 (clone DT106) and vector-transfected P815 were generously provided by Dr. Lewis Lanier (DNAX, Palo Alto, CA). B7-2-transfected P815 (clone HTR.C/B7-2) and vector-transfected control (clone HTR.C/C) were generously provided by Dr. Thomas Gajewski (University of Chicago Medical Center, Chicago, IL). Transfected cells were maintained *in vitro* in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS; Rehatuin, Intergen, Purchase, NY) and 1 mM gentamicin at 37° C in 5% CO_2.

Antibodies

The anti-CD4 monoclonal antibody (mAb; clone GK1.5) used for T cell depletion studies was kindly provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Hanover, NH). Purified rat anti-murine B7-1 mAb (1G10) and rat anti-murine B7-2 mAb (GL1) were purchased from Pharmingen (San Diego, CA) and used to block B7-1 and B7-2 in vivo. Rat IgG was purchased from Cappel (Organon Teknika, West Chester, PA) and used as an Ig control for T cell depletion and in in vivo blocking studies. Spleen cell phenotypes were determined by flow cytometric analysis using the following antibodies: fluorescein (FITC)-conjugated anti-B220 (RA3-6B2), FITC-conjugated anti-CD62L

(MEL-14), phycoerythrin (PE)-conjugated anti-B7-1 (1G10), PE-conjugated anti-B7-2 (GL1), PE-conjugated anti-CD8α (53-6.7), and biotin-conjugated anti-Mac-1 (M1/70) from Pharmingen; and RED670-conjugated anti-CD44 (IM7.8.1) and the second step reagent streptavidin-Red670™ from GIBCO (Grand Island, NY).

Animal Treatment

TCDD, purchased as a certified reference standard (>98% purity; Cambridge Isotope Laboratories, Woburn, MA), was dissolved in anisole and diluted in peanut oil. Male C57Bl/6 mice were treated with an immunosuppressive dose of TCDD (15 μg/kg body weight) or vehicle by gavage one day prior to *i.p.* injection of $1x10^7$ P815 tumor cells. At various times after P815 injection, mice were killed by CO_2 asphyxiation. Blood was collected by heart puncture using syringes prefilled with 12 U sodium heparin. Plasma was separated by centrifugation and stored at -70°C.

In vivo depletion of CD4± spleen cells

Mice were injected *i.p.* with 0.25 mg of anti-CD4 mAb or a control rat IgG on day -2 relative to B7-P815 injection on day 0. Preliminary studies determined that >99% of naive ($CD44^{high}CD45RB^{low}$) $CD4^+$ T cells were depleted for 10 days using this treatment protocol.

Treatment of mice with anti-B7-1 and anti-B7-2 mAb

Mice were injected i.p. with 50 μg of anti-B7-1, anti-B7-2 or control rat IgG on days 0, 3 and 6 relative to P815 injection. An additional group of mice received 50 μg of both anti-B7-1 and anti-B7-2 mAb.

Spleen cell preparation

Spleens were removed aseptically. Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Cells were washed once and resuspended in HBSS supplemented with 2.5% FBS, 20 mM HEPES, 1 mM gentamicin, and 1.5 mM sodium pyruvate.

Three-color flow cytometric analysis

Spleen cells ($1x10^6$) were incubated on ice in round-bottom 96-well plates (Flow Laboratories, McLean, VA) in PBS containing 1.0% bovine serum albumin and 0.1% azide. Nonspecific staining through the Fc receptor was blocked with excess rat IgG (Organon Teknika). Cells were then stained with FITC-labelled anti-B220, PE-labelled anti-B7-1 or anti-B7-2, and biotin-anti-Mac-1 followed by streptavidin-Red670. CTL_E were identified by the expression of PE-labelled anti-CD8, RED670-labelled anti-CD44 and FITC-labelled anti-CD62L as previously described (Mobley and Dailey, 1992). Appropriately-labeled, isotype-matched Igs were used to determine nonspecific staining. After staining, cells were passed through a 40 μm nylon mesh to remove clumps and analyzed immediately on an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL). Fluorescent measurements were collected on 20,000-100,000 stained cells by listmode acquisition and analyzed using Cyclops software (Cytomation, Fort Collins, CO) or WinList (Verity Software House, Inc., Topsham, ME).

CTL assay

The cytolytic activity of spleen cells against P815 tumor cells was measured in a standard 4 hr ^{51}Cr-release assay as previously described (DeKrey et al., 1993). The

percent cytotoxicity at each effector to target (E:T) ratio was calculated using the following equation:

$$\% \text{ cytotoxicity} = \frac{Er - Nr}{Mr - Sr} \times 100$$

where Er = the experimental release using spleen cells from P815-challenged mice, Nr = the nonspecific release using splenocytes from naive mice, Mr = maximum release of ^{51}Cr from cells incubated with sodium dodecyl sulfate, and Sr = spontaneous release of ^{51}Cr incubated in media alone. E:T ratios from 200:1 to 6.25:1 were tested in duplicate.

Cytotoxic Ab response

Plasma samples were heat-inactivated for 30 min at 56° C and centrifuged prior to assay. Two-fold dilutions (1:10 to 1:2560) of plasma were incubated with $1x10^4$ ^{51}Cr-labelled P815 tumor cells for 20 min at 37° and 5% CO_2 in the wells of 96-well round-bottom plates. After the cells were washed, 100 µl of Low-Tox-M rabbit complement (Accurate Chemical & Scientific Corporation, Westbury, NY) (diluted 1:12) were added to each well and plates were incubated for an additional 45 min at 37° C. Supernatant (50 µl) was collected and the amount of ^{51}Cr released was measured by gamma scintillation counting. The percent cytotoxicity was calculated for each plasma dilution using the following equation:

$$\% \text{ cytotoxicity} = \frac{Er - Cr}{Mr - Sr} \times 100$$

where Er = the experimental release using plasma from P815 injected mice and Cr = the release of ^{51}Cr due to complement alone. Mr and Sr are as previously defined. All samples were run in duplicate on separate plates. The Ab titer was defined as the highest dilution of plasma at which a minimum of 30% specific cytotoxicity was measured. The titer was transformed to $\log_2$ dilution for statistical analysis.

RESULTS

Effect of TCDD exposure on B7-1 and B7-2 expression on B220± and Mac-1± spleen cells in mice challenged with allogeneic tumor cells.

To determine whether TCDD exposure altered B7 expression *in vivo*, a timecourse study was performed in which B7-1 and B7-2 expression were examined 4-10 days after P815 injection. This timecourse was selected since CTL development is first detected on day 6 and peaks on days 9-10 following the *i.p.* injection of viable P815 tumor cells (Kerkvliet et al., 1996; Devens and Webb, 1995). We first identified B cells ($B220^+$) and macrophages ($Mac\text{-}1^+$), two recognized APC populations, from the spleen of P815-challenged mice. In vehicle-treated mice, the total number of B cells ($B220^+$) increased on days 5 and 6, then decreased out to day 10, whereas in TCDD-treated mice, the number steadily decreased between days 6 and 10 after P815 injection (figure III-1A). In contrast to $B220^+$ cells, the number of $Mac\text{-}1^+$ spleen cells remained relatively constant in vehicle-treated mice 4-10 days after P815 injection (figure III-1B). Interestingly, in TCDD-treated mice, the number of $Mac\text{-}1^+$ cells increased 4-5 fold by day 9 followed by a sharp decrease on day 10.

After identifying the $B220^+$ and $Mac\text{-}1^+$ cells, we examined these populations for the expression of B7-1 and B7-2. As shown in the representative histograms in figure III-2, little or no B7-1 staining was detected on either $B220^+$ or $Mac\text{-}1^+$ cells from nonimmune mice or from mice injected with P815 tumor cells, whereas B7-2 was constitutively expressed at low levels in nonimmune mice and increased following P815 injection. As shown in figure III-3A and III-3B, TCDD treatment did not alter B7-1 expression on any day following P815 injection. In contrast, TCDD exposure significantly decreased the percentage of $B220^+$ and $Mac\text{-}1^+$ cells that expressed B7-2 on days 6-10 and 6-9,

Figure III-1. Effect of TCDD exposure on the total number of B220$^+$ and Mac-1$^+$ spleen cells in mice challenged with allogeneic tumor cells.

C57Bl/6 (H-2^b) mice were treated with a single oral dose of vehicle control (open circles) or 15 μg TCDD/kg body weight (filled circles) one day prior to intraperitoneal (i.p.) injection of $1x10^7$ P815 (H-2^d) tumor cells. Spleen cells were stained and analyzed by flow cytometry 4-10 days after P815 injection for the expression of B220 and Mac-1 as described in *Materials and Methods*. The total number and percentage (inset) of spleen cells expressing either B220 (A) or Mac-1 (B) are shown. Data points represent mean ± SEM of 4-6 animals per treatment group per day.

Figure III-1.

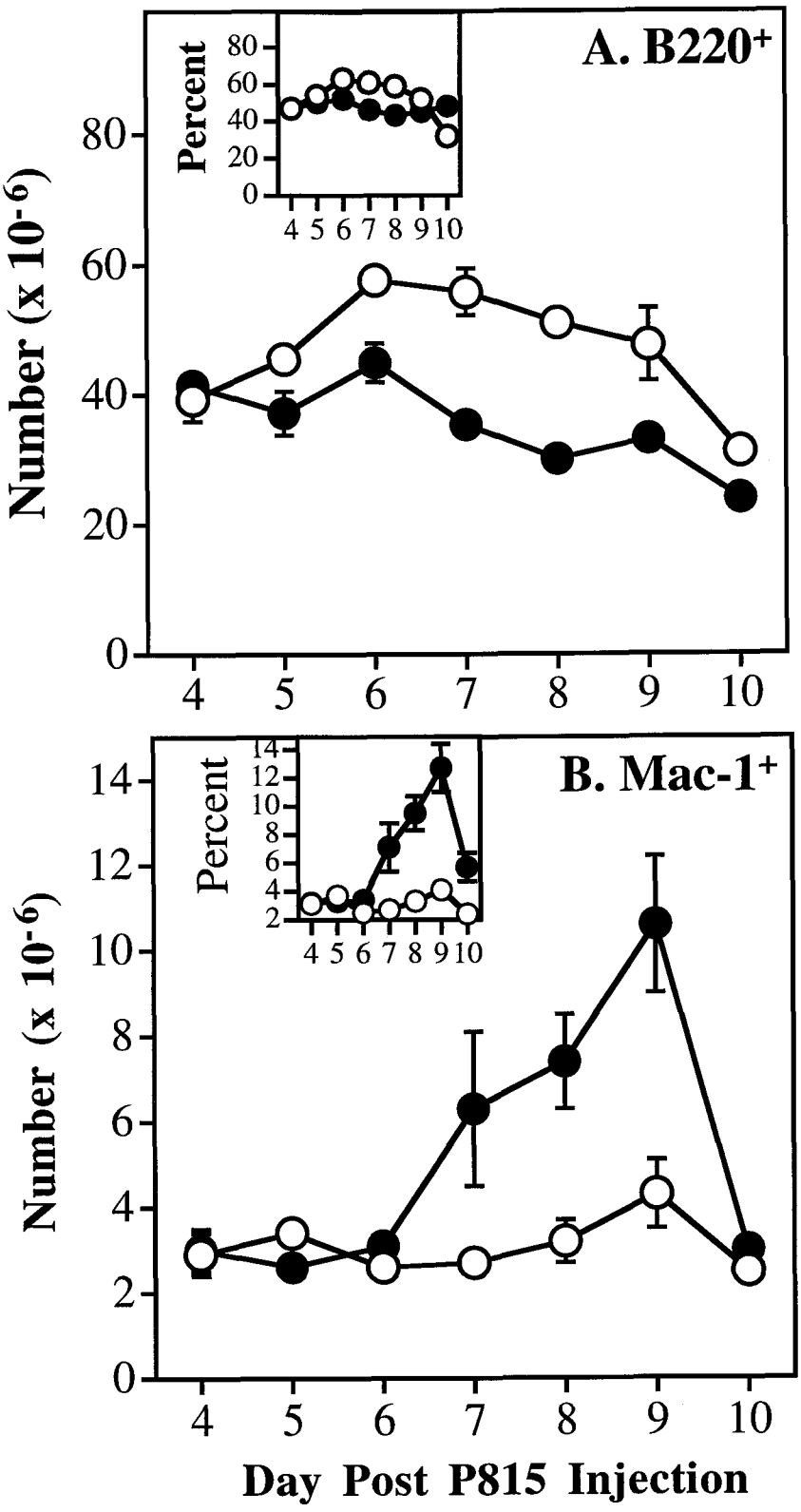


Figure III-2. Representative histogram showing the expression of B7-1 (solid line) and B7-2 (dashed line) on B220+ or Mac-1+ spleen cells from nonimmune mice or mice injected with P815 tumor cells 6 days previously.

Cells were stained and analyzed by three color flow cytometry as described in *Materials and Methods*. Staining with an isotype-matched control Ab is indicated by the dotted line in each histogram.

Figure III-3. Effect of TCDD exposure on the kinetics of B7-1 and B7-2 expression on B220+ and Mac-1+ spleen cells in mice challenged with allogeneic tumor cells.

C57Bl/6 (H-2^b) mice were treated with a single oral dose of vehicle control (open circles) or 15 μg TCDD/kg body weight (filled circles) one day prior to intraperitoneal (i.p.) injection of $1x10^7$ P815 (H-2^d) tumor cells. Spleen cells were stained with FITC-conjugated anti-B220, PE-conjugated anti-B7-1 or PE-conjugated anti-B7-2, and biotin-anti-Mac-1 followed by the second step reagent, streptavidin-Red670, and analyzed by three color flow cytometric analysis. These graphs represent the percentage of B220+ cells (A and C) and Mac-1+ cells (B and D) co-expressing B7-1 (A and B) or B7-2 (C and D). Data points represent mean ± SEM of 4-6 animals per treatment group per day.

Figure III-2.

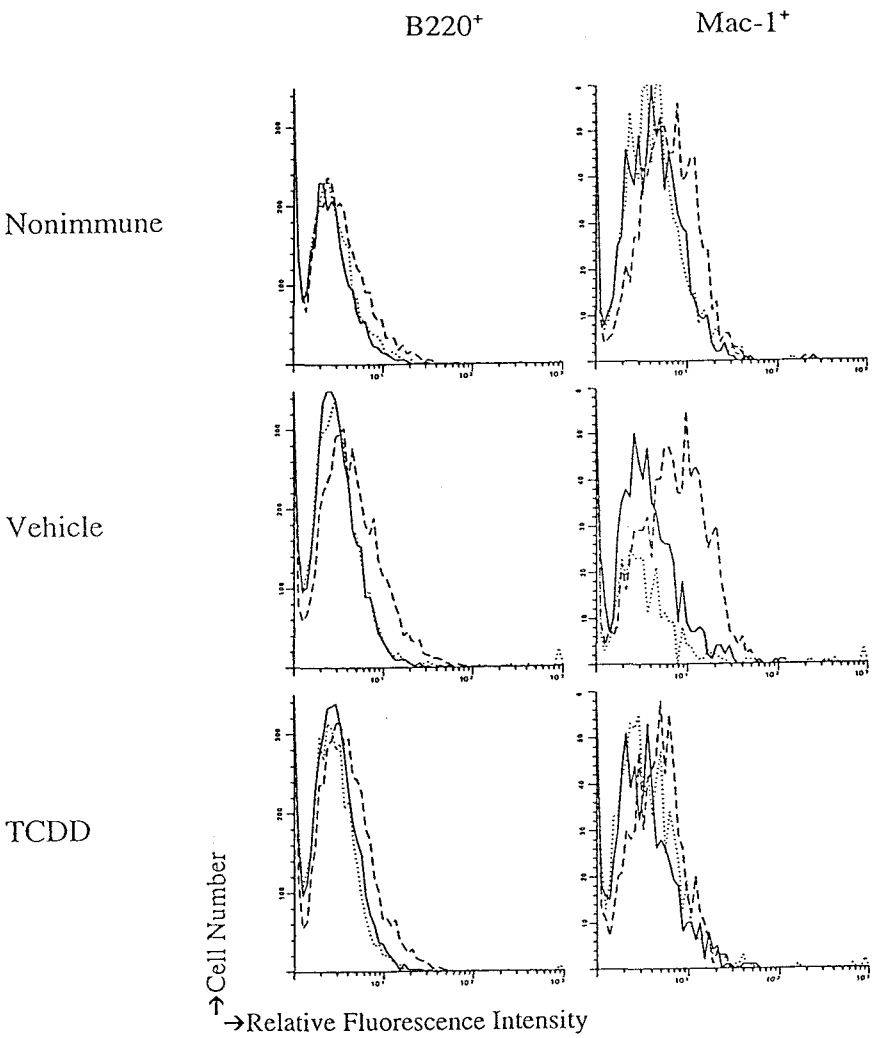
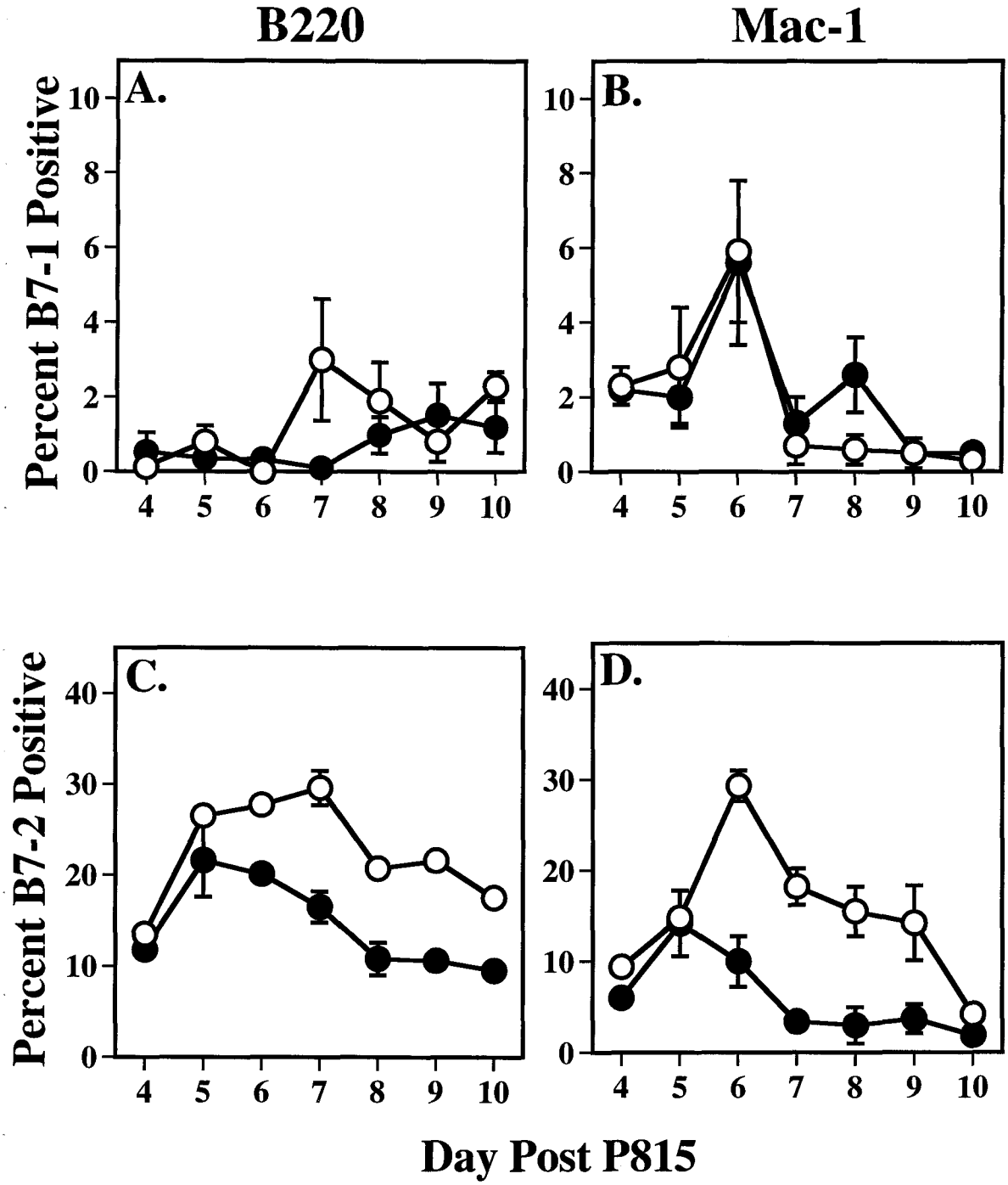

B220+
Mac-1+
Nonimmune
Vehicle
TCDD
→Cell Number
→Relative Fluorescence Intensity

Figure III-3.


B220
Mac-1
A.
B.
C.
D.
Percent B7-1 Positive
Percent B7-2 Positive
Day Post P815
0
2
4
6
8
10
10
20
30
40
4
5
6
7
8
9
10

respectively, when compared to vehicle-treated controls (figure III-3C and III-3D). These data suggest that B7-2 represents the predominant B7 molecule expressed in response to P815 tumor challenge and that one possible mechanism by which TCDD suppresses the immune response to P815 tumor cells is through reduced B7-2 expression.

Differential effects of anti-B7-1 and anti-B7-2 in P815 tumor rejection in vivo

To further address the individual roles of B7-1 and B7-2 in the rejection of allogeneic P815 tumor cells, we used antibodies to B7-1 and B7-2 to selectively block B7-1/CD28 and/or B7-2/CD28 interactions and measured the effects on CTL activity and cytotoxic alloantibody titers. As shown in figure III-4A, CTL activity in mice treated with anti-B7-1 was unaffected when compared to mice treated with control rat IgG. In contrast, administration of anti-B7-2 significantly suppressed CTL activity at all effector to target ratios when compared to controls. CTL activity in mice injected with both anti-B7-1 and anti-B7-2 was also highly suppressed when compared to rat IgG- or anti-B7-1-treated mice; however the degree of suppression was not significantly different from anti-B7-2-treated mice. Similarly, anti-B7-2, and to a lesser degree anti-B7-1, administration suppressed the cytotoxic alloantibody titers when compared to rat IgG-treated controls (figure III-4B). Interestingly, however, injection of both anti-B7-1 and anti-B7-2 suppressed antibody titers more than either antibody alone. These data further indicate that B7-2 represents the major B7 molecule involved in the generation of an immune response to allogeneic P815 tumor cells but also suggests that B7-1 may play a minor role in the alloantibody response.

Injection of B7-transfected P815 tumor cells induces CTL activity in TCDD-treated mice

To determine whether providing exogenous B7-mediated costimulation could induce CTL activity in TCDD-treated mice we challenged vehicle- and TCDD-treated mice with either B7-1- or B7-2-transfected P815 and the appropriate vector-transfected control P815 and measured CTL activity 9 days later. As expected, exposure to 15 μg TCDD/kg body weight significantly suppressed CTL activity following injection of vector-transfected control P815 (figure III-5A and III-5B). In contrast, a high level of CTL activity was detected in TCDD-treated mice challenged with either B7-1$^+$ or B7-2$^+$ P815 tumor cells. Interestingly, the level of CTL activity detected in TCDD-treated mice challenged with B7-transfected tumor cells was comparable to vehicle-treated mice injected with non-transfected P815. We also detected a significant increase in CTL activity following injection of B7-transfected P815 when compared to non-transfected P815, an affect that has been previously reported (Yang et al., 1995).

Flow cytometric analysis of spleen cells showed that CTL activity in mice injected with B7-2$^+$ tumor cells correlated with increased frequency of CTL_E, defined by the expression of CD44^{hi} CD62L^{lo} on CD8$^+$ cells (figure III-6). This subset of cells has been previously characterized by Mobley and Dailey (1992) in a mouse skin allograft model as the effector cells that express cytotoxic activity in a ^{51}Cr-release assay. Similar results were observed following injection of B7-1$^+$ P815 tumor cells (data not shown). In addition, cytotoxic activity generated in mice injected with B7-transfected tumor cells was shown to be allospecific based on the lysis of H-2^d but not H-2^k or H-2^b target cells (Table III-1). These data indicate that exogenous B7, in the form of either B7-1 or B7-2, is capable of inducing an allospecific CTL response in TCDD-treated mice following P815 injection.

Figure III-4. Differential involvement of B7-1 and B7-2 in P815 rejection.

C57Bl/6 mice received 50 μg of control rat IgG, anti-B7-1, or anti-B7-2 on days 0, 3, and 6 relative to P815 injection. Mice treated with both mAb received 50 μg of each mAb on days 0, 3, and 6. On day 10, CTL activity and cytotoxic alloantibody titers were measured as described in *Materials and Methods.* Data points represent mean ± SEM of 6 animals per treatment group.

Figure III-5. Injection of B7-1 or B7-2 transfected P815 tumor cells induces CTL activity in TCDD-treated mice.

C57Bl/6 mice were treated with a single oral dose of vehicle or 15 μg/kg TCDD one day prior to the injection of $1x10^7$ vector-transfected P815, B7-1-transfected P815 (A) or B7-2-transfected P815 tumor cells (B). Nine days later splenic CTL activity was measured in a standard 4-hr ^{51}Cr-release assay as previously described (DeKrey et al., 1993). Cytotoxicity data are shown for the E:T ratio of 100:1. Cytotoxicity of splenocytes from naive mice was less than 1%. Data represents mean ± SEM of 6 mice per treatment group.

Figure III-6. Injection of B7-2-transfected P815 tumor cells induces CTL_E phenotype ($CD8^+CD44^{high}CD62L^{low}$) in TCDD-treated animals.

C57Bl/6 mice were exposed to a single oral dose of vehicle or TCDD on day -2 relative to vector-transfected P815 or B7-2-transfected P815 injection. Nine days later spleen cells were stained and analyzed for the CTL_E phenotype as describe in *Materials and Methods.* The region identified represents the population of $CD8^+$ spleen cells that express the CTLE phenotype as defined by Mobley and Dailey (1992).

Figure III-4.

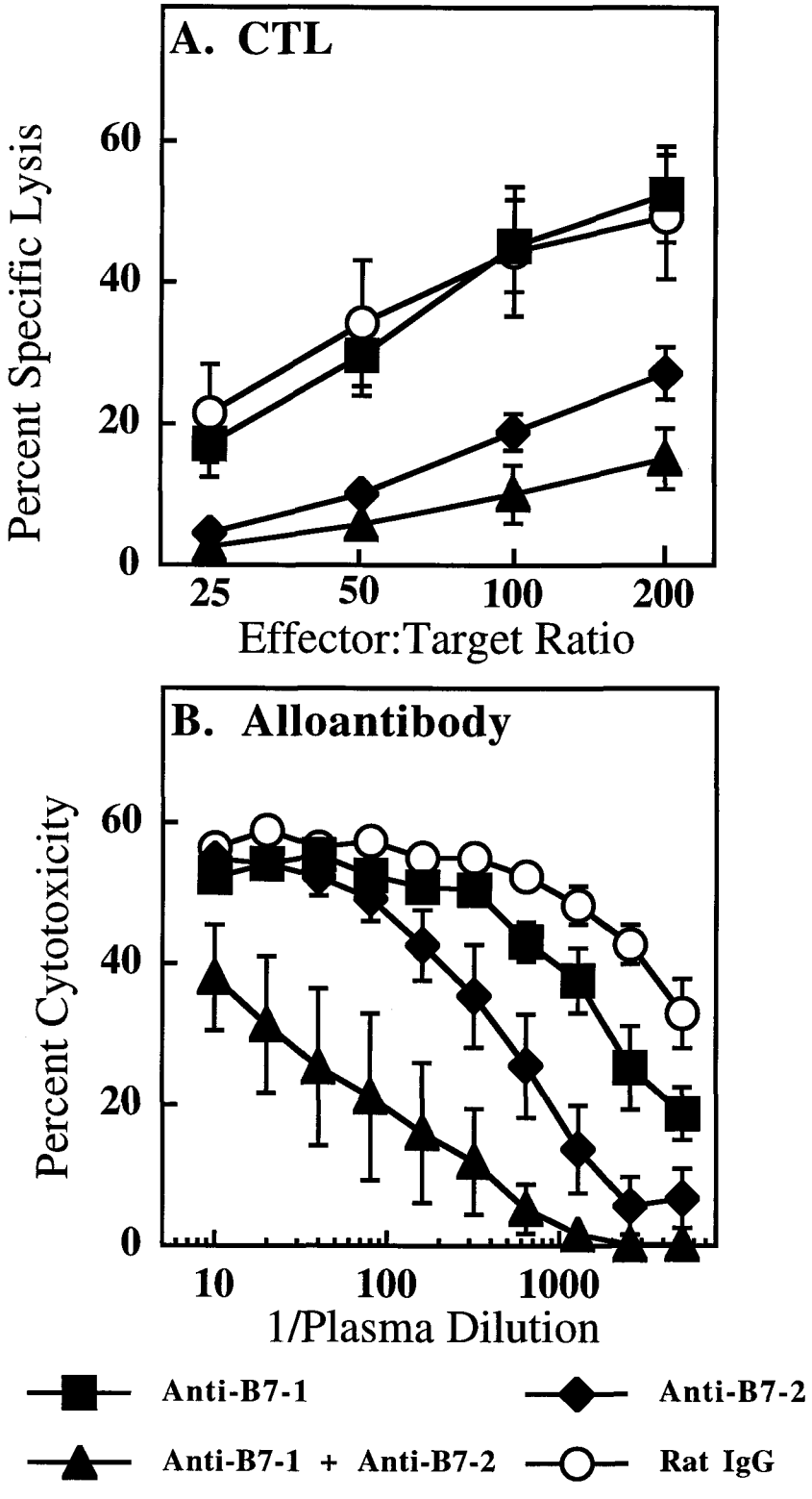

A. CTL
Percent Specific Lysis
60
40
20
0
25
50
100
200
Effector:Target Ratio
B. Alloantibody
Percent Cytotoxicity
60
40
20
0
10
100
1000
1/Plasma Dilution
Anti-B7-1
Anti-B7-2
Anti-B7-1 + Anti-B7-2
Rat IgG

Figure III-5.

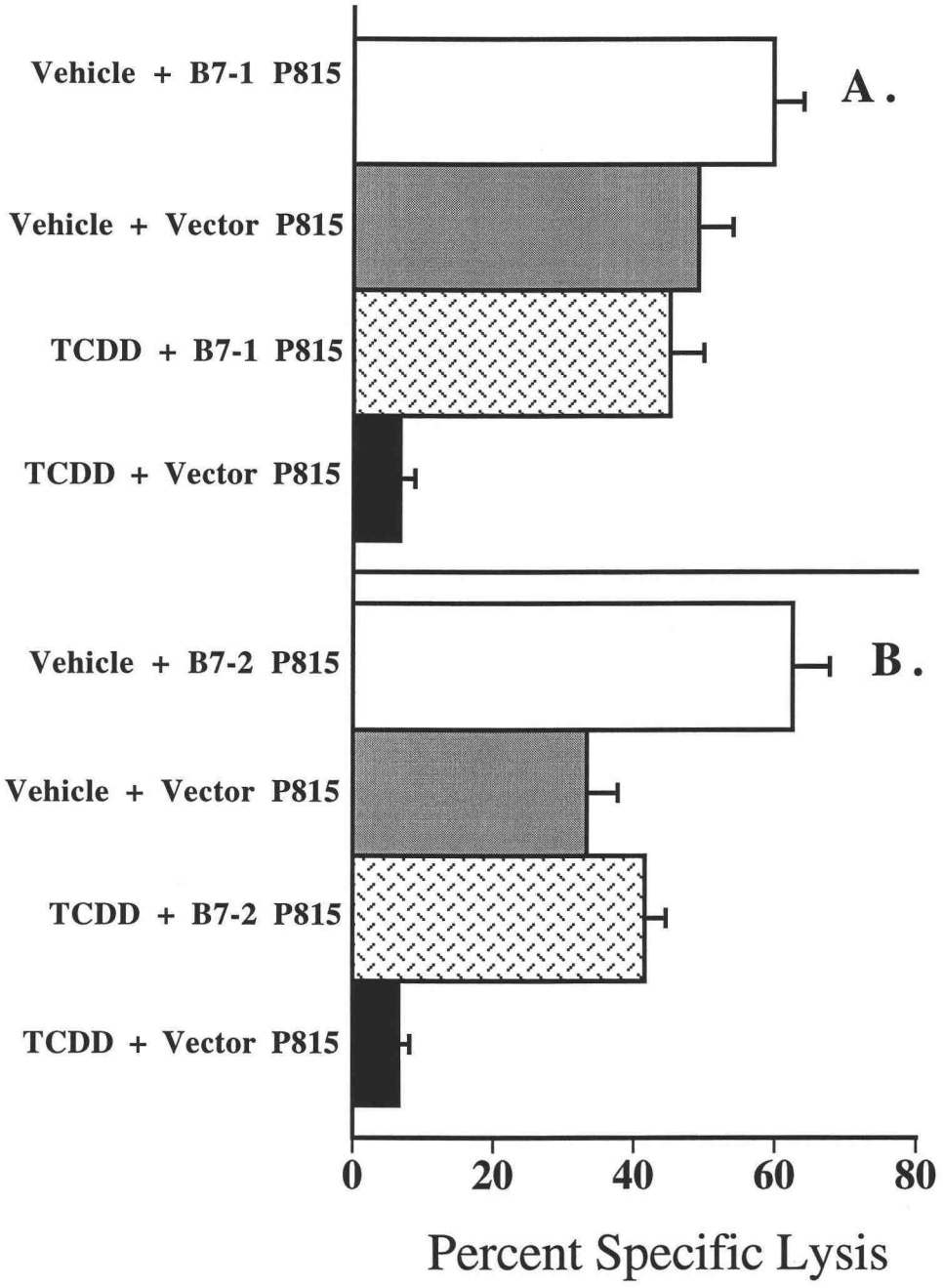
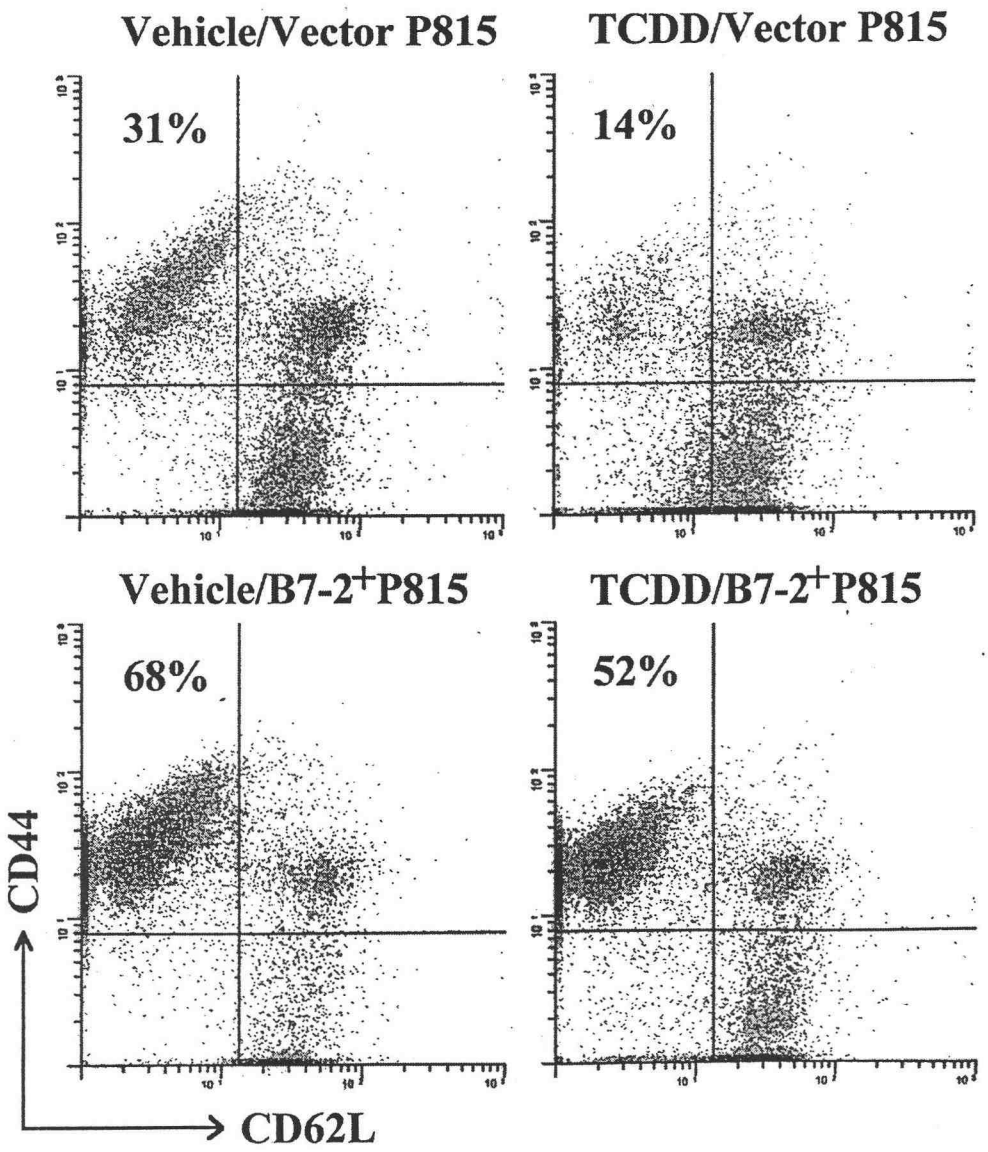

Vehicle + B7-1 P815
A.
Vehicle + Vector P815
TCDD + B7-1 P815
TCDD + Vector P815
Vehicle + B7-2 P815
B.
Vehicle + Vector P815
TCDD + B7-2 P815
TCDD + Vector P815
0
20
40
60
80
Percent Specific Lysis

Figure III-6.


Vehicle/Vector P815
TCDD/Vector P815
31%
14%
Vehicle/B7-2+P815
TCDD/B7-2+P815
68%
52%
CD44
CD62L

B7-transfected P815 induce CTL activity independent of CD4± T cells

We recently reported that CD4+ T cells were required for the generation of allo-specific CTL activity to nontransfected P815 mastocytoma (Kerkvliet et al., 1996). However, the rejection of B7-transfected tumor cells has been shown to occur independently of CD4+ T helper cells *in vitro* and *in vivo* via direct activation of CD8+ CTL_P (Harding and Allison, 1993; Gajewski et al., 1995; Townsend and Allison, 1993). Therefore, we depleted mice of CD4+ cells prior to B7-P815 injection to determine if TCDD directly affected the ability of CTL_P to differentiate into CTL_E. Interestingly, as shown in figure III-7, both vehicle- and TCDD-treated mice previously depleted of CD4+ T cells generated high levels of CTL activity following B7-P815 injection. These results demonstrate that, like other B7-transfected tumor cells (Townsend and Allison, 1993; Chen et al., 1992), rejection of B7-P815 by allogeneic hosts is independent of CD4+ T helper cells. Furthermore, the data indicate that TCDD does not directly affect the ability of CTL_P to differentiate into CTL_E.

B7-P815 challenge does not restore the cytotoxic alloantibody response in TCDD-treated mice

In order to determine if challenge with B7-P815 could also restore the cytotoxic antibody response in TCDD-treated mice, antibody titers were measured in the same animals in which the CTL response was generated (see figure III-5A and III-5B). Antibody titers in TCDD-treated mice were highly suppressed (3 $\log_2$ dilutions) when compared to titers from vehicle-treated mice following injection of vector-transfected P815. However, unlike the CTL response, neither B7-1 nor B7-2-transfected P815 tumor cells altered the suppression of the cytotoxic antibody response in TCDD-treated animals. In

Table III-I. Allospecificity of cytotoxic T lymphocyte activity in response to B7-1+ P815 challenge [a].

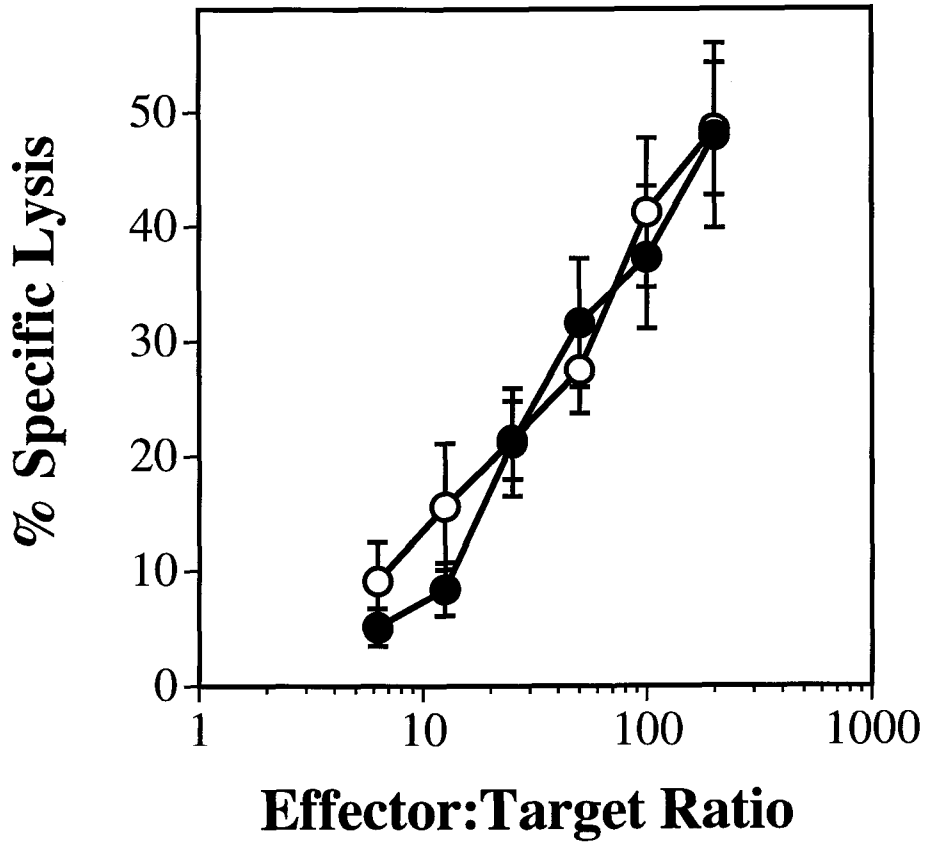
	Percent Specific Lysis	
Target cells	Vector -P815	B7-1 P815
DBA	14	18
C57Bl/6	<1	<1
C3H/HeJ	<1	<1
P815	32	37

[a] C57Bl/6 mice were injected with $1x10^7$ vector-transfected or B7-1-transfected P815 tumor cells. Nine days later Con A stimulated spleen cells from DBA ($H2^d$), C57Bl/6 ($H2^b$), and C3H/HeJ ($H2^k$) or P815 tumor cells were used as target cells in a standard 4-h ^{51}Cr release assay. Data represent mean ± SEM CTL activity at an effector:target ratio of 100:1 of 4 mice per group.

Figure III-7. TCDD exposure does not affect CTL activity in CD4-depleted mice challenged with B7-P815.

C57Bl/6 mice were injected with 0.25 mg anti-CD4 (mAb GK1.5) on day -2 and exposed to a single oral dose of vehicle (open circles) or 15 μg/kg TCDD (filled circles) on day -1 relative to B7-P815 ($1x10^7$) injection. Nine days later CTL activity was measured in a standard 4-hr ^{51}Cr-release assay (DeKrey et al., 1993). $CD4^+$ T cell depletion was verified on the days 0 and 9 using flow cytometric analysis (data not shown). Data points represent mean ± SEM of 4-6 mice per group. In an independent experiment, a dose of 0.25 mg GK1.5/mouse one day prior to the injection of non-transfected P815 tumor cells was shown to inhibit the CTL response by >90% (data not shown).

Figure III-7.


% Specific Lysis
50
40
30
20
10
0
1
10
100
1000
Effector:Target Ratio

fact, antibody titers were equally suppressed (3 $\log_2$ dilutions) in TCDD-treated mice after challenge with vector- or B7-transfected P815 tumor cells (figure III-8A and III-8B).

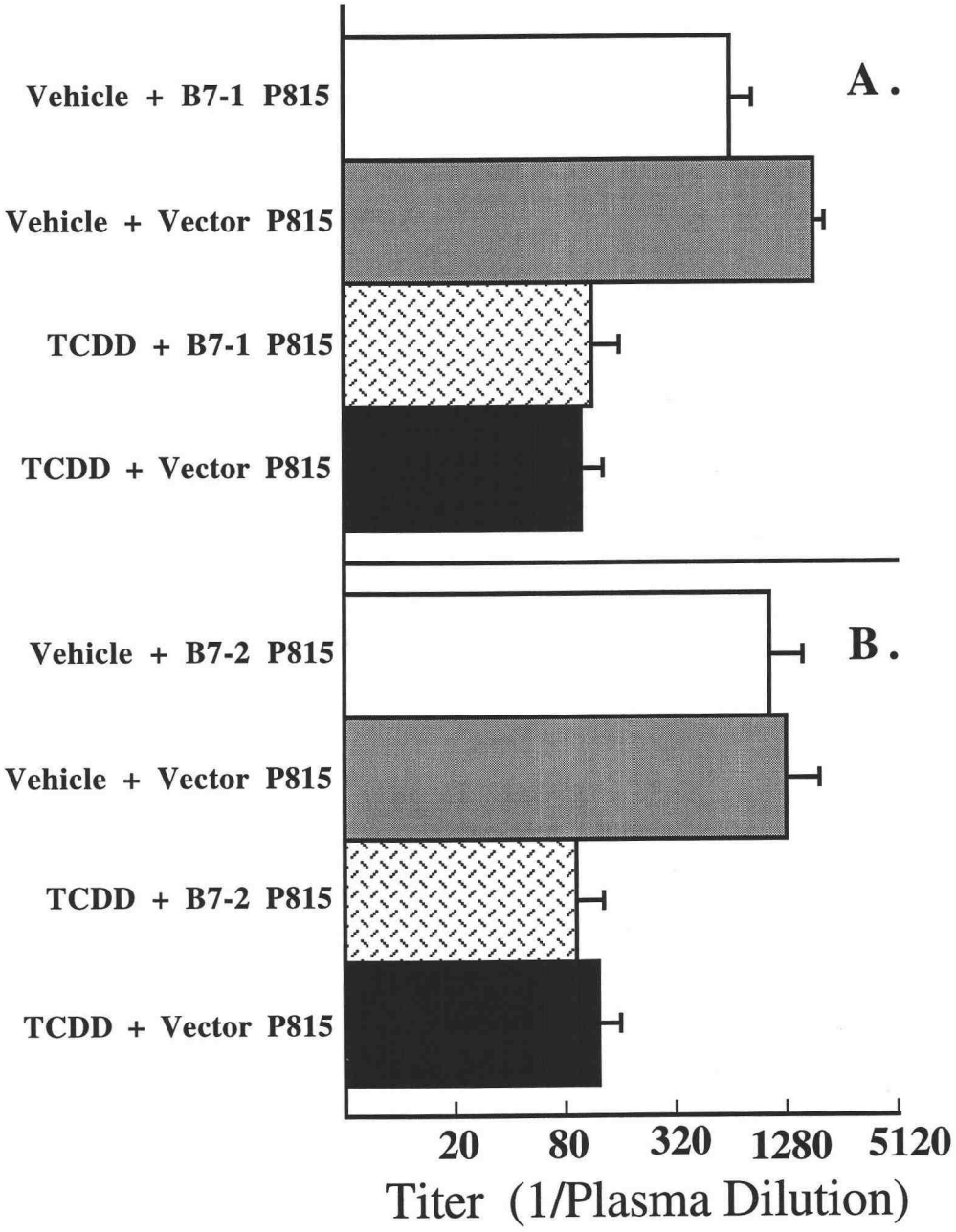
DISCUSSION

It is generally accepted that a B7-mediated costimulatory signal, in conjunction with a TCR generated signal, is required for complete T cell activation (Allison, 1994; Boussiotis et al., 1994; Walter et al., 1994). It has been proposed that B7-2 represents the primary B7 molecule *in vivo* for initiating T-dependent immune responses since it is constitutively expressed on dendritic cells and monocytes and is upregulated earlier than B7-1 on activated B cells (Freeman et al., 1993c; Bluestone, 1995). However, recent studies suggest that B7-1-mediated costimulation preferentially induces T_H1 type cytokines (IL-2, IFN-γ) whereas B7-2 preferentially induces T_H2 type cytokines (IL-4, IL-5, IL-10) (Freeman et al., 1995; Kuchroo et al., 1995). This has led to a controversy over the relative contributions of B7-1 and B7-2 *in vivo* during an immune response (Bluestone, 1995). Evidence suggests that B7-2 is the dominant costimulatory ligand in certain *in vivo* models, such as rejection of allogeneic pancreatic islet cells (Lenschow et al., 1995b) and the development of diabetes in NOD mice (Lenschow et al., 1995a), whereas B7-1 appears to be more dominant in other *in vivo* models such as the development of murine experimental allergic encephalomyelitis (EAE) (Kuchroo et al., 1995). Two hypotheses have evolved to explain these paradoxical results. First, it has been shown that B7-1 and B7-2 bind to CTLA-4, and presumably CD28, with different kinetics (Linsley et al., 1994) which may result in the recruitment of distinct intracellular signalling pathways that could preferentially induce T_H1 or T_H2 responses. Alternatively, Natesan et al. (1996) proposed that these differences may simply reflect the relative abundance and/or distribution of B7-1

Figure III-8. Injection of B7-transfected P815 tumor cells does not restore the cytotoxic alloantibody response in TCDD-treated mice.

The cytotoxic alloantibody response was measured in plasma collected from the same vehicle- and TCDD-treated animals shown in Figure 5 using a complement-dependent ^{51}Cr-release assay as described in *Materials and Methods*. Data represents the highest plasma dilution at which a minimum of 30% cytotoxicity was measured. Background killing of plasma from naive mice was not detected at the lowest dilution.

Figure III-8.


Vehicle + B7-1 P815
Vehicle + Vector P815
TCDD + B7-1 P815
TCDD + Vector P815
A.
Vehicle + B7-2 P815
Vehicle + Vector P815
TCDD + B7-2 P815
TCDD + Vector P815
B.
20
80
320
1280
5120
Titer (1/Plasma Dilution)

and B7-2 in the respective models. Consistent with this interpretation, Miller et al. (1995) recently reported that B7-1 appears to be the primary B7 molecule expressed during the progression of murine EAE which could explain why B7-1 mAb blocked the progression of the disease, whereas B7-2 mAb exacerbated the disease (Kuchroo et al., 1995).

Gajewski et al (1996) recently demonstrated that a host-derived CTLA4 ligand is required for the *in vivo* rejection of certain P815 tumor variants. However, this study used a murine construct of CTLA4 Ig which binds both B7 molecules, and therefore could not distinguish between the relative contribution of B7-1 and B7-2 *in vivo* . In the allogeneic P815 tumor model, we found that the percentage of B cells and macrophages that expressed B7-2 was approximately 10 fold and 6 fold higher than the percentage that expressed B7-1, respectively. In addition, injection of anti-B7-2 but not anti-B7-1 mAb inhibited both CTL and cytotoxic antibody responses to allogeneic P815 tumor cells. Taken together, these data do not support a preferential expression of B7-1 in T_H-1 type responses. Rather, they suggest that B7-2 represents the predominant B7 molecule involved in the immune response to allogeneic P815 tumor cells which is consistent with the results reported by Lenschow et al. (1995b).

We found that TCDD-induced immune suppression was associated with reduced expression of B7-2 on $B220^+$ and Mac-1^+ spleen cells *in vivo* (figure III-3C and III-3D) and injection of B7-2-transfected P815 tumor cells induced a significant CTL response in TCDD-treated mice (figure III-5B). Interestingly, injection of B7-1-transfected P815 also induced a significant CTL response in TCDD-treated mice (figure III-5A). These results are intriguing since blocking B7-1 in vivo did not alter the CTL response which would suggest that B7-1 plays little if any role in generating a CTL response to P815 tumor cells (figure III-4A). However, a recent study by Lanier et al. (1995) demonstrated that B7-1 and B7-2 provide similar costimulatory signals for the generation of CTL activity and cytokine production *in vitro* using APC depleted spleen cells as responder cells. In addition, it has been shown that both B7-1 and B7-2-transfected tumor cells are rejected in

syngeneic mice (Yang et al., 1995; Gajewski et al., 1996). Therefore, these results suggest that either B7-mediated signal, whether B7-1 or B7-2, can provide the appropriate costimulatory signal which is critical for the generation of anti-P815 specific CTL activity in allogeneic mice.

The ability of B7-P815 to restore the CTL response in TCDD-treated mice previously depleted of $CD4^+$ T helper cells (figure III-7) suggests that the primary target(s) of TCDD are the cells responsible for activating the CTL_P (e.g., APC and/or $CD4^+$ T cells) rather than the CTL_P itself. This conclusion is supported by reports that B7-transfected P815 are capable of generating P815-specific CTL_E independent of $CD4^+$ T helper cells *in vitro* (Harding and Allison, 1993; Gajewski et al., 1995) and *in vivo* (Yang et al., 1995; Gajewski et al 1996; and this paper) by directly activating the CTL_P. Therefore, had TCDD directly affected the ability of the CTL_P to differentiate and clonally expand, CTL activity should have still been suppressed regardless of direct B7-stimulation.

In contrast to the CTL response, B7-transfection of P815 tumor cells was unable to bypass the suppression of the alloantibody response. These results could be interpreted to suggest that B7-mediated costimulation is not involved in alloantibody production to P815 cells. However, we demonstrate that anti-B7-2, and to a lesser degree, anti-B7-1 monoclonal antibodies suppressed the antibody response to allogeneic P815 tumor cells (figure III-4), which is in agreement with other reports in which B7/CD28 interactions have been shown to play a critical role in antibody production to T dependent antigen. For example, Linsley et al. (1992) reported that *in vivo* treatment with CTLA4-Ig suppressed T cell-dependent antibody responses to sheep red blood cells and keyhole limpet hemocyanin. Also, Damle et al. (1991) showed that blocking the direct interaction between CD28 on alloactivated T helper cells and B7 on allospecific B cells with either anti-CD28 or anti-B7 antibodies inhibited contact-dependent B cell differentiation into Ig secreting cells. Therefore this interpretation is unlikely.

Alternatively, we believe that these data strengthen our hypothesis that reduced B7 expression on APC may represent one underlying mechanism of TCDD-induced immunosuppression. Because B7-P815 tumor cells do not express MHC Class II on their cell surface, they lack the ability to deliver the TcR mediated signal required for $CD4^+$ T cell activation. Therefore, $CD4^+$ T cells would depend on resident APC to deliver both the TcR and costimulatory signals required for activation. If, however, resident APC in TCDD-treated mice are unable to provide sufficient costimulation, $CD4^+$ T helper cells would not receive the signals required for complete activation, even if mice were injected with B7-transfected P815 cells. As a result, the unactivated $CD4^+$ T cells would not be able to activate B cells, via cytokines or cognate interactions, necessary for alloantibody production. This interpretation could explain why B7-transfected P815 tumor cells preferentially restored the cell-mediated and not the humoral immune response in TCDD-treated mice.

It is interesting to note that we detected a 2-3 fold increase in the number of Mac-1^+ cells in the spleens of TCDD-treated mice 7-9 days after P815 injection that was not present in vehicle-treated mice (figure III-1B). These cells apparently did not express B7-1 or B7-2 since the percentage of $B7^+$ macrophages did not increase in TCDD-treated mice relative to vehicle-treated mice (figure III-3B and III-3D). The role, if any, of these Mac-1^+ cells in the suppression of CTL activity in TCDD-treated mice is not known at present. Interestingly, however, their increased numbers occurred at the same time that CTL activity failed to increase in TCDD-treated mice. Watson and Lopez (1995) recently described a population of Mac-1^+ Mac-2^+ cells in the spleen of tumor-bearing Balb/c mice. This Mac-1^+2^+ cell population appeared in parallel with tumor progression and was found to potently suppress T cell responses in a non-MHC-specific T cell/macrophage contact-dependent interaction. It is possible that TCDD induces a similar population of

suppressor macrophages in P815-injected mice. This possibility is consistent with a lack of expression of costimulatory molecules like B7.

In summary, we have shown that injection of anti-B7-2, and to a lesser degree anti-B7-1, suppressed alloimmune responses to P815 tumor cells. We have also shown that the suppressed immune response to P815 tumor cells in TCDD-treated mice was associated with decreased expression of B7-2 on B cells and macrophages, suggesting insufficient costimulation as a possible mechanism of TCDD-induced immunosuppression. In addition, injection of either B7-1- or B7-2-transfected tumor cells were able to induce the CTL but not the alloantibody response in TCDD-treated mice. These results support a model in which the MHC Class II^- B7-P815 can directly activate the $CD8^+$ CTL_P but not the $CD4^+$ T helper cell. Thus reduced B7 expression may represent a central component of TCDD-induced suppression of both cell mediated and humoral immune responses. Although it is unlikely that B cells and macrophages represent the APC responsible for initiating the immune response to P815 tumor cells, since this function likely belongs to dendritic cells (Moser et al., 1995), they may represent the primary APC responsible for the expansion of allospecific CTL_E. Experiments to investigate the effect of TCDD exposure on B7 expression on dendritic cells are currently in progress.

CHAPTER IV

TCDD-INDUCED IMMUNE SUPPRESSION: TOLERANCE AS A POTENTIAL MECHANISM

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ABSTRACT

We have previously shown that exposure to the immunotoxicant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreases the expression of the critical costimulatory molecule CD86 (B7-2) during the development of an immune response to allogeneic P815 tumor cells. In addition, the production of IL-2 and IFN-γ was significantly suppressed by TCDD. It has been suggested that blocking B7-mediated costimulation in vivo induces tolerance, possibly through a mechanism of antigenic nonresponsiveness (anergy). It has also been reported that providing exogenous IL-2 can reverse or prevent the induction of tolerance in both in vivo and in vitro models. Alternatively, a second means to induce in vivo tolerance is by peripheral deletion of antigen-specific T cells. Studies were designed to address the hypothesis that exposure to TCDD induces a state of in vivo tolerance. TCDD-treated mice were injected with 50, 500, or 2500 U IL-2 twice daily on days 7-9 relative to P815 injection. Administration of IL-2 dose-dependently increased CTL activity and the generation of effector CTL (CTL_E) in TCDD-treated mice. Similarly, IL-2 (2500 U) increased CTL activity and the generation of CTL_E in vehicle-treated mice challenged with P815 tumor cells. In contrast, administration of IFN-γ at 10, 100, or 1000 ng/day on days 6-9 relative to P815 injection did not restore CTL activity in TCDD-treated mice. To test whether exposure to TCDD enhanced peripheral deletion, vehicle or TCDD-treated mice were injected with the superantigen, staphylococcal enterotoxin A (SEA). SEA causes the initial expansion of T cells which contain Vß3$^+$ T cell receptors. This expansion is followed by the peripheral deletion the same Vß3$^+$ T cells. TCDD exposure did not affect the expansion or deletion of the Vß3$^+$ T cells. These data suggest that enhanced peripheral deletion does not play a major role in TCDD-induced immune suppression. In contrast, the ability of IL-2 but not IFN-γ to induce CTL activity in TCDD-treated mice is consistent with the reported ability of IL-2 to reverse T cell tolerance and suggests that the lack of optimal IL-2 production may play a role in TCDD-induced immune suppression.

INTRODUCTION

Previous studies have shown that exposure to TCDD suppresses the immune response to allogeneic P815 tumor cells (Clark et al., 1981; De Krey and Kerkvliet, 1995; Kerkvliet et al., 1990b). TCDD-exposed mice have suppressed cytotoxic T lymphocyte (CTL) activity as well as suppressed cytotoxic alloantibody titers. Recently, we demonstrated that the decrease in CTL activity correlated with the inability of precursor CTL (CTL_P) to mature and differentiate into a population of effector CTL (CTL_E) (Kerkvliet et al., 1996). Furthermore, the production of interleukin (IL)-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α was suppressed when spleen cells from TCDD-exposed animals were restimulated with P815 tumor cells in vitro (Kerkvliet et al., 1996). More recently, we reported that exposure to TCDD decreased the expression of the critical costimulatory molecule CD86 (B7-2) on B cells and macrophages after injection of P815 tumor cells (Prell and Kerkvliet, 1997). The importance of the decreased CD86 expression was demonstrated by the fact that providing exogenous B7, in the form of B7-1 or B7-2 transfected P815 tumor cells, prevented the TCDD-induced suppression of the CTL response.

B7/CD28 interactions are critical for generating a functional immune response. Binding of B7 to CD28 up-regulates the production of several cytokines, including IL-2, IFN-γ and TNF, that are essential for T cell activation and acquisition of effector functions. Blocking B7/CD28 interactions in vivo with CTLA4-Ig, a CD28 receptor antagonist, has been shown to induce T cell tolerance to xenografts and allografts and to inhibit antibody production sheep red blood cells (reviewed in June et al., 1994 and Bluestone, 1995). CTLA4-Ig-induced tolerance is also associated with an 80-90% decrease in IL-2 and IFN-γ production (Judge et al., 1996; Onodera et al., 1997).

The two principle mechanisms for the induction of adult peripheral tolerance are clonal anergy and peripheral deletion (Dallman et al, 1993). Anergy, which appears to be a phenomenon exclusive to lymphocytes, is one consequence of insufficient costimulation

that leads to abortive T cell activation. Studies indicate that anergy is associated with decreased production of certain cytokines such as IL-2 and IFN-γ (Schwartz, 1990; Dallman et al., 1991; Harding et al,. 1992; Sayegh et al., 1995) and providing exogenous IL-2 has been reported to reverse anergy in vitro as well as in some in vivo models (Jenkins et al., 1987; Essery et al., 1988; Dallman et al., 1991; Heath et al., 1992).

In contrast, peripheral deletion as a mechanism of tolerance results from the physical elimination of antigen-specific T cells. Injection of superantigens (SA), such as SEA, have been used extensively to study the induction of in vivo peripheral tolerance and factors which influence this phenomenon. These models are useful because SA bind to specific Vβ chains of the TCR and the in vivo responses of antigen-specific T cells can be easily tracked using anti-TCR antibodies (McCormack et al., 1994; Rellahan et al., 1990; Vella et al., 1995). Peripheral tolerance following challenge with superantigen has been reported to be a consequence of both peripheral deletion and clonal anergy (reviewed by Huber et al., 1996). It has been shown that administration of cytokines including TNF-α, IFN-γ, IL-1 and IL-2 can delay or prevent SEA-induced peripheral deletion (Vella et al., 1995; Belfrage et al., 1997).

In the present studies we demonstrate that providing exogenous IL-2 dose dependently increased CTL activity and the generation of CTL_E in TCDD-treated mice challenged with P815 tumor cells. We also demonstrate that administration of exogenous IL-2 partially rescued the CTL response in CTLA4-Ig-treated mice. In contrast, exogenous IFN-γ did not affect CTL activity in TCDD-treated mice. Exposure to TCDD did not affect the expansion or deletion of $V\beta3^+$ T cells following challenge with SEA. These results suggest that enhanced peripheral deletion may not be involved in TCDD-induced immune suppression. However, TCDD-induced T cell tolerance may share a common mechanism with CTLA4-Ig since CTLA4-Ig induced tolerance could also be partially reversed by exogenous IL-2 administration.

MATERIALS AND METHODS

Animals

Six-week-old C57Bl/6 (H-2^b) and DBA/2 (H-2^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57Bl/6 mice were used in experimental studies between 8 to 12 weeks of age. Four-week-old B10.BR/SgSnJ (H-2^k) were purchased from The Jackson Laboratory and used in experimental studies at 5 weeks of age. Mice were housed in front of a laminar flow unit and given food and water *ad libitum.*

Reagents

TCDD, purchased as a certified reference standard (>98% purity; Cambridge Isotope Laboratories, Woburn, MA), was dissolved in anisol and diluted in peanut oil. P815 tumor cells, derived from a methylcholanthrene-induced mastocytoma of DBA origin, were maintained in vivo as an ascites tumor by weekly i.p. passage in DBA/2 mice. CTLA4-Ig and the isotype-matched control chimeric antibody, L6-Ig were generously provided by Dr. Peter Linsley (Bristol-Myer Squibb, Seattle, WA). Staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology (Madison, WI).

Animal treatment

C57Bl/6 or B10BR/SgSnJ mice were treated with a known immunosuppressive dose of TCDD or vehicle control by gavage one day prior to i.p. injection of $1x10^7$ viable P815 tumor cells (DeKrey and Kerkvliet, 1995). On days 7, 8, and 9 after P815 injection C57Bl/6 mice were injected i.p. two times per day with 100, 500, or 2500 U of recombinant murine IL-2 (Genzyme, Cambridge, MA). For IFN-γ treatment, TCDD-treated C57Bl/6 mice were injected i.p. with 10, 100, or 1000 ng of recombinant IFN-γ (Pharmingen) on days 6, 7, 8, and 9 relative to P815. On day 10, mice were killed by CO_2 asphyxiation.

Treatment of mice with CTLA4-Ig

Mice were injected i.p. with 200 μg of CTLA4-Ig or L6-Ig on days 0, 1, and 2 relative to P815 injection. Mice then received i.p. injections twice per day of 2500 U of recombinant murine IL-2 on days 7, 8, and 9 after injection of P815 tumor cells. On day 10, mice were killed by CO_2 asphyxiation.

Treatment of mice with SEA

B10BR/SgSnJ mice received 15 μg TCDD/kg body weight or vehicle control via gavage one day prior to the i.p. injection of 0.15 μg SEA in Hanks Balanced Salt Solution (HBSS; Sigma, St. Louis, MO). Animals were killed at various times after SEA injection via CO_2 asphyxiation.

Cell processing and staining

Spleens were removed and processed into single cell suspensions by pressing between the frosted ends of two microscope slides. Red blood cells were removed by hypotonic lysis. Cells were resuspended in 3 mls of HBSS containing 5% fetal bovine serum (HyClone, Logan, UT) (HBSS-5). For SEA experiments, T cells from spleen were enriched on nylon wool columns as previously described (Julius et al., 1973).

Popliteal, inguinal, axillary and superficial dorsal axillary nodes were removed at various times after SEA injection. Lymph nodes were pooled and single cell suspensions of lymph node cells (LNC) were prepared as described for spleen cells. LNC were washed once in HBSS-5 and resuspended in 0.5 mls.

Three-color flow cytometric analysis

Spleen cells (2×10^6) were incubated on ice in V-bottom 96 well plates (Costar Corporation, Cambridge, MA) in PBS containing 1.0% BSA and 0.1% azide. Nonspecific staining through the Fc receptor was blocked with excess rat IgG (Organon Teknika). Cy

Chrome-conjugated CD8α (53.6.7), PE-conjugated anti-CD62L (MEL-14), and FITC-conjugated anti-CD44 (IM7.8.1) were purchased from Pharmingen and used to identify CTL_E as previously described (Prell and Kerkvliet, 1997). Nonspecific staining was determined by staining with the appropriately labelled, isotype matched Igs. After staining, cells were immediately analyzed on an EPICS V (Coulter Electronics, Hialeah, FL) or an EPICS XL-MCL flow cytometer (Coulter, Miami, FL). Fluorescent measurements were collected on 10,000 CTL_E by listmode acquisition and analyzed using WinList (Verity Software House, Topsham, ME).

To identify $V\beta3^+$ T cells, LNC or T cell-enriched spleen cells (1×10^6) were incubated in V-bottom plates on ice for 30 min in the presence of 5% normal mouse serum to prevent nonspecific binding of the staining antibody as previously described (Vella et al., 1995). Cells were stained with Red613-labelled anti-CD8α (Gibco, Grand Island, NY), PE-labelled anti-CD4 (Gibco) and FITC-labelled anti-TCRVβ3 (KJ25-607.7; Pullen et al., 1988) and incubated on ice in the dark for an additional 15 minutes. Cells were washed once in PBS containing 1.0% BSA and 0.1% azide, resuspended in 200 μl, and immediately analyzed on an EPICS XL-MCL flow cytometer. Fluorescent measurements were collected on 10000 viable cells and analyzed by System II software (Coulter, Miami, FL).

Stimulation assay

Lymph nodes were isolated from SEA-injected or naive mice and processed under sterile conditions as described above. $CD4^+$ LNC were purified using Cellect $CD4^+$ T cell columns according to manufacturers recommendation (Biotex Laboratories, Edmonton, Alberta, Canada). T cell populations were cultured in DMEM supplemented with 10% FBS, sodium bicarbonate, gentamicin, 2-mercaptoethanol, sodium pyruvate and nonessential amino acids (DMEM-10). $CD4^+$ LNC cells were titrated in triplicate into a 96-well flat-bottomed plate (Corning) containing 5×10^5 T cell-depleted, irradiated (3000 rads)

splenocytes/well. SEA was added to each well to give a final concentration of 1 μg/ml. Cells were cultured at 37° C, 5% CO_2 for 72 hr after which 1μCi of ^{3}H-thymidine (ICN Pharmaceuticals, Costa Mesa, CA) was added to each well. After an additional 8 hour incubation, cells were harvested onto glass fiber filters using a Skatron cell harvester (Skatron, Sterling, VA). ^{3}H-Thymidine incorporation was determined by scintillation counting using a model 1600 TR Packard liquid scintillation analyzer (Packard Instruments, Meridian, CT).

CTL assay and cytotoxic alloantibody response

^{51}Cr-labelled P815 tumor cells were used as targets to determine CTL activity and cytotoxic alloantibody titers as previously described (Kerkvliet et al., 1996, Prell and Kerkvliet, 1997).

RESULTS

In vivo administration of IL-2, but not IFN-γ, dose dependently increases CTL activity in TCDD-treated mice.

We recently showed that IL-2 and IFN-γ production was suppressed on days 7-9 and 6-10 in spleen cell cultures from TCDD-treated mice (Kerkvliet et al., 1996). To test the hypothesis that the TCDD-induced immunosuppressive effects might be a result of decreased production of IL-2 or IFN-γ, we administered either IL-2 or IFN-γ to TCDD-treated mice during the time their production was inhibited.

On the day of sacrifice (day 10), gross pathology of the peritoneal cavity, spleen weight and spleen cell recovery were recorded as an indication of the in vivo efficacy of cytokine administration. Vehicle-treated mice had cleared most of the tumor cells from the peritoneal cavity by day 10 as previously observed. In contrast, the tumor progressively invaded the peritoneum of TCDD-treated mice. Injection of IFN-γ did not appear to affect

the gross pathology whereas administration of IL-2 reduced the size of the tumor mass in the peritoneal cavity of TCDD-treated mice although not to the extent of that observed in vehicle-treated animals. Spleen weight was also significantly suppressed in TCDD-treated mice injected with PBS when compared to vehicle-treated control mice (Table IV-1). Interestingly, spleen weights were not significantly altered by IL-2 injection. However, administration of the highest dose of IL-2 (2500 U/injection) significantly increased total spleen cell recovery (Table IV-1). Similarly, injection of IFN-γ did not significantly alter spleen weight or cellularity in TCDD-treated mice except at the highest dose of IFN-γ (1000 ng/injection), indicating that IFN-γ had an in vivo effect.

As shown in figure IV-1, exposure to TCDD significantly suppressed CTL activity following injection of P815 tumor cells and administration of IL-2 dose-dependently increased CTL activity in TCDD-treated mice. Flow cytometric analysis of spleen cells showed that IL-2 dose-dependently increased the generation of CTL_E in TCDD-treated mice, as defined by the phenotype of $CD8^+CD44^{high}CD62L^{low}$ (figure IV-1B). In contrast, IFN-γ did not affect CTL activity in TCDD-treated mice (figure IV-2). In fact, the CTL activity was equally suppressed in TCDD-treated mice which received 1000 ng IFN-γ/day when compared to TCDD-exposed mice injected with PBS.

Influence of IL-2 on the generation of CTL_E in vehicle-treated mice

Because IL-2 dose dependently increased CTL_E development in TCDD-treated mice, we were interested in seeing if vehicle-treated mice also respond to exogenous IL-2. Therefore, we administered 2500 U IL-2 to on days 7-9 relative to P815 injection. As shown in Table IV-2, injection of IL-2 significantly increased CTL activity as well as the frequency and total number of CTL_E in vehicle-treated animals.

In order to determine if naive CTL_P could be driven to mature CTL_E during the 3 days of treatment with IL-2 we administered 2500 U IL-2 to mice on days 1-3, relative to

P815 challenge. Interestingly, no detectable cytotoxic activity was measured on the fourth day even at a effector to target ratio of 200:1 (Table IV-2). These data suggest that the increased CTL activity in IL-2-treated mice is most likely due to the increased expansion of partially activated CTL_P. Furthermore, these data indicate that the cytotoxic activity is not a function of non-specific cytotoxicity since administration of IL-2 for three consecutive days immediately after P815 injection did not induce any detectable lytic activity on the fourth day.

In vivo IL-2 administration increases CTL activity in CTLA4-Ig-treated mice

Administration of the CD28 receptor antagonist CTLA4-Ig induces in vivo T cell tolerance by blocking B7/CD28 interactions. Since TCDD-induced immune suppression has also been associated with altered B7/CD28-mediated costimulation, we wanted to determine if IL-2 administration could augment CTL activity in CTLA4-Ig-treated mice. As shown in figure IV-3A, the immunosuppressive effect of CTLA4-Ig treatment was readily apparent by the death of 5 out of 6 mice prior to day 10. In contrast, all 6 of the CTLA4-Ig-treated mice that received IL-2 survived to day 10 (figure IV-3A). In addition, IL-2 treatment resulted in a significant induction of CTL activity toward P815 tumor cells, whereas the one surviving CTLA4-Ig-treated mouse had no detectible CTL activity (figure IV-3B).

Exposure to TCDD does not alter SEA-induced expansion or deletion of $V\beta3^{\pm}$ T cells

Injection of the bacterial superantigen SEA into mice causes the clonal expansion followed by the peripheral deletion of T cells containing the Vβ3 chain of the TCR. In order to specifically address deletion as a mechanism of TCDD-induced immune

Table IV-I. Effects of in vivo cytokine treatment on spleen weight and cellularity.

Experiment 1			TCDD + IFN-γ (ng)		
	Vehicle/PBS	TCDD/PBS	10	100	1000
Spleen Wt (mg)	139.4±3.6[c]	115.5±6.6[b]	126.5±4.5	126.3±3.7	139.1±9.3[c]
No. SC (x 10^{-7})	9.05±0.4[c]	7.95±0.5[b]	8.77±0.5	8.67±0.4	9.90±0.6[c]
Experiment 2			TCDD + IL-2 (units)		
	Vehicle/PBS	TCDD/PBS	50	500	2500
Spleen Wt (mg)	155.7±4.2[c]	118.2±9.0[b]	120.3±5.8[b]	115.3±3.7[b]	115.7±10.6[b]
No. SC (x 10^{-7})	10.1±0.5	7.6±0.4[b]	8.3±0.5[b]	8.6±0.5	9.1±0.5[c]

[a] Vehicle- and TCDD-treated mice were injected with $1x10^7$ P815 tumor cells. TCDD-treated mice were subsequently injected i.p. with IFN-γ or IL-2 as described in Materials and Methods. Data represent mean ± SEM of 5-6 mice per group.
[b] Significantly different from Vehicle/PBS, $p<0.05$
[c] Significantly different from TCDD/PBS, $p<0.05$

Figure IV-1. Administration of exogenous IL-2 dose dependently increases CTL activity and CTL_E phenotype ($CD8^+CD44^{high}CD62L^{low}$) in TCDD-treated mice.

C57Bl/6 mice were gavaged with a single oral dose of vehicle or 7.5 μg/kg TCDD 1 day prior to the i.p.injection of $1x10^7$ P815 tumor cells. TCDD-treated mice were injected i.p. two times per day with 50, 500, or 2500 U of recombinant murine IL-2 (rmIL-2) on days 7-9 relative to P815 injection. On day 10, A) CTL activity was measured in a standard 4-hr ^{51}Cr release assay as previously described (DeKrey and Kerkvliet, 1995). B) Spleen cells were stained and analyzed for the CTL_E phenotype ($CD8^+CD44^{high}CD62L^{low}$) as described in the Materials and Methods.

Figure IV-2. Administration of IFN-γ does not affect CTL activity in TCDD-treated mice.

C57Bl/6 mice were treated with a single oral dose of vehicle or 3.75 μg/kg TCDD 1 day prior to the injection of P815 tumor cells. TCDD-treated mice were injected i.p.with 10, 100, 1000 ng of recombinant IFN-γ on days 6-9 relative to P815 injection. Vehicle-treated and one group of TCDD-treated mice were injected with 0.1% BSA in PBS as a carrier control. On day 10, CTL activity was measured in a standard 4-hr ^{51}Cr release assay as previously described (DeKrey and Kerkvliet, 1995).

Figure IV-1.

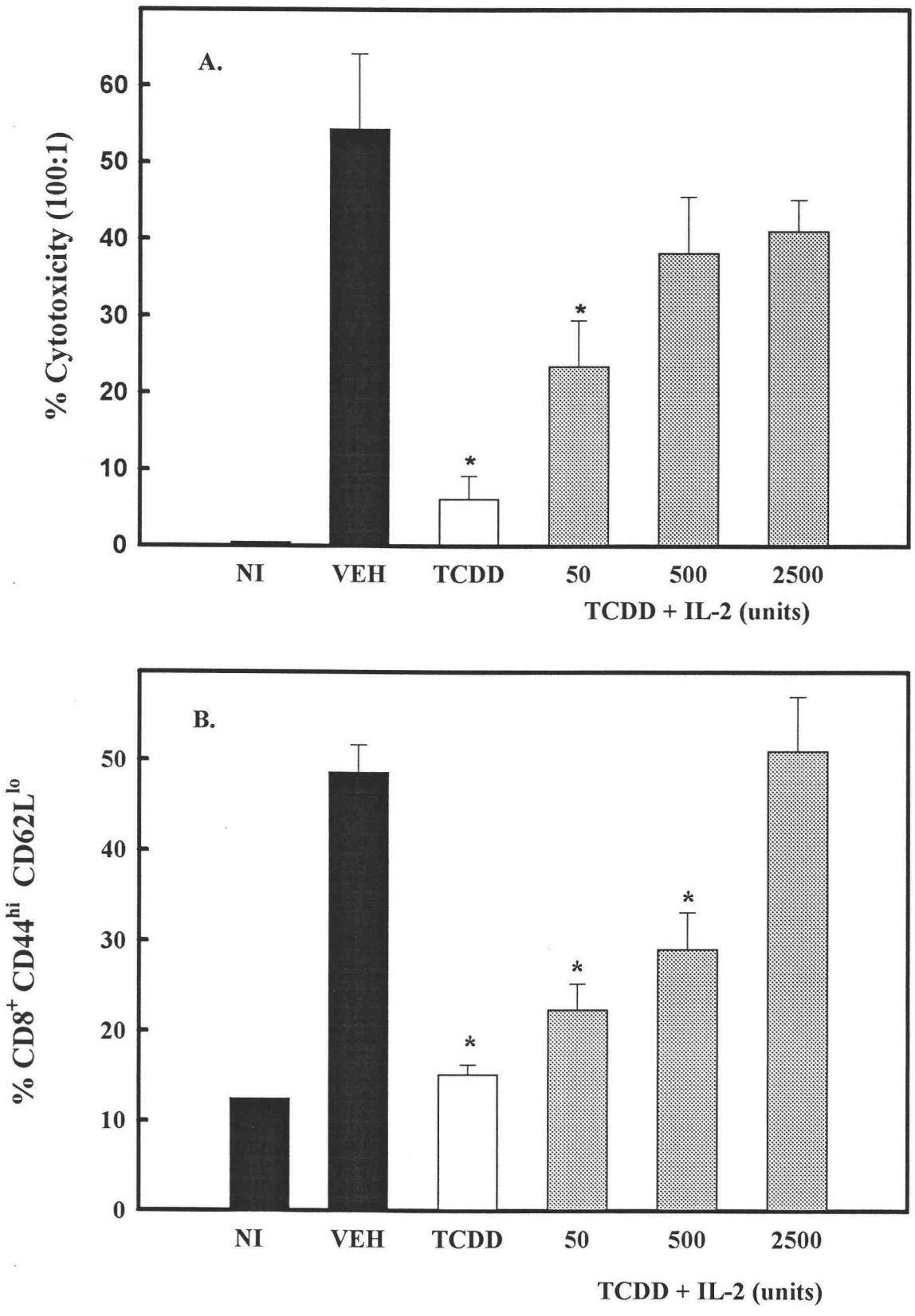

A.
% Cytotoxicity (100:1)
0
10
20
30
40
50
60
*
*
NI
VEH
TCDD
50
500
2500
TCDD + IL-2 (units)
B.
% CD8+ CD44hi CD62Llo
0
10
20
30
40
50
*
*
*
NI
VEH
TCDD
50
500
2500
TCDD + IL-2 (units)

Figure IV-2.

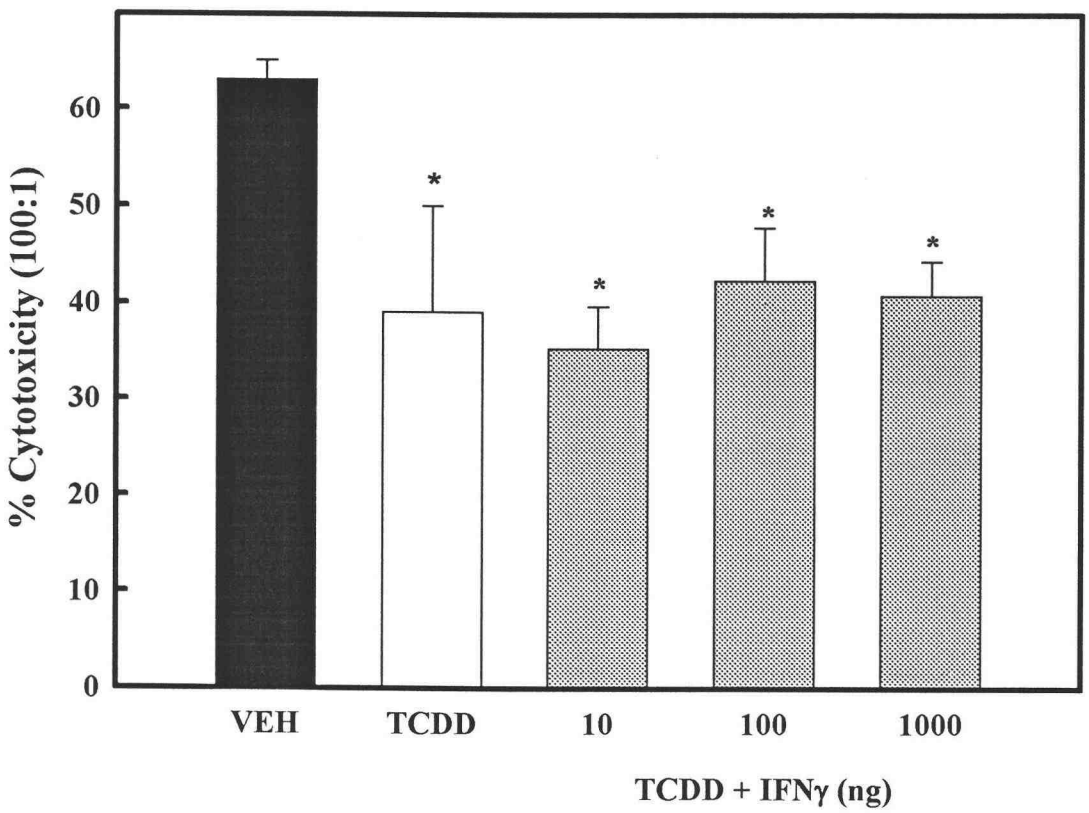

% Cytotoxicity (100:1)
60
50
40
30
20
10
0
*
*
*
*
VEH
TCDD
10
100
1000
TCDD + IFNγ (ng)

Table IV-2. Influence of exogenous IL-2 on the cytotoxic T cell response to allogeneic P815 tumor cells.

	Vehicle/PBS[a] (days 7-9)	Vehicle/IL-2[a] (days 7-9)	Vehicle/IL-2[b] (days 1-3)
% Specific Lysis (25:1)	57.8±3.2	93.5±7.8	0
% CTL_E	62.2±2.1	88.2±0.8	ND
No. of CTL_E (x 10^{-7})	21.59±2.1	109.3±14.2	ND

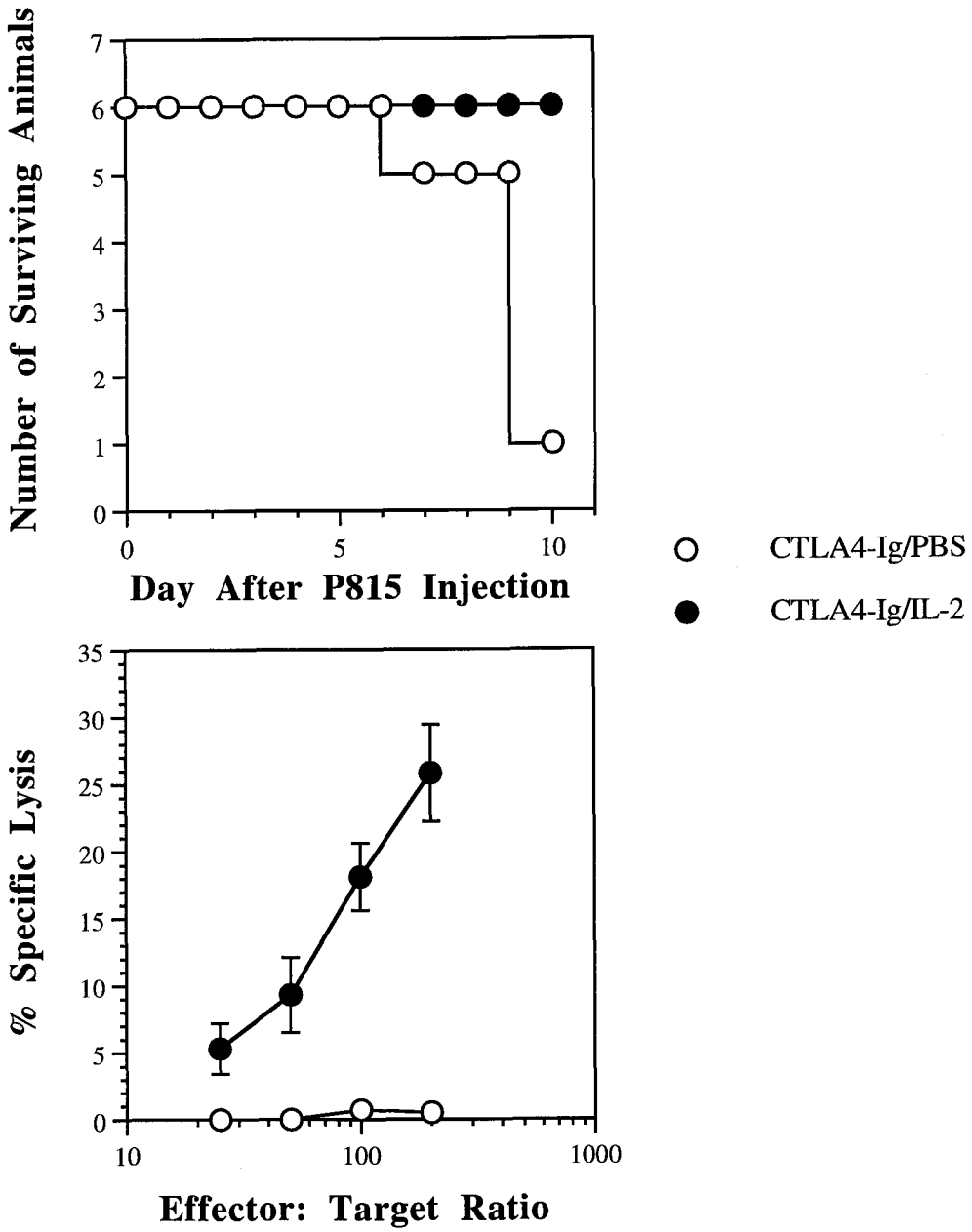
[a] Mice were injected i.p. two times per day with PBS or IL-2 (2500 U/injection) on days 7-9 and CTL activity was measured on day 10 relative to P815 injection.

[b] Mice were injected two times per day with IL-2 (500 U/injection) on days 1-3 and CTL activity was measured on day 4 relative to P815 injection.

Figure IV-3. Effects of IL-2 administration on CTLA4-Ig-induced immune suppression.

C57Bl/6 mice were treated with 200 μg of CTLA4-Ig on days 0, 1, and 2 relative to P815 injection. Mice then received i.p. injections two times per day of 2500 U rmIL-2 on days 7, 8, and 9 after P815 injection. A) Overall survival of CTLA4-Ig-treated mice injected with IL-2 or PBS. B) Day 10 splenic CTL activity in the surviving mice (n=1 for CTLA4-Ig + PBS; n=6 for CTLA4-Ig + IL-2).

Figure IV-3.


Number of Surviving Animals
7
6
5
4
3
2
1
0
0
5
10
Day After P815 Injection
CTLA4-Ig/PBS
CTLA4-Ig/IL-2
% Specific Lysis
35
30
25
20
15
10
5
0
10
100
1000
Effector: Target Ratio

suppression, we tracked the fate of antigen-specific $CD4^+$ and $CD8^+$ T cells in TCDD-treated B10BR mice following the injection of SEA. As shown in figure IV-4, injection of SEA induced a 3-5 fold increase in the frequency and total number of spleen and LNC expressing $V\beta3^+$ T cell receptor. Peak expansion occurred by day 2 and was followed by a rapid deletion of the $V\beta3^+$ T cells by day 5. Both the frequency and total number of $V\beta3^+$ T cells remained low after SEA injection. Exposure to TCDD one day prior to SEA injection did not affect the expansion or subsequent deletion of $V\beta3^+$ T cells with the exception of one point. In this case, TCDD treatment increased the percentage and total number of $CD8^+V\beta3^+$ spleen cells 3 days after SEA injection. However, 5 days after SEA injection there was no difference between vehicle- or TCDD-treated mice in either the frequency or total number of $CD8^+V\beta3^+$ spleen cells. Consistent with a previous report, the remaining $V\beta3^+$ T cells on day 9 after SEA injection were as responsive as cells from naive mice (Vella et al., 1995). In addition, cells from TCDD-treated mice were equally responsive to SEA in vitro.

Since TCDD did not affect the SEA-induced response, it was of interest to determine if B10BR/SgSnJ mice were sensitive to TCDD-induced immunotoxicity. Therefore, we challenged vehicle- and TCDD-treated B10BR/SgSnJ mice with allogeneic P815 tumor cells. As shown in figure IV-6, treatment of B10BR/SgSnJ mice with the same dose of TCDD used in the SEA study significantly suppressed CTL activity and the generation of CTL_E 10 days after P815 injection. These data demonstrate that B10BR/SgSnJ mice are extremely sensitive to TCDD-induced immunotoxicity and suggest that the lack of TCDD-induced alterations in the SEA model was not due to the strain of mouse tested.

DISCUSSION

Studies in laboratory animals indicate that the immune system is one of the most sensitive targets of TCDD-induced toxicity. In mice, exposure to very low doses of TCDD results in decreased resistance to bacterial, viral and parasitic infections as well as suppression of specific effector functions associated with T helper cells, cytotoxic T cells, and B cells (Kerkvliet and Burleson, 1994). Most if not all of the immunotoxic effects of TCDD are thought to be mediated via binding to the AhR, a ligand activated transcription factor, that binds to consensus DNA sequences known as dioxin responsive elements (DREs) (Hankinson, 1995). Recently, several cytokine genes have been identified as containing putative DRE sequences (Lai et al., 1996). Therefore, alterations in the transcription of such genes may play a role in many of the immunotoxic effects of TCDD (Döhr et al., 1994; Lai et al., 1996).

We have previously reported that exposure to TCDD suppressed CTL activity and the generation of CTL_E against allogeneic P815 tumor cells (Kerkvliet et al., 1990b; De Krey and Kerkvliet, 1995; Kerkvliet et al., 1996). TCDD-induced suppression of the CTL response was associated with a profound decrease in the production of TC-1 cytokines (IL-2, IFN-γ and TNF-α), while the production of other cytokines (IL-4, IL-6, IL-1β) was not affected. Furthermore, the expression of the critical costimulatory molecule CD86 was significantly decreased on B cells and macrophages in P815-challenged mice exposed to TCDD (Prell and Kerkvliet, 1997). The potential significance of the decreased CD86 expression was demonstrated by the ability of CD86-transfected P815 tumor cells to prevent the suppressed CTL activity in TCDD-treated mice.

Figure IV-4. Exposure to TCDD does not affect SEA-induced expansion and deletion of Vβ3⁺ T cells.

B10BR/SgSnJ mice received a single oral dose of vehicle (squares) or 15 μg/kg TCDD (diamonds) one day prior to the i.p. injection of 0.15 μg SEA. At day 2, 3, 5, and 9 after SEA injection, the frequency and total number of $CD4^+$ (A-D) and $CD8^+$ (E-H) spleen and LN T cells expression of Vβ3 were identified as described in Materials and Methods. Each data point represents mean ± sem from 6 mice per group with the exception of day 3, TCDD-treated mice, where n=5.

Figure IV-5. T cells from TCDD-treated mice respond to SEA in vitro.

$CD4^+$ T cells were purified from the LN of vehicle- or TCDD-treated mice that were injected with PBS or 0.15 μg SEA. T cells were isolated 9 days after SEA injection and titrated in triplicate into tissue culture wells containing irradiated, T cell-depleted spleen cells. SEA was added to each well to give a final concentration of 1 μg/ml. 3H-thymidine was added 3 days later and incubated for an additional 8 hours. Cultures were harvested and ^{3}H-Thymidine incorporation was determined by liquid scintillation. The data show the mean cpm ± sem from the triplicate samples.

Figure IV-6. Exposure to TCDD suppresses the alloimmune response in B10BR/SgSnJ mice.

B10BR/SgSnJ mice were gavaged with a single oral dose of vehicle or 15 μg/kg TCDD 1 day prior to P815 injection. On day 10, A) CTL activity was measured in a standard 4-hr ^{51}Cr release assay as previously described (DeKrey and Kerkvliet, 1995). B) Spleen cells were stained and analyzed for the CTL_E phenotype ($CD8^+CD44^{high}CD62L^{low}$) as described in the Materials and Methods.

Figure IV-4.

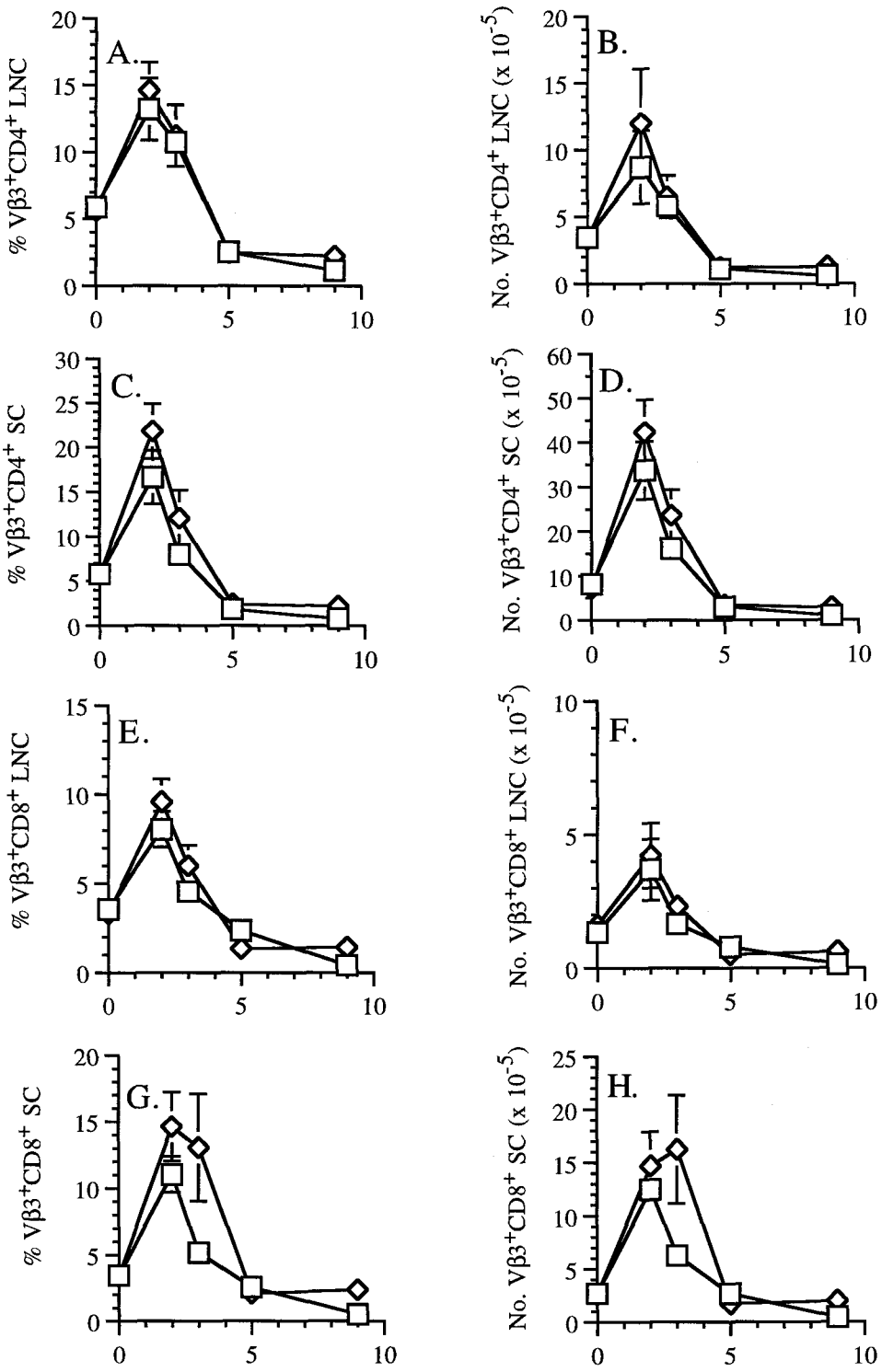

A.
B.
C.
D.
E.
F.
G.
H.
% Vβ3+CD4+ LNC
No. Vβ3+CD4+ LNC (x 10-5)
% Vβ3+CD4+ SC
No. Vβ3+CD4+ SC (x 10-5)
% Vβ3+CD8+ LNC
No. Vβ3+CD8+ LNC (x 10-5)
% Vβ3+CD8+ SC
No. Vβ3+CD8+ SC (x 10-5)
Day after SEA injection

Figure IV-5.

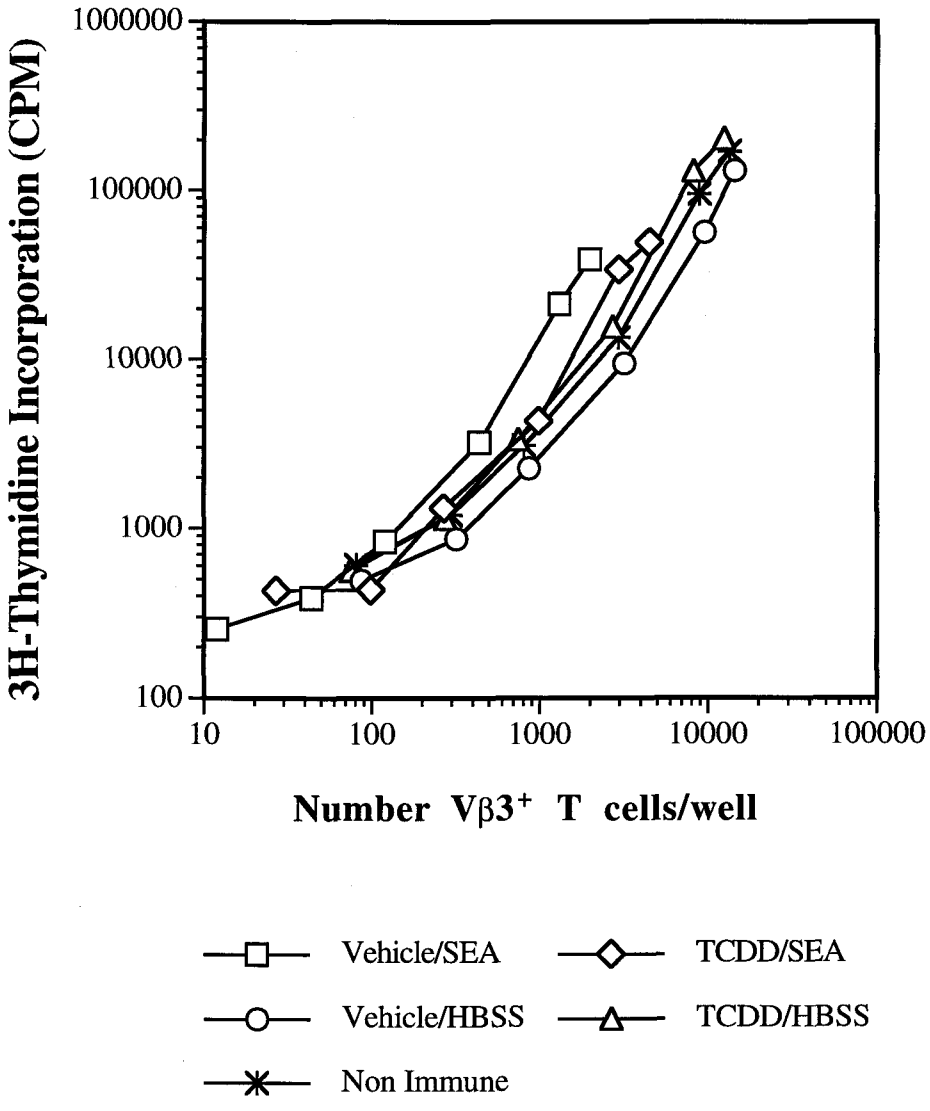
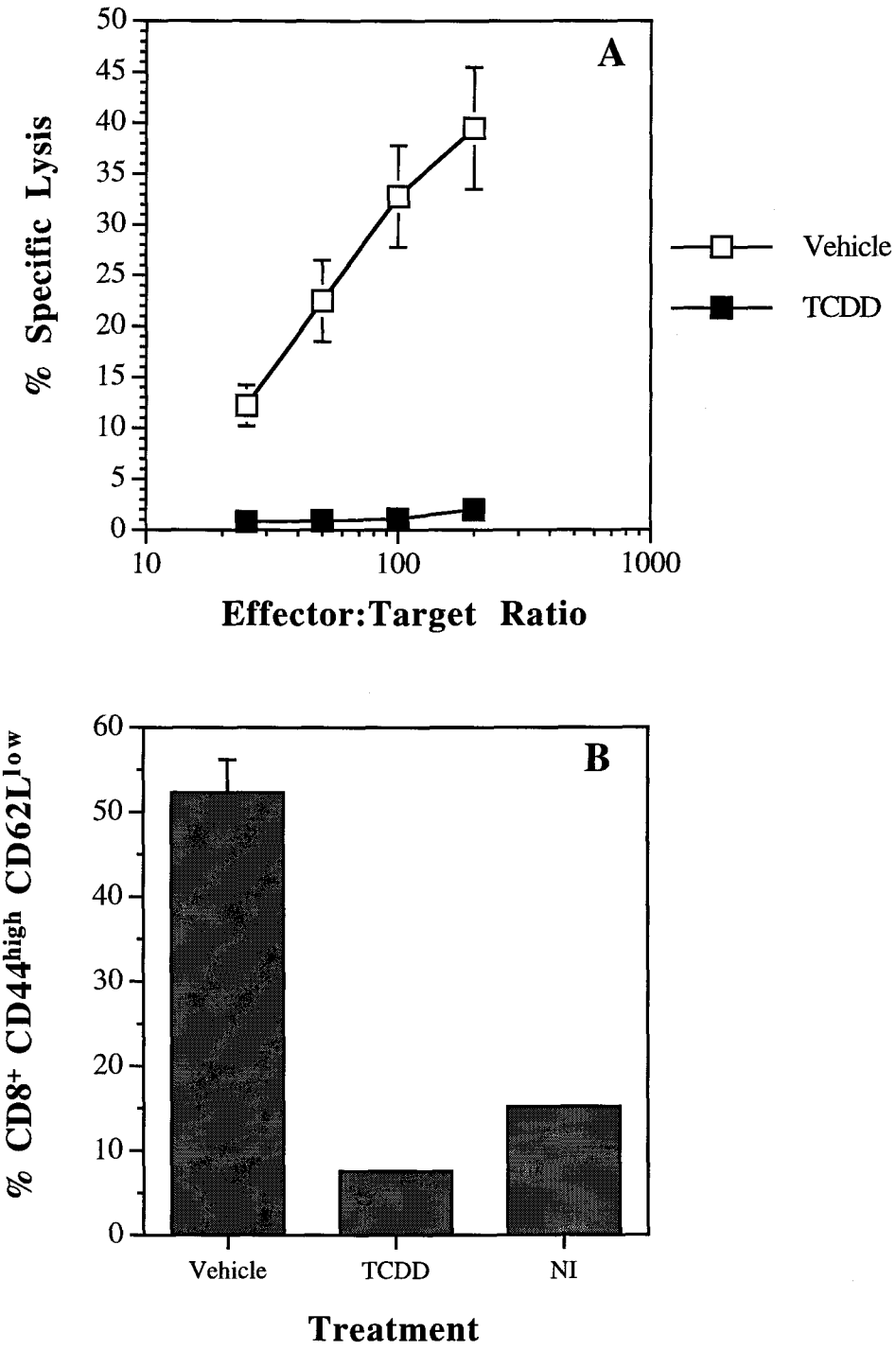

3H-Thymidine Incorporation (CPM)
1000000
100000
10000
1000
100
10
100
1000
10000
100000
Number Vβ3+ T cells/well
Vehicle/SEA
TCDD/SEA
Vehicle/HBSS
TCDD/HBSS
Non Immune

Figure IV-6.


A
% Specific Lysis
50
45
40
35
30
25
20
15
10
5
0
10
100
1000
Effector:Target Ratio
Vehicle
TCDD
B
% CD8+ CD44high CD62Llow
60
50
40
30
20
10
0
Vehicle
TCDD
NI
Treatment

In this report we show that administration of exogenous IL-2 dose dependently increased CTL activity and the generation of CTL_E in TCDD-treated mice. At 2500U/injection, the frequency of CTL_E was restored to the frequency seen in vehicle-treated mice. Interestingly, administration of IL-2 also increased the CTL response in mice treated with the potent immunosuppressive agent CTLA4-Ig. However, IL-2 treatment was less effective in CTLA4-Ig-treated mice as compared to TCDD-treated mice. This most likely reflects differences in the degree of inhibition of B7/CD28 interactions between the two immunosuppressive compounds. CTLA4-Ig completely blocks all B7/CD28 interactions whereas TCDD only partially inhibits the induction of CD86 on APCs (Prell and Kerkvliet, 1997). These results suggest that exposure to TCDD, like CTLA4-Ig, may induce peripheral tolerance by interfering B7/CD28-mediated costimulation thereby inhibiting IL-2 production (Judge et al., 1996; Onodera et al., 1997). This interpretation is also consistent with the reported ability of IL-2 to overcome T cell anergy in vitro (Jenkins et al., 1987; Essery et al., 1988).

To specifically address whether TCDD alters peripheral T cell deletion we utilized the SEA model to follow the expansion and deletion of antigen-specific T cells. We were surprised to find that exposure to TCDD one day prior to SEA injection did not inhibit clonal expansion of $V\beta3^+$ T cells since SEA-induce expansion has been shown to be dependent on the B7/CD28 costimulatory pathway (Vella et al., 1997). These results suggest that in contrast to the allogeneic tumor model, TCDD may not interfere with the B7/CD28 pathway following injection of SEA. Alternatively, this discrepancy may reflect the different requirements of B7-mediated costimulation in the respective models. Consistent with this idea, the strength of the antigen-specific signal through the TCR has been suggested to determine the level of costimulation required for complete T cell activation with low TCR occupancy being more dependent on costimulation than high TCR occupancy (Lenschow et al., 1996). Injection of SEA, which delivers a strong TCR signal to both $CD4^+$ and $CD8^+$ T cells bearing Vβ3 may require only a minimum of costimulation

that can be achieved by constitutively expressed levels of B7-2 on APCs (reviewed in Huber et al., 1996). In contrast, T cell activation following injection of P815 tumor cells may deliver a weaker TCR-mediated signal. Thus, the amount costimulation required for T cell activation would depend on higher levels of B7 expression, which TCDD has been shown to inhibit (Prell and Kerkvliet, 1997).

Similarly, exposure to TCDD did not affect the peripheral deletion of Vβ3$^+$ T cells following injection of SEA. Since peripheral deletion of antigen specific T cells following injection of superantigen is thought to occur primarily through the induction of apoptosis, our results indicate that exposure to TCDD does not affect SEA-induced apoptosis. However, this is in contrast to data previously reported by Prell et al (1995) in which exposure to TCDD enhanced anti-CD3 induced peripheral deletion of CD4$^+$ T cells. Flow cytometric analysis suggested that peripheral cell death occurred through an apoptotic mechanism (Prell et al., 1995). Furthermore, exposure to TCDD has been shown to induce apoptosis in thymocytes possibly through the activation of a Ca^{2+}-dependent endonuclease (McConkey et al., 1988) or by a FAS-dependent mechanism (Rhile et al., 1996). One possible explanation is that B10BR/SgSnJ mice used in the SEA study are not sensitive to TCDD-induced toxicities. However, this is unlikely, since exposure to TCDD significantly suppressed the CTL response to P815 tumor cells.

An alternative explanation for the lack of effects of TCDD is that SEA-induced peripheral deletion may not allow the detection of increased deletion of antigen-specific Vβ3$^+$ T cells. As we have reported here and others have reported elsewhere, approximately 5% of the CD4$^+$ and CD8$^+$ LNC contain the Vβ3 chain of the TCR in naive B10BR/SgSnJ mice (Vella et al., 1995; Vella et al., 1997). Upon activation with SEA, these cells expand approximately 3-4 fold within the first 3 days. These same antigen-specific T cells are subsequently deleted from the peripheral lymphoid tissue such that the remaining population of Vβ3$^+$ T cells represents approximately 1% of CD4$^+$ and CD8$^+$ T cells. Therefore, it may be difficult to detect any significant increase in peripheral deletion.

In summary, we have shown that administration of exogenous IL-2, but not IFN-γ, dose dependently increased CTL activity in TCDD-treated mice which was associated with a profound expansion of $CD8^+$ T cells expressing the CTL_E phenotype. Similarly, administration of IL-2 partially reversed the immunosuppressive effects of CTLA4-Ig. These results suggest that the TCDD may induce T cell tolerance by inhibiting IL-2 production. These results also suggested that the CTL_P in TCDD-treated mice were not deleted since injection of IL-2 caused clonal outgrowth into a CTL_E population. The lack of deletion is consistent with the apparent lack of effects of TCDD on the expansion or peripheral deletion of Vβ3 bearing T cells following SEA injection. Taken together, these results suggest that the immunosuppressive mechanisms of TCDD and CTLA4-Ig may share certain characteristics such as inhibiting IL-2 production. However, in contrast to CTLA4-Ig, exposure to TCDD did not influence SEA-induced expansion of $V\beta3^+$ T cells and therefore may represent a novel mechanism of immune suppression or peripheral tolerance.

CHAPTER V

CONCLUSIONS

The findings in these studies suggest that TCDD-induced alterations of T cell functions are a consequence of inappropriate signals delivered by APCs. This conclusion is based on the following lines of evidence: 1) Exposure to TCDD did not significantly alter plasma cytokine levels following anti-CD3 injection with the exception of a small decrease in IFN-γ at 24 hours and a small increase in IL-6 at 48 hours. 2) Exposure to TCDD did not significantly alter SEA-induced clonal expansion or peripheral deletion of Vβ3$^+$ T cells with one exception. A small increase in the expansion of splenic CD8$^+$Vβ3$^+$ T cells was observed 3 days after SEA injection in TCDD-treated mice. 3) The remaining Vβ3$^+$ T cells from TCDD-treated mice proliferated when restimulated with SEA in vitro, suggesting that these cells were not rendered anergic. 4) The TCDD-induced suppression of the alloimmune response to P815 tumor cells was associated with a significant decrease in the expression of the CD86 on B cells and macrophages. The importance of the decreased CD86 expression was demonstrated by the fact that providing exogenous CD86 prevented the TCDD-induced immune suppression independent of APCs by directly activating CD8$^+$ CTL_P. 5) Providing exogenous IL-2 to TCDD-treated mice within the timeframe that IL-2 production is inhibited, dose dependently increase CTL activity and the generation of CTL_E. Taken together, these results suggest that exposure to TCDD has relatively few effects on T cell function following activation with antigens that directly bind to external components of the TCR in an unprocessed form. In addition, these results suggest that T cells in TCDD-treated mice are functional if they receive sufficient costimulation via CD86 or receive the critical growth factor IL-2. Moreover, since exposure to TCDD primarily altered T cell activation and effector functions that are highly dependent on APC, these

results indicate that an APC, rather than the T cells, may be the primary target or TCDD. Further experiments will be required to determine whether APCs, such as dendritic cells and/or macrophages are directly affected by TCDD exposure.

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