Immunotoxicity of TCDD: Role of Fas Expression and MHC Phenotype on TCDD-Mediated Thymic Atrophy and Decrease in Peripheral T Cell Responsiveness

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IMMUNOTOXICITY OF TCDD: ROLE OF FAS EXPRESSION AND MHC PHENOTYPE ON TCDD-MEDIATED THYMIC ATROPHY AND DECREASE IN PERIPHERAL T CELL RESPONSIVENESS

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(ABSTRACT)

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is well known for its immunotoxic effects particularly on the thymus as well as on T and B lymphocyte functions. Previous studies have suggested that TCDD may induce apoptosis in thymocytes although its demonstration *in vivo* has met with limited success. TCDD has also been shown to alter the major histocompatiblity complex (MHC)-encoded molecules, however, its role in immunotoxicity is not clear. In this study, we investigated the role of Fas (CD95), an important molecule involved in the induction of apoptosis, on TCDD-mediated immunotoxicity using mice bearing homozygous *lpr* mutation which leads to failure of expression of Fas. When TCDD was administered orally at 0, 0.1, 1.0, or 5.0 μg/kg body weight for 11 days, it was found to be less toxic to the thymocytes from C57BL/6 *lpr/lpr* mice (Ah-responsive, Fas^-) when compared to C57BL/6 +/+ mice (Ah-responsive, Fas^+). Similar results were obtained when peripheral T cell responsiveness to antigenic challenge with conalbumin was studied in these mice. When mice that differed only at the MHC were compared for immunotoxic effects of TCDD, it was noted that B10.D2 (Ah-responsive, $H\text{-}2^d$) were more sensitive to

TCDD-mediated thymic atrophy and peripheral T cell dysfunction when compared to B10 mice (Ah-responsive, H-2^b). In all TCDD-sensitive strains tested, the thymic atrophy was accompanied by a uniform depletion of all four subsets of T cells ($CD4^+$, $CD4^+CD8^+$, $CD4^-CD8^-$, and $CD8^+$). Furthermore, in these strains, TCDD suppressed the antigen-specific peripheral T cell responsiveness but not the responsiveness of naive resting T cells to polyclonal mitogens. Lastly, using cell-mixing experiments, it was demonstrated that TCDD directly affected the T cells responding to conalbumin but not the antigen presenting cells (APCs). Together our studies demonstrate that although Ah locus plays the primary role determining the toxicity of TCDD on the T cells, there are secondary factors such as expression of Fas or the MHC-phenotype which may play an important role in TCDD-mediated immunotoxicity. The role of Fas further suggests that TCDD may induce toxicity in T cells by triggering apotosis.

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1.0 General Introduction and Review of Literature

1.1 The Immune System:

The origins of immunology begin with the phenomenon of immunity in ancient peoples. Thucydides, author of The Peloponnesian War, noticed that people who had a disease and recovered from it did not get the disease again (Golub, 1991). While it is not known when immunization to prevent disease began, it has been a practice of African tribes to place pulverized poison glands from venomous snakes under the skin to produce resistance to snake bites. Similarly, Mithridates VI, King of Pontus from 120 to 63 B.C., was known to consume small quantities of poison daily to protect himself from an assassin's poison (Bibel, 1988). As early as 1000 A.D., the Chinese were inoculating healthy people with material from the pustule of a person infected with smallpox (Golub, 1991).

Edward Jenner's introduction of immunization against smallpox in 1798 was the beginning of an era of immunology that places Louis Pasteur as the father of both immunology and microbiology. Pasteur discovered the use of

attenuated strains of cholera bacillus in chickens as well as vaccines against anthrax bacillus and the rabies virus. The work that followed in French and German schools of immunologists headed by Pasteur and Robert Koch, respectively, led to the establishment of the germ theory of disease and the isolation of many disease causing agents (Golub, 1991).

While the work of Paul Ehrlich focused on immunochemistry and, thus, humoral immunity, the concept of cellular immunity was first observed by Elie Metchnikoff. Metchnikoff, a Russian scientist, observed the phagocytosis of a microbe and proposed this mechanism as the basis for the immune response. Later, Almroth Wright showed that the immune system used both humoral and cellular elements in combating microorganisms (Clark, 1980).

The immune system is the third and most specific line of defense of the body to protect against foreign substances. The first line of defense is simply the physical barrier provided by the skin, cilia, and epithelial linings. The second line involves the mechanisms of lysozymes, inflammatory reactions, and other nonspecific methods of clearing microbes. The immune system is a complex collection of organs, tissues, and cells that are capable of a highly specific and adaptive response (Hildemann, 1984). The primary organs of the immune system are the bone marrow and thymus while the spleen and lymph nodes play important secondary roles.

The bone marrow is of major importance to the body since it is the site of ultimate origin for both lymphoid and erythroid cells. Bone marrow is found

in almost all bones of the body but is most abundant in the long bones. It is here that pluripotant stem cells arise that can later differentiate into a number of different types of cells. Stem cells undergo a series of maturations and differentiations to form three major groups of cells known as granulocytes, monocytes, and lymphocytes (Clark, 1980).

The thymus is a bi-lobed organ that is subdivided into smaller compartments. The cortex or periphery is the region of rapidly dividing lymphocytes The cells of the medulla are primarily epithelial cells though some lymphocytes are present. The thymus is large at birth and continues enlargement until puberty at which time it begins to atrophy. With age, the thymus is filled with fatty and fibrous tissue though it remains functional throughout life. The main function of the thymus involves the maturation of lymphocytes into mature, functional T lymphocytes or T cells.

Both T and B cells express a number of different surface glycoproteins and receptors during the maturation process and as mature cells. These markers are referred to as clusters of differentiation (CD) and are used to characterize cells. During the course of maturation some of these markers are lost while others are gained. The CD8 marker is expressed early in T cell maturation followed by the co-expression of CD4 and CD8 as well as CD3 and T-cell receptor (TCR). This is followed by the loss of CD4 or CD8 and results in the commitment of the cell to the role of either a helper T cell ($CD4^+CD8^-$) or a cytotoxic T cell ($CD4^-CD8^+$). The CD3 molecule is retained throughout the lifetime of the T cell as is the TCR (Golub, 1991).

In the thymus, T cells are selected both positively and negatively based on their responsiveness to self-antigens. Of the cells entering the thymus, only a small portion will leave as mature T cells. This is due to the removal of T cells that recognize self-antigens. These auto-reactive T cells are activated to undergo programmed cell death or apoptosis. Also, those T cells that fail to recognize self MHC are removed during the positive selection process. It is in this manner that T cells are 'educated' to discriminate between self and non-self. It is possible, however, that some T cells may escape these selection processes and are thus able to mount autoimmune responses in the body (Golub, 1991).

The spleen plays an important role in the body although it is not indispensable. The spleen functions as a blood filterer in the removal of dead and damaged red blood cells (RBCs) and foreign particles from the bloodstream. Large numbers of phagocytic cells reside in the spleen and enable it to perform these functions. Another role fulfilled by the spleen is to allow the interaction of T and B cells with macrophages and antigen to elicit immune responses.

The lymph nodes are also important secondary organs in the immune system. Lymph nodes are small, bean-shaped structures located throughout the body and are involved in trapping foreign antigens and in promoting lymphocyte-antigen interaction. The cortex of the lymph node is populated primarily with B cells while the paracortex contains mostly T cells. The medulla is the less populated central region containing both B and T cells. Antigen is trapped in

the fibrous tissue of the lymph node thereby facilitating interaction with lymphocytes in the surrounding area (Clark, 1980).

The granulocytes include neutrophils, eosinophils, and basophils. Neutrophils are primarily phagocytic cells and contain degradative enzymes in their granules. Eosinophils are also phagocytic although their primary role is in the release of substances to counter the effects of histamines and thus reduce inflammation. Basophils possess granules that contain the pharmacologically active substances serotonin, heparin, and histamine (Roitt, 1985).

The monocytes perform two main functions. The first is as phagocytic cells and the second is as antigen presenting cells (APCs). Monocytes are found in the blood and various organs and migrate into tissues such as the lung, brain, spleen, and kidney to become macrophages. Monocytes and macrophages possess lysosomes that contain hydrolases and peroxidases used in the killing of microorganisms. They have strong adherent properties aided by the expression of complement and Fc receptors. Macrophages also produce important monokines such as interleukin-1 (IL-1), prostaglandins, tumor necrosis factor (TNF), interferon, and colony stimulating factor (CSF). These monokines are involved in stimulating other cells of the immune system and also play a role in inflammation processes. APCs have high class II Major Histocompatibility Complex (MHC) molecule expression that is necessary in presentation of antigen to $CD4^+$ T cells (Roitt, 1985).

Lymphocyte populations consist of both T and B cells which can be distinguished by both function and their expression of cell surface markers. B cells are categorized by the presence of immunoglobulins (Ig) or antibodies (Ab) on their membrane surface. These antibodies on the cell surface are endogenously synthesized and act as antigen specific receptors. There are five classes of immunoglobulins including IgM, IgD, IgG, IgE, and IgA. Most B cells express both IgM and IgD on their cell surface. B cells also express complement receptors and Fc receptors to which antibody may bind.

T cells are characterized by expression of TCR/CD3 complex. The TCR consists of a heterodimer of polypeptides held together by disulfide bonds. The chains of the TCR consist of a number of different regions including a highly polymorphic region known as the variable (V) region that enables T cells to interact specifically with the many distinct antigens that may be encountered in the body. T cells may express a TCR consisting of α and β chains or a TCR composed of γ and δ chains. The group of T cells bearing the γ/δ TCR are $CD4^-CD8^-$ cytotoxic T cells but their exact role in the immune system is not yet fully understood. T cells bearing the α/β TCR can be divided into $CD4^+CD8^-$ T Helper (T_H) cells, $CD4^-CD8^+$ cytotoxic T cells (T_C), and T suppressor cells (T_S) (Golub, 1991).

The last major group of immune cells are natural killer (NK) cells. NK cells are $CD3^-$, $CD16^+$, $CD56^+$, $CD2^+$ large granular lymphocytes involved in the non-specific killing of tumor cells as well as in the lysis of virally infected cells. Recently, NK cells were shown to bear the NK 1.1 surface molecule.

Unlike T_C, NK cells are MHC-unrestricted and can effectively lyse target cells lacking class I MHC. NK cells therefore play an important role in immunosurveillance by destroying tumor cells that lack class I MHC expression and cannot be effectively lysed by cytotoxic $CD8^+$ T cells.

The nature of the immune response to a particular foreign antigen varies. The body may mount a humoral or cell-mediated response depending on the way the antigen is presented to the lymphocytes (Roitt, 1985). A humoral immune response is one that involves the secretion of antibodies by B cells while a cell-mediated response involves antigen presentation and activation of T cells. It should be noted that these reactions do not necessarily occur independently of each other. Often, an immune response involves both of these mechanisms.

Humoral immunity involves the production of antibody by B cells. The primary role of antibodies is to bind antigen. This is accomplished through the variable domain that, like the TCR, is highly polymorphic and, thus, antibody to any antigen that is encountered in the body may be produced. Antibodies express a constant region that is involved in binding to immune cells that express Fc receptor and, thereby, aid effector cells in target recognition. Additionally, the constant domain is essential in the complement pathway. Antibodies also act by directly neutralizing bacterial toxins and can coat bacteria to enhance phagocytosis by macrophages. Antibodies consist of two identical light chains and two identical heavy chains each having a constant and variable region. There are five major classes of immunoglobulins that are distinguished by their heavy chains.

IgG makes up the bulk of immunoglobulin in the human immune system and is the major antibody of secondary immune responses and the only antibody that has anti-toxin capability (Roitt, 1985). IgA is found mostly in secretions such as tears, saliva, colostrum, and mucus. IgM is present in highest concentration in the blood and is the first antibody to appear in the immune response. IgE is the antibody responsible for immediate hypersensitivity reactions though it is found in only trace amounts. Finally, IgD is found bound to the membranes of B cells and the exact function of it is not clear.

Cell-mediated immunity is a phenomenon involving T cell activation in response to antigen presentation. The primary actions of cell-mediated immunity involve destruction of intracellular parasites, tumor responses, graft rejection, and delayed-type hypersensitivity. These types of immune responses entail not only T cells but the cooperation of numerous immune cells as well as the production of various lymphokines. Lymphokines can produce more efficient immune responses and also downregulate the immune system.

T cells cannot recognize soluble antigen and, therefore, require the presence of accessory cells that can present antigen in association with Major Histocompatibility Complex (MHC) molecules on their cell surfaces. In antigen recognition, T cells are restricted by their ability to interact with MHC products. Cytotoxic T cells (T_C) can recognize antigen only in the association with class I MHC while helper T cells (T_H) interact with cells

bearing class II MHC plus antigen. Class I MHC is expressed on all nucleated cells and thus allows T_C to destroy any virally infected cell in the body. Class II MHC is present on the cell surface of antigen presenting cells (APCs).

There are two main routes of antigen presentation that occur. Antigen presentation with class II MHC involves the uptake of antigen by endocytosis or by binding to surface immunoglobulins in the case of B cells. The antigen is fragmented by proteolytic enzymes and becomes associated with class II molecules. This complex is then presented on the cell surface for presentation to $CD4^+$ T_H cells. In the case of class I MHC association, the antigen is primarily endogenous such as a viral protein. These antigens are degraded in the cytoplasm and then associated with class I MHC molecules for recognition by $CD8^+$ T_C cells. Macrophages assume the major role as APCs but other cells such as B cells and dendritic cells can also function as APCs.

The role of T_H cells is crucial to the immune response. These cells play an important regulatory role in the immune system. Upon activation, T_H cells secrete a number of chemical messengers known as lymphokines that can mediate cellular responses. These molecules include a class of lymphokines that are referred to as interleukins (IL). In particular; IL-2, IL-4, interferon-gamma (INF-γ), and IL-6 are important factors produced by T_H cells that serve to modulate immune responses. T_H cells are also needed in the production of antibody by B cells.

The development of a cytotoxic response involves both sets of T cells. The T_H cells are needed to produce IL-2 in order to drive differentiation into T_C cells and enhance their cytotoxic ability. Cytotoxic T cells bind targets bearing both antigen and the class I MHC molecule. This is followed by the release of cytotoxic granules that contain perforins and lymphotoxins. These substances disrupt the membrane of the target cell leading to its lysis. These functions are essential in the destruction of virally infected cells and some tumors.

1.2 Immunotoxicology:

The field of toxicology that deal with the effects of chemicals on the immune system is called immunotoxicology. This area of research strives to understand how the immune system is involved in the metabolism and elimination of xenobiotics, or foreign compounds, from the body. Because the immune system is vital in host defense mechanisms, any compound that is able to alter immune functions may directly or indirectly affect the survival of the host. Therefore, it is important to study any chemical encountered in the environment or taken therapeutically that may modulate the immune system.

The field of immunotoxicology as a distinct area of interest is very young. In

1977, Vos compiled information regarding immune suppression and its relation to toxicology. He noted that toxicological testing was neglecting the role of the immune system and suggested that more attention be paid to this vital system in the body during toxicity evaluation. Sharma and Zeeman published their findings about a number of environmental contaminants in 1980 and suggested several possible mechanisms by which immunotoxic agents may act. Later contributions by Sharma include a two-volume collection of reviews of immunotoxicologic literature and a classification of xenobiotics. Many other investigators have also contributed to the evolution of this field including Dean and Luster who have compiled numerous volumes on the classification and effects of various immunotoxic compounds (Burrell *et al.*, 1992).

While the effects of some chemicals may be obvious in that they cause severe organ or tissue damage, the effects of xenobiotics on the immune system often occur at levels that do not otherwise show toxic symptoms. Impairment of the immune system may manifest itself in a number of ways. This includes alterations in lymphoid organ weights or histology, changes in cellularity of lymphoid tissue, peripheral leukocytes, or bone marrow; impairment of cell functions, increased susceptibility to infectious agents or tumors, allergy, and autoimmunity (Dean *et al.*, 1985). Although many of these effects result in the suppression of immune functions, it is also possible for xenobiotics to stimulate the immune system in a potentially unfavorable fashion.

Understanding the immunotoxic effects of xenobiotics also provides valuable information on the nature of the normal immune response. In studying

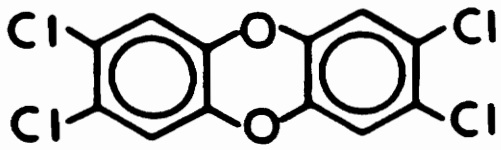
chemically induced immune responses, immunotoxicologists must discern the pathways involved in chemical-biological interactions, determine the specific cell types involved, examine the role of cellular receptors and messengers, and also consider the possibilities of cellular interactions via chemical mediators. All of these considerations make the comprehension of the exact nature of xenobiotic-immune interactions difficult to grasp. However, as the various steps in these mechanisms are unraveled, we gain a better understanding of the basic functions of the immune system.

A number of different types of xenobiotics are of interest to the immunotoxicologist. These include industrial products and by-products to which workers may be exposed, food additives and preservatives, air and water contaminants, and environmental pollutants that may be incorporated into the food chain. Another important area of attention involves those chemicals taken therapeutically. Important considerations must be given to the possibility of compromising the immune system while treating for another ailment. The end result could be disastrous in such a situation.

Although many xenobiotics have been studied with great intensity, the exact location of action is unclear and the mechanisms are often speculative. Some of the possible mechanisms of immunotoxic agents implicate damage to bone marrow, inhibition of protein or nucleic acid synthesis, selective and non-selective destruction of lymphocytes, inhibition of enzymes, complement deficiencies, delayed maturation, induction of autoantibodies, and inhibition of antibody production (Sharma, 1981). Any of these mechanisms is capable

of severely immunocompromising the host animal. As a result, the immunocompromised individual has increased susceptibility to opportunistic infection and tumor incidence.

1.3 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD):



TCDD

2,3,7,8-Tetrachlorodibenzo-p-dioxin, commonly called TCDD or dioxin is a biologically potent environmental contaminant in the class of halogenated aromatic hydrocarbons (HAH). TCDD is produced by a number of primary sources including chemical, thermal, photochemical, and enzymatic reactions. High levels of TCDD are associated with production of certain chlorinated phenols, chlorophenoxy herbicides, and polychlorinated biphenyls (PCBs) (Rappe, 1991). The bleaching of pulp using chlorine is a major source of aquatic contamination in North America and TCDD is found in sediment outside pulp mills as well as in commercial products made of bleached pulp such as coffee filters, milk cartons, facial tissue, and diapers (Rappe, 1991). TCDD is also produced during the incineration of wood, garbage, plastics, and other wastes.

The toxicity of TCDD has been widely studied and characterized. The most commonly described human symptom is chloracne, in which hyperplasia and hyperkeratinization of the epidermis has been noted (Poland and Kende, 1976). Common effects in laboratory animals due to TCDD exposure include body weight loss, thymic atrophy, reproductive toxicity, teratogenicity, endocrine effects, dermal lesions, and induction of several hepatic xenobiotic-metabolizing enzymes (Poland and Knutson, 1982; Thomas and Faith, 1985; Davis and Safe, 1988). TCDD has been shown to have profound effects on the immune system even at very low levels that do not induce organ toxicity. Studies of the effects of TCDD on T-cell mediated immunity have shown that TCDD can suppress cell-mediated immunity (CMI). TCDD has also been shown to inhibit humoral immunity involving antibody (Ab) production by B lymphocytes (Faith and Moore, 1977; Faith and Luster, 1979; Sharma *et al.*, 1978). Other immunomodulatory effects of TCDD include suppression of B cell differentiation (Fine *et al.*, 1988), dose-dependent suppression of cytotoxic T lymphocytes (Thigpen *et al.*, 1975), thymic atrophy (Thomas and Faith, 1985), impairment of delayed type hypersensitivity responses, and increased susceptibility to certain infectious agents (Clark *et al.*, 1981). Though it is clear that TCDD alters these immune functions, the mode(s) of toxicity is unclear.

Biological responses to TCDD as well as a vast array of other foreign chemicals, or xenobiotics, are regulated by the major histocompatibility complex (MHC) and the Ah (aryl hydrocarbon) locus (reviewed by Nebert *et*

al., 1982). The Ah locus is responsible for the induction of metabolizing enzymes such as cytochrome P_1-450 and aromatic hydrocarbon hydroxylase (AHH). It is thought that HAH congeners bind to a cytosolic receptor that is encoded by the Ah locus. The receptor-ligand complex is translocated to the nucleus where binding to specific "dioxin regulatory element" (DRE) sites causes pleiotropic gene induction leading to a measurable increase in mRNA for various enzymes and gene products regulated at that site (Clark *et al.*, 1981; Nebert *et al.*, 1982; Nebert, 1989). Studies focusing on the Ah locus have used aryl hydrocarbon hydroxylase (AHH) to establish the phenotype of the Ah locus in particular strains of inbred mice. It was discovered that about half of all inbred strains of mice are Ah-responsive meaning that enzymes such as cytochrome P_1-450 are inducible by polycyclic aromatic hydrocarbons. In non-responsive strains of mice cytochrome P_1-450 is non inducible or less inducible. It has been determined that Ah^b is a dominant allele for responsiveness and Ah^d is the recessive allele. Therefore, experimental results have shown C57BL/6 (Ah^b/Ah^b) to be a TCDD-responsive strain and DBA/2 (Ah^d/Ah^d) to be unresponsive (Nebert *et al.*, 1982; Nebert, 1989).

It has been established that responsiveness to TCDD is linked to segregation with the Ah locus. Clark *et al.* showed that suppression of cytotoxic T lymphocytes (CTLs) induced by TCDD segregated with the Ah locus (Whitlock, 1987; Clark *et al.*, 1983, Nagarkatti *et al.*, 1984). Poland and Glover deduced that TCDD-induced thymic atrophy segregated with the Ah-receptor (Ah-R) (Poland and Glover, 1980). It has been shown using congenic

mice that TCDD could induce enhanced suppression in Ah^{bb} C57BL/6 mice when compared to Ah^{dd} C57BL/6 mice (Kerkvliet *et al.*, 1990). Studies of the Ah-R have shown that different cell types within the same organ can possess different levels of Ah-R (Greenlee *et al.*, 1985), though it is not clear whether the degree of specific binding by TCDD to different cell types determines the degree of immunotoxicity. It should be noted that, although induction of AHH and associated enzymes has been shown to be related to the Ah-R (Safe, 1986; Whitlock, 1990; Okey *et al.*, 1983), Okey *et al.* demonstrated that the Ah-R was necessary but not sufficient for the induction of AHH (Okey, Mason, and Vella, 1983).

The role of the Ah locus in TCDD-induced suppression of humoral immunity is a more controversial issue. Vecchi *et al.* showed that Ab response to SRBC could be suppressed in C57BL/6 mice with very low, single doses of TCDD (1.2 μg/kg) while higher doses (6 μg/kg) were required in DBA/2 mice to give partial suppression and longer exposure did not increase susceptibility of DBA/2 mice (Vecchi *et al.*, 1983). A good correlation between Ah-R binding and suppression of humoral immunity was also shown by Davis and Safe using congeners of TCDD (Davis and Safe, 1988). In contrast, it has been observed that the toxicity of TCDD may be mediated by Ah-independent mechanisms. Studies using 2,7-DCDD, a TCDD congener with a weak affinity for Ah-R, showed a suppressed Ab response to SRBC with no effect on the thymus or AHH activity while TCDD caused thymic atrophy and increases in AHH activity as well as suppression of Ab responses (Holsapple *et al.*, 1988).

Although these studies suggest the possibility of Ah-independent mechanisms for immunosuppression, they do not put aside the role played by the Ah locus in TCDD-mediated suppression of B cell responses.

Studies involving B cell responses to T-dependent and T-independent antigens are also a source of controversy. Using both T cell-dependent and independent antigens, Tucker *et al.* (Tucker, Vore, and Luster, 1986) and Luster *et al.* (Luster *et al.*, 1988) demonstrated that B cells from Ah-responsive mice were susceptible to TCDD-induced suppression while Ah-nonresponsive mice were resistant. Also, House (House *et al.*, 1990) demonstrated that TCDD in Ah-responsive mice inhibited the B cell antibody response to sheep red blood cells (SRBC) which is a T-dependent antigen. However, studies of two different T-independent antigens using TCDD-treated mice showed suppression of B cell response against TNP-ficoll but not against TNP-LPS. The investigators reasoned that TNP-ficoll requires antigen processing by accessory cells while TNP-LPS does not. Therefore, the observed results may be explained by the effect of TCDD on macrophage functions. In contrast, Holsapple *et al.* (Holsapple, McCay, and Barnes, 1988) demonstrated that dioxins suppressed the antibody response to LPS similarly in both Ah-responsive and Ah-nonresponsive mice. In the above studies, TCDD was added directly to *in vitro* cultures in contrast to previous studies (Nagarkatti *et al.*, 1984; Poland and Glover, 1980; Vecchi *et al.*, 1983) in which TCDD was administered *in vivo*. This suggests that differential sensitivity based on the Ah locus is seen only when TCDD is

administered *in vivo*. Later studies from Luster (Luster *et al.*, 1988) investigated the effect of TCDD added to *in vitro* cultures on B cell antibody responses to TNP-LPS. Their results showed that TCDD suppressed B cell antibody response in Ah-responsive mice but not in Ah-nonresponsive mice. The reason for the discrepancies in these studies mentioned is unclear.

These studies illustrate the controversy surrounding the role of the Ah locus on TCDD-induced suppression of humoral immunity. This controversy may in part be due to the role played by accessory cells in humoral immunity. DNP-ficoll is classified as a T-independent antigen, however, it has been shown that macrophages (Cushed *et al.*, 1976) and even T cells (Mond *et al.*, 1980) may be necessary for optimal responses. This illustrates the need to study individual subpopulations of the immune system since many T and B cell responses involve the interaction of various lymphocytes and macrophages.

Also important in governing immune system responses is the MHC (major histocompatiblity complex). MHC genes have been shown to regulate susceptibility to autoimmune diseases, development of tumors, and responsiveness to certain antigens as well as the immunotoxicity of some environmental contaminants such as mercury and gold (Laurence, 1985; Sapin *et al.*, 1981; Charpertier *et al.*, 1981). However, research has not been done to examine the relationship between the MHC and TCDD-induced toxicity. MHC genes may be a contributing factor in TCDD immunotoxicity and is therefore a subject of interest in the current investigation.

It is important to understand the role of each cell of the immune system in response to a particular xenobiotic so that one can see the exact mechanism by which a specific compound is inducing toxicity. Since the immune system is an interactive network of T cells, B cells, and macrophages, one must examine each of these cell types individually. The function of each of these subpopulations directly affects the activities and proper functioning of the others. In order to gain an understanding of TCDD-induced immunosuppression, we wish to examine individual cell types of the immune system and to study the roles played by both the MHC and Ah loci.

1.4 Specific Aims:

Although the toxicity of TCDD has been widely studied, the exact mechanisms are unclear. It is important to understand how the immune system deals with the introduction of xenobiotics into the body because the immune system is exposed to such chemicals on a daily basis. By gaining knowledge of immune responses to toxic chemicals, we can further understand the basic mechanisms of the immune system. Because TCDD has been implicated in the induction cancer, studies focusing on cellular mechanisms may give insights into the complex actions that precede the onset of cancer.

The specific aims of the research were to determine:

1. The *in vitro* toxic effects of TCDD on the ability of splenic T and B cells to respond to mitogens.

2. The role of Fas, a molecule involved in apoptosis, on TCDD-induced thymic atrophy and suppression of peripheral T cell responsiveness to conalbumin or mitogens.

3. The role of MHC phenotype on the regulation of immunotoxic effects of TCDD in Ah-responsive mice.

4. To investigate whether TCDD acts at the level of antigen-specific T cells or the antigen presenting cells (APC).

2.0 Immunotoxicity of TCDD: Role of Fas expression and MHC phenotype in TCDD-mediated suppression of thymocyte differentiation and peripheral T cell responsiveness.

2.1 Introduction:

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a highly toxic environmental contaminant formed as a by-product in the manufacturing of products from chlorinated phenols or during the combustion of chlorinated materials. TCDD has been shown to cause generalized wasting syndrome, thymus involution, hepatotoxicity, gastric lesions, tumor promotion, teratogenicity, and embryo toxicity (Reviewed by Holsapple *et al.*,1991; Safe, 1986). One of the most characteristic features of TCDD-induced toxicity involves its action on the immune system. TCDD alters the functions of the immune system at doses that do not produce organ toxicity. TCDD has been shown to induce thymic involution as well as suppress T and B lymphocyte functions (Holsapple *et al.*,1991). Despite extensive studies, the exact mechanism by which TCDD

alters the functions of the immune system is not clear. The bulk of the literature on the immunotoxicity of TCDD has shown that this effect may be regulated by the aryl hydrocarbon (Ah) locus (Silkworth and Grabstein, 1982; Nagarkatti *et al.*,1984; Kerkvliet *et al.*,1989). While several investigators have demonstrated that the immunotoxicity of T cell-mediated immune responses segregates with the Ah locus, other investigators have also demonstrated that the suppression of B cell activation may involve Ah-independent mechanisms (Holsapple *et al.*,1991).

Thymus is a highly sensitive organ to TCDD-mediated immunotoxicity and several hypotheses have been proposed to explain the mechanism of thymic atrophy triggered by TCDD. McConkey *et al.* (1988) demonstrated using rat thymocytes that TCDD may kill immature thymocytes by initiating apoptosis. However, in a recent study, it was noted that TCDD administration failed to trigger apoptosis in thymocytes (Silverstone *et al.*,1994). Whether TCDD-induced thymic atrophy *in vivo* is triggered by apoptosis is difficult to detect due to rapid clearing of apoptotic cells *in vivo* (Gerschenson and Rotello, 1992) and remains a possibility which needs further consideration.

Recently, several molecules involved in signal transduction leading to apoptosis have been characterized and Fas (CD95) is considered to be the most important among such receptors (Itoh *et al.*,1991; Oehm *et al.*,1992). Mice with homozygous lpr mutation develop lymphoproliferative and autoimmune disease resulting from a defect in the expression of Fas antigen

(Watanabe-Fukunaga *et al.*,1992). Thus, lpr mutation offers an excellent tool to study apoptosis in the thymus.

Also important in governing responses of the immune system is the major histocompatibility complex (MHC). Recent studies have shown that immunotoxicity of certain heavy metals is under the influence of MHC genes (Laurence, 1985). Whether the immunotoxic effects of TCDD is regulated by the MHC genes has not been previously investigated. In this context, it is interesting to note that TCDD has been shown to induce a significant increase in Ia positive cells in the skin of mice as well as on macrophages (Puhvel and Sakamoto 1989; Kerkvliet and Oughton, 1993).

In the current study, therefore, we investigated the effect of Fas expression and MHC phenotype on the immunotoxicity induced by TCDD. Our results demonstrated that mice expressing Fas molecule were more susceptible to TCDD-mediated immunosuppression when compared to mice that lack the Fas antigen. Secondly, using B10 congenic strains we observed that the MHC phenotype significantly influenced the TCDD-mediated thymic atrophy and peripheral T cell dysfunction. Moreover, our results demonstrated that TCDD acts at the time when T cells are differentiating in response to antigenic challenge whereas TCDD fails to act on naive and resting T cells that have not been triggered by antigenic challenge. Together, our data suggested that TCDD may mediate its immunotoxicity, at least in part, through the Fas molecule leading to apoptosis in activated T cells and that MHC-phenotype may also play an important role in regulating TCDD-mediated

immunotoxicity.

2.2 Experimental Procedures

2.2.1 Mice:

Adult, female C57BL/6 and DBA/2 mice were purchased from the National Cancer Institute, Bethesda, MD. C57BL/10, B10.D2, and C57BL/6 lpr/lpr mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in our animal facilities as described (Seth *et al.*,1988; Kakkanaiah *et al.*,1990). C57BL/6 lpr/lpr mice used were always less than 2 months of age, prior to onset of autoimmunity. The mice were housed in polyethylene cages containing wood shavings in laminar flow units (Animal Storage Isolators, Nu Aire Inc., Plymouth, MN) and given rodent chow and tap water *ad libitum*. Mice were housed in rooms maintaining a temperature of 74±2 °F and on a twelve hour light/dark cycle.

2.2.2 TCDD exposure:

2,3,7,8-Tetrachlorodibenzo-p-dioxin was generously donated by Dr. K. Chae of NIEHS, Research Triangle Park, NC and stored at -20 °C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone. Serial dilutions were done to reach the desired concentrations. Mice were administered 0.1, 1.0, or 5.0 μg/kg body weight TCDD or the vehicle via oral gavage in 0.1 ml volume daily for 11 days.

TCDD used in *in vitro* studies was dissolved in dimethyl sulfoxide (DMSO) and diluted in media to reach final dilutions of 0.1 nM to 50 μM.

2.2.3 Cell preparation:

Mice were euthanized after TCDD or vehicle treatment and thymus, spleen, and lymph nodes were removed by dissection. Organs were place in RPMI-1640 medium (GIBCO Lab., Grand Island, NY) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 10 mM HEPES, 1 mM glutamine, 40 μM/ml of gentamicin sulfate and 50 μM 2-mercaptoethanol. Single cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation and resuspended in a 0.83% ammonium chloride solution to lyse erythrocytes. Cells were further washed two times in medium.

2.2.4 Total cellularity:

Thymus, spleen, or lymph node cells were prepared as described above and resuspended in medium. Twenty-five microliters of the single cell suspension was added to 100 or 500 μl of trypan blue dye and viable cells were enumerated by exclusion of trypan blue under an inverted phase contrast microscope (Olympus model T041).

2.2.5 In vitro studies with TCDD:

Whole spleen suspensions from DBA/2 and C57BL/6 mice were prepared as above and tested for their ability to respond to T and B cell mitogens as described (Clary *et al.*, 1990) in the presence of media containing TCDD (0.1 nM-50 μM) or control media containing DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). The anti-CD3 and anti-αβ TCR mAbs were purified from hybridoma culture supernatants as described (Kakkanaiah *et al.*,1990; Dean *et al.*,1990). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell suspensions were cultured in flat-bottom 96-well plates at concentrations of 4 x 10^5 cells/well in 0.2 ml medium for 48 hours in an incubator maintained at 37 °C and 5% CO_2. The plates were pulsed with 2 μCi ^{3}H-thymidine during the last 8 hours of incubation and the cells were harvested using an automated cell harvester (Skatron, Sterling, VA). The amount of radioactivity was determined using a scintillation counter and mean cpm±S.E.M. of triplicate cultures were calculated.

2.2.6 Cell proliferation to mitogen stimulation:

The draining lymph node cells from conalbumin immunized mice were tested for their ability to respond to T cell mitogens as described (Clary *et al.*,

1990). T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). The anti-CD3 and anti-αβ TCR mAbs were purified from hybridoma culture supernatants as described (Kakkanaiah *et al.*,1990; Dean *et al.*,1990). Cell suspensions were cultured in flat-bottom 96-well plates at concentrations of 4 x 10^5 cells/well in 0.2 ml medium for 48 hours in an incubator maintained at 37 °C and 5% CO_2. The plates were pulsed with 2 μCi ^{3}H-thymidine during the last 8 hours of incubation and the cells were harvested using an automated cell harvester (Skatron, Sterling, VA). The amount of radioactivity was determined using a scintillation counter and mean cpm±S.E.M. of triplicate cultures were calculated. The data were depicted as Δ cpm= (cpm in cultures stimulated with mitogens) - (cpm in cultures incubated with medium alone).

2.2.7 Flow cytometry:

The cells were washed twice with medium consisting of phosphate buffered saline containing 0.1% sodium azide and the cells (3 x 10^6) were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb and phycoerythrin (PE)-conjugated anti-CD8 mAb (Pharmigen, San Diego, CA) for 30 minutes at 4 °C. Fluorescence of the cell populations was

measured by flow cytometric analysis as described (Kakkanaiah *et al.*,1990; McKallip *et al.*,1995). The analysis was performed by an Epics V, model 752, flow cytometer. Ten thousand cells were analyzed per sample.

2.2.8 In vivo T cell stimulation:

The T cell responsiveness to conalbumin was studied as described (McKallip *et al.*,1995; Dean *et al.*,1990). Mice were treated with TCDD orally for 11 days. They were immunized with 100 μg conalbumin and Fruend's complete adjuvant (1:1) in the rear footpads on the fourth day of TCDD or vehicle treatment to stimulate T cells. Following treatment, on day 12, the draining lymph nodes were removed and single cell suspensions were prepared. Cells (4×10^5) were cultured in 96 well plates in 0.2 ml of medium with 100 μg/ml conalbumin for 3 days to stimulate antigen-specific T cells. Cultures were pulsed with ^{3}H-thymidine during the last 18 hours and harvested to determine the proliferative response as measured by DNA synthesis. The data were depicted as Δ cpm= (cpm in cultures stimulated with conalbumin) - (cpm in cultures incubated with medium alone). The cultures were carried out in triplicate and the mean Δ cpm ± S.E.M. was calculated and depicted.

2.2.9 Cell-mixing experiment to study the effect of TCDD on T cells or antigen presenting cells (APC):

To investigate whether TCDD affects T cells or APC, cell-mixing experiments were carried out. To this end, T cells were purified from draining lymph node cells of TCDD or vehicle-treated mice immunized with conalbumin. Similarly, splenic macrophages were purified from TCDD or vehicle-treated mice as a source of APC. The T cells were purified by passing the lymph node cells over nylon wool followed by plastic adherence to deplete any macrophages as described (Dean *et al.*,1990). To isolate macrophages, the spleen cells were allowed to adhere to the plastic plates for 1 hour at 37 °C. The nonadherent cells were discarded and the adherent cells were removed by forceful pipetting (Dean *et al.*,1990). The APC were irradiated at 2000 rads and used in the assays. To study the T cell responsiveness to conalbumin, 4×10^5 T cells from TCDD or vehicle-treated mice were mixed with 6×10^5 irradiated APC from TCDD-treated or untreated mice in the presence of 100 μg/ml of conalbumin. The cultures were incubated at 37 °C for 72 hours and pulsed with ^{3}H-thymidine as described above to study the T cell proliferation.

2.2.10 Statistical Analysis:

In the current study, groups of 4-5 mice were used throughout, for the control and TCDD-treated groups. The data was statistically analyzed using Tukey-Kramer multiple comparison test or Student's t-test and differences with p values less than 0.05 were considered to be statistically significant. All

assays were repeated at least twice with consistent results.

2.3 Results:

2.3.1 *In vitro effect of TCDD on whole spleen populations:*

In the current study, whole spleen populations from DBA/2 and C57BL/6 mice were prepared and cultured *in vitro* with various concentrations of TCDD ranging from 0.1 nM to 50 μM in the presence of T and B cell mitogens. Control splenocytes were cultured with media containing DMSO equivalent to the amount found in the corresponding TCDD solution. Initial studies using concentrations of 0.1, 1.0, 10, 100, or 1000 nM TCDD showed no consistent and dose dependent effect on mitogen responsiveness in either DBA/2 (Fig. 1) or C57BL/6 splenocytes (Fig. 2). Subsequent studies used higher concentrations of TCDD such as 1.0 (Figs. 3,4), 5.0 (Figs. 5,6), 10 (Figs. 7,8), 20 (Figs. 9,10), or 50 μM (Figs. 11,12). Only in very high concentrations of TCDD, such as those above 5 μM, was suppression of T and B cell responsiveness observed in both DBA/2 and C57BL/6 splenocytes. However, it should be noted that the toxicity observed at 20 and 50 μM of TCDD, may have resulted at least in part from DMSO used as the solvent in dilution of TCDD. These studies demonstrated that at physiologically relevant concentrations, TCDD did not decrease the ability of naive T and B cells to respond to mitogens.

2.3.2 *Role of Fas and MHC phenotype on TCDD-induced thymic atrophy:* Initially, we investigated the effect of TCDD administration on

the total cellularity of the thymus in different strains of mice to investigate the effect of Ah and MHC loci as well as in mice that were deficient in the expression of Fas. To this end, mice were orally administered with 0 (vehicle), 0.1, 1.0, or 5.0 μg/kg body weight of TCDD daily for a period of 11 days. Twenty-four hours after the last dose, the mice were sacrificed and the total viable cellularity of the thymus was determined by trypan blue dye exclusion assay. The data presented in Table 1 demonstrated that administration of TCDD in C57BL/6 (Ah-responsive) mice induced a dose dependent decrease in the total viable cellularity of the thymus. In contrast, similar administration in Ah-nonresponsive mice such as DBA/2 led to no significant decrease in total cellularity at 0.1 and 1.0 μg/kg body weight of TCDD. However, when TCDD was administered at 5.0 μg/kg body weight, there was a significant decrease in total cellularity. These data are similar to previous studies that have demonstrated that the thymocytes of Ah-responsive mice are more susceptible to TCDD-mediated toxicity when compared to Ah-nonresponsive mice (Holsapple *et al.*,1991).

Next, we investigated the effect of TCDD in C57BL/6 lpr/lpr mice which are deficient in the expression of Fas and compared these data to the wild type C57BL/6 mice which express the Fas molecule. Interestingly, these results demonstrated that C57BL/6 lpr/lpr mice were more resistant to thymic atrophy induced by TCDD when compared to C57BL/6 mice. For example, at 0.1 μg/kg body weight of TCDD, C57BL/6 lpr/lpr mice did not demonstrate a significant decrease in thymic cellularity and at 1.0 μg/kg, the percent decrease in the total cellularity of the thymus was relatively less (42%) when

compared to the decrease in cellularity observed at 1.0 μg/kg body weight (81%) in C57BL/6 mice. However, at highest concentration tested such as 5.0 μg/kg body weight, both C57BL/6 lpr/lpr and C57BL/6 mice demonstrated a similar degree of thymic atrophy. These data suggested that the thymocytes from C57BL/6 lpr/lpr mice were relatively more resistant to TCDD-mediated toxicity at lower concentrations when compared to C57BL/6 mice and, since the difference between C57BL/6 lpr/lpr and C57BL/6 mice rests on a single gene mutation leading to nonexpression of Fas in the former, our data suggested that Fas expression may regulate the immunotoxicity of TCDD in the thymus.

Similar studies were undertaken in B10 and B10.D2 strains which are both Ah-responsive and differ only at their MHC, the former bearing the H-2^b haplotype and the latter H-2^d. We reasoned that if DBA/2 strain (H-2^d) was relatively more resistant to TCDD-mediated thymic atrophy due to the specific MHC phenotype, which plays an important role in negative and positive selection of thymocytes, it should be possible to test this using an Ah-sensitive H-2^d strain and comparing it with another congenic strain that differs only at the MHC. The results shown in Table 1 demonstrated that B10.D2 mice were in fact more sensitive than B10 mice to TCDD-induced thymic involution at 0.1 and 1.0 μg/kg. These data suggested that the reason why DBA/2 thymus is resistant to the immunotoxicity of TCDD is not due to the specific MHC phenotype (H-2^d) but was due to Ah-nonresponsiveness. These data also suggested that within the Ah-sensitive strains of mice, some MHC phenotypes such as H-2^d may be more sensitive than other phenotypes

such as H-2^b.

Further studies were undertaken to investigate whether TCDD would affect the different subsets of T cells that are found in the thymus. To this end, double-staining using anti-CD4 and anti-CD8 mAbs was carried out and the cells were analyzed flow cytometrically. Representative experiments have been shown in Figs. 13-15 and the results of multiple experiments have been summarized in Table 2. These data indicated that administration of TCDD at 0.1, 1.0, or 5.0 μg/kg body weight did not induce any statistically significant difference in the various subpopulations of T cells in all of the strains examined. Furthermore, based on these percentages, the absolute numbers of various T cell subsets were calculated by using the total cellularity as depicted in Table 1. It was observed that at concentrations of TCDD that caused a significant decrease in the total cellularity of the thymus there was also a proportionate decrease in all four subsets of T cells (Table 1). These data together demonstrated that TCDD does not exhibit any preferential effect on any one subset of T cells and that all subsets were uniformly susceptible to TCDD-induced toxicity. It should be noted that in C57BL/10 and B10.D2 strains, the T cell subsets were enumerated only at the highest concentration namely 5.0 μg/kg body weight and even at this highest dose, there was no significant change in the percentage of T cell subsets in the thymus (Table 2).

It should be noted that although Fas played an important role, it was secondary only to the Ah locus because MRL +/+ (Fas^+) and MRL-lpr/lpr (Fas^-) mice which were both Ah-nonresponsive failed to exhibit susceptibility to

TCDD-mediated thymic atrophy (Table 3).

2.3.3 *Role of Fas and MHC-phenotype on peripheral T cell responsiveness to conalbumin or mitogens in TCDD-treated mice:*

In order to find out whether the expression of Fas and MHC would also influence peripheral T cell responsiveness to antigenic challenge, different strains of mice were administered for 11 days with vehicle or 0.1, 1.0, 5.0 μg/kg body weight TCDD and on day 4, the mice were challenged with 100 μg conalbumin mixed with Fruend's adjuvant into the rear footpads. Eight days later, after completion of treatment with TCDD, the draining lymph nodes were harvested and cultured either with conalbumin or a variety of T cell mitogens *in vitro*. The data on the peripheral T cell responsiveness to conalbumin as depicted in Figure 16 demonstrated that C57BL/6 mice exhibited significant decreases in T cell responsiveness to conalbumin at 1.0 and 5.0 μg/kg body weight of TCDD but not at 0.1 μg/kg. In contrast, DBA/2 mice were totally resistant to decreases in peripheral T cell responsiveness to conalbumin at all three concentrations of TCDD tested (Fig. 17). Furthermore, C57BL/6 lpr/lpr mice were more resistant to TCDD-mediated suppression of T cell responsiveness to conalbumin when compared to C57BL/6 mice (Fig. 18). For example at 1.0 μg/kg of TCDD, there was only 24% decrease in the T cell responsiveness to conalbumin in lpr mice (Fig. 18), when compared to 58% decrease in C57BL/6 mice at a similar dose (Fig. 16), although at the highest dose tested, this effect was not seen.

In similar studies, splenic T cells were purified from TCDD-treated DBA/2

and C57BL/6 mice immunized with conalbumin and tested as above for responsiveness to antigenic challenge. As observed using lymph node T cells, splenic T cells from C57BL/6 mice exhibited significant decreases in T cell responsiveness to conalbumin at 1.0 and 5.0 μg.kg body weight of TCDD but not at 0.1 μg/kg (Fig. 19). In contrast, splenic T cells from TCDD-treated DBA/2 mice were totally resistant to decreases in peripheral T cell responsiveness to conalbumin at all three concentrations of TCDD tested (Fig. 20). These data demonstrated that TCDD exerts similar effects on splenic and lymph node T cells.

The draining lymph nodes immunized with conalbumin were also tested for their ability to respond to various T cell mitogens. Interestingly, as shown in Figures 21-23, in this assay, none of the strains tested, exhibited decrease in responsiveness to mitogens, following TCDD administration. These data demonstrated that TCDD may be acting only on those T cells which are responding to the antigenic challenge *in vivo* but not the remaining naive resting T cells.

When B10.D2 and B10 mice were compared for their ability to respond to conalbumin following TCDD administration it was observed that both strains demonstrated similar decreases in T cell responsiveness to conalbumin at 1.0 and 5.0 μg/kg body weight TCDD (Figs. 24,25). However, at 0.1 μg/kg of TCDD, B10.D2 demonstrated significant decrease in T cell responsiveness to conalbumin whereas at this dose, B10 failed to exhibit any suppression. These data demonstrated that the reason why the DBA/2 strain was

resistant to TCDD-mediated peripheral T cell dysfunction (as shown in Fig. 20) was due to the Ah phenotype rather than the MHC (H-2^d) phenotype. However, these data also suggested that within the Ah-sensitive congenic strains, H-2^d phenotype was more sensitive than H-2^b to TCDD-mediated peripheral T cell dysfunction. Furthermore, the draining lymph node T cells from conalbumin-immunized, TCDD-treated mice did not exhibit any consistent and dose dependent decrease in their responsiveness to mitogens when compared to the controls (Figs. 26,27).

2.3.4 *TCDD acts directly on the T lymphocytes but not on antigen presenting cells (APC):* In order to address whether the decrease in T cell responsiveness to conalbumin resulted from the direct action of TCDD on T lymphocytes or through its indirect action on APC, cell-mixing studies were undertaken in which purified lymph node T cells from TCDD-treated or untreated (control) mice were incubated *in vitro* with normal untreated or TCDD-treated splenic macrophages as a source of APC, in the presence of conalbumin. The data shown in Figs. 28 and 29 demonstrated that when conalbumin-specific T cells from TCDD-treated (1.0 or 5 μg/kg) mice were incubated with macrophages from vehicle-treated mice in the presence of conalbumin, the T cells demonstrated a significant decrease in their proliferative response when compared to similarly stimulated conalbumin-specific T cells from vehicle-treated mice (Fig. 28). These data suggested that TCDD was able to act directly on the conalbumin specific T cells. Furthermore, when conalbumin-specific T cells from vehicle-treated

mice were studied for their ability to respond to conalbumin *in vitro* in the presence of macrophages from TCDD-treated or vehicle-treated mice, there was no decrease in the ability of T cells to respond to conalbumin when TCDD-treated APC were used (Fig. 29). These data together demonstrated that TCDD acted directly on conalbumin activated T cells rather than on antigen presenting cells.

2.4 Discussion:

The current study demonstrates that, although TCDD-mediated thymic involution and decrease in T cell responsiveness to antigenic challenge in the lymph nodes is regulated primarily by the Ah locus, there may be other factors in addition to the Ah locus which might play an important role in TCDD-mediated immunological alterations. Our studies suggest that the toxicity seen in the thymus and in the periphery may depend on the expression of Fas, a molecule involved in apoptosis, thereby suggesting that TCDD may exert its effect by triggering apoptosis *in vivo*. Secondly, it was noted that within the Ah-responsive strains, the immunosuppressive effect of TCDD on T cells may depend on the MHC phenotype. This was demonstrated using B10 congenic mice differing in MHC. Thirdly, our studies also demonstrate that the immunotoxicity of TCDD may depend on the state of activation of T cells. Thus, TCDD may act on T cells that are differentiating or responding to antigens *in vivo* rather than those that are naive and in an inactive state. Lastly, it was also noted that TCDD directly affected the

antigen-triggered T cells rather than the APC.

Bulk of the literature on the immunotoxicity of TCDD has shown that this effect may regulated by the Ah locus (Holsapple *et al.*,1991; Silkworth and Grabstein, 1982; Nagarkatti *et al.*,1984; Kerkvliet *et al.*,1989). However, studies particularly with B cells, demonstrated that the immunotoxicity of TCDD maybe mediated by Ah-independent mechanisms (Holsapple *et al.*,1991), although the exact mechanism remains unresolved. In the current study, therefore we addressed whether MHC, which has been previously shown to regulate the immunotoxicity of certain heavy metals (Laurence, 1985), and Fas, an important molecule involved in triggering apoptosis (Itoh *et al.*,1991; Oehm *et al.*,1992), would influence the immunotoxicity of TCDD. It should be noted that while making the comparisons between different strains we considered the lower dose of 0.1 and 1.0 μg/kg of TCDD because at the highest dose tested (5 μg/kg), even Ah-nonresponsive mice (DBA/2) exhibited thymic atrophy. Studies using congenic strains, namely B10 which is Ah-responsive with H-2^b haplotype and B10.D2 which is Ah-responsive with H-2^d haplotype, demonstrated that B10.D2 is more sensitive than B10 to immunotoxicity caused by TCDD. For example, at 0.1 μg/kg of TCDD, the thymus of B10.D2 mice demonstrated 51% decrease in cellularity whereas in B10 mice this dose induced only a 26% decrease. Similar observation was also made while studying the peripheral T cell responsiveness to conalbumin at this dose of TCDD exposure. These data also demonstrated that the reason why DBA/2 (Ah-nonresponsive, H-2^d) exhibits resistance to TCDD is not due to the H-2^d MHC haplotype but primarily due to the Ah

nonresponsiveness.

Despite extensive research, the exact mechanism by which TCDD triggers thymic atrophy is not clear. Inasmuch as, the thymus has higher concentrations of Ah-R, TCDD may directly act on the thymocytes (Okey *et al.* 1983). In the current study we also observed that thymus was more sensitive to TCDD-mediated immunotoxicity when compared to antigen-specific peripheral T cells. Fine *et al.* (1990) demonstrated that prothymocyte activity was severely impaired in TCDD-treated mice thereby suggesting that thymic atrophy may result, at least in part, from impaired thymic seeding of prothymocytes. Although TCDD has been shown clearly to affect the prothymocytes, its direct effect on thymocytes is also possible as demonstrated by the ability of TCDD to alter T cell differentiation of the thymus *in vitro* (Lundberg *et al.*,1990; Cook *et al.*,1987). Furthermore, some studies have demonstrated that TCDD treatment leads to significant decrease in the percentage of double-positive thymocytes and a significant increase in double-negative thymocytes. This effect is particularly conspicuous after prenatal exposure (Blaylock *et al.*,1992; Holladay *et al.*,1991) but was less marked in adult mice (Kerkvliet and Brauner, 1990). The current studies suggested that TCDD treatment in adult mice causes thymic involution in Ah-responsive but not Ah-nonresponsive mice and, furthermore, the proportions of various subpopulations of T cells in the thymus of adult mice are not significantly altered. Our data are in agreement with a recent report which demonstrated that TCDD-induced thymic atrophy resulted from a proportional loss of all classes of thymocytes (Silverstone *et*

al.,1994).

It has also been postulated that TCDD may trigger thymic atrophy via induction of apoptosis. McConkey *et al.* (1988) showed using rat thymocytes that TCDD kills immature thymocytes by initiating apoptosis similar to that previously described for glucocorticoid hormones. These authors demonstrated that TCDD evoked a sustained increase in cytosolic free Ca^{2+} concentration leading to possible activation of endonuclease and DNA fragmentation in thymocytes. Similar increases in Ca^{2+} content caused by TCDD has been reported in various tissues by other investigators (Al-Bayati *et al.*,1988; Canga *et al.*,1988) which may account for induction of apoptosis. However, in a recent study, a single dose of TCDD which induced thymic atrophy *in vivo* failed to exhibit apoptosis (Silverstone *et al.*,1994). It is not clear whether this was due to failure to study apoptosis immediately after TCDD administration, during the initial 48 hours, or whether TCDD triggers a slow thymic atrophy, leading to efficient clearing of apoptotic cells by the phagocytic cells (Gerschenson and Rotello, 1992). Currently, kinetic studies are in progress in our laboratory to directly correlate the Fas expression on thymocytes with ability of TCDD to induce apoptosis *in vivo*. It should be noted that in the current study, at higher doses such as 5 μg/kg body weight, the thymus of most strains including that from Ah-nonresponsive mice and Fas^- (*lpr/lpr*) mice was susceptible to TCDD-mediated immunotoxicity. These data suggested that, at high TCDD doses, mechanisms other than those involving Ah or Fas may be involved. Also, thymic atrophy may also result from the effect of TCDD on bone marrow stem cell precursors (Silverstone *et al.*,1994).

Since it is difficult to demonstrate apoptosis *in vivo,* due to rapid clearing of apoptotic cells, we used mice bearing homozygous *lpr* mutation which has been shown to affect the expression of the Fas molecule and therefore cells from this mutation exhibit defective apoptosis (Watanabe-Fukunaga *et al.*,1992). Our data demonstrated that both the thymic atrophy and the peripheral T cell responsiveness to conalbumin was regulated by the Fas molecule inasmuch as Fas^- (C57BL/6 *lpr/lpr*) mice were less sensitive to TCDD-mediated immunosuppression when compared to Fas^+ (C57BL/6) mice. For example, at 0.1 μg/kg of TCDD, C57BL/6 *lpr/lpr* thymus did not exhibit significant change unlike the C57BL/6 thymus and furthermore at 1.0 μg/kg of TCDD, the proportion of decrease in C57BL/6 *lpr/lpr* thymus cellularity was comparable to the effect of 0.1 μg/kg of TCDD in C57BL/6 mice. Thus, C57BL/6 *lpr/lpr* mice needed 10 fold more TCDD to induce similar toxicity in the thymus as the C57BL/6 mice. The difference between C57BL/6 *lpr/lpr* and C57BL/6 mice was less dramatic when peripheral T cell responsiveness was studied. This may be because thymus is more sensitive than peripheral T cells to TCDD-mediated immunotoxicity. For example, at 0.1 μg/kg of TCDD, none of the strains studied exhibited a significant decrease in antigen-specific responsiveness in the periphery, whereas, at this dose, there was marked decrease in thymic cellularity in many strains.

The exact mechanism by which Fas regulates TCDD-induced immunotoxicity is not clear. This can accounted for by the fact that TCDD may activate

certain cytokines involved in the upregulation of Fas or its ligand thereby facilitating the thymocyte or T cell interaction with other cells leading to apoptosis. It is believed that apoptosis plays a major role in the negative selection of thymocytes and a defect in the expression of Fas as seen in *lpr/lpr* mice, leads to lymphoproliferative disease with the accumulation of abnormal $CD4^{-}CD8^{-}$ T cells (Hammond *et al.*,1993). In this context, it is interesting to note that TCDD can increase serum TNF levels following endotoxin treatment and that the endotoxin hypersensitivity induced by TCDD can be inhibited by anti-TNF antibodies (Clark, Lucier *et al.*,1991; Clark, Taylor *et al.*,1991). Because Fas belongs to the TNF receptor family, it is possible that TCDD may affect the regulation of Fas or its ligand thereby mediating toxicity.

MHC genes have been shown to regulate the susceptibility to autoimmune diseases, development of tumors, responsiveness to antigens as well as immunotoxicity of certain compounds (Laurence, 1985). Also, class II MHC molecules play an important role in the presentation of foreign peptide by B cells and macrophages to T helper cells. It has been previously suggested that TCDD may upregulate MHC expression (Puhvel and Sakamoto; Kerkvliet and Oughton, 1993). Such events may alter the negative or positive T cell selection in the thymus. Also, there is a possibility that the decrease in immune responsiveness of T cells to antigenic challenge may be caused by TCDD interfering with antigen processing or association of the peptide with MHC molecules or interleukin secretion, thereby failing to activate the T cells efficiently. In order to address this, we used T cells from TCDD-treated or

untreated mice and stimulated them in culture with macrophages from TCDD-treated or untreated mice in the presence of conalbumin. These results demonstrated that TCDD acts at the T cell level rather than at the level of antigen presenting cells. These findings are consistent with the earlier observation that macrophage functions are not altered by TCDD (Holsapple *et al.*,1991). Together, our studies therefore demonstrated that TCDD may directly affect T cell functions *in vivo.*

In the current study, it was also noted that TCDD did not affect resting T cells but caused significant decrease in the responsiveness of T cells that were undergoing activation *in vivo* upon challenge with an antigen such as conalbumin. These results were clearly demonstrated using the same lymph nodes which contained a mixture of naive T cells and antigen specific T cells. Thus, TCDD, while affecting the responsiveness of sensitized T cells to conalbumin *in vitro,* failed to decrease the responsiveness to polyclonal mitogens. Similar results were recently reported in another study (Lundberg *et al.*,1992). These findings may also explain why thymus in which T cells are actively differentiating is more susceptible to TCDD-mediated toxicity when compared to peripheral lymphoid organs of unimmunized mice.

2.5 Conclusions:

1. In the current study, we investigated factors which regulate the immunotoxicity of TCDD. The data demonstrated that although the immunotoxicity of TCDD at lower doses was dependent on the Ah responsiveness, other secondary factors such as expression of Fas, a molecule involved in apoptosis and MHC, an important locus regulating immune reactions, played an important role in regulating the degree of toxicity.

2. Mice with homozygous *lpr* mutation which leads to non-expression of Fas, were more resistant to TCDD-mediated thymic atrophy and decrease in peripheral T cell responsiveness to antigenic challenge with conalbumin. These data indirectly suggested that TCDD may mediate toxicity by inducing apoptosis in thymocytes and antigen-activated T cells.

3. Using B10 congenic strains it was noted that B10.D2 (H-2^d) mice were more sensitive to TCDD-mediated immunotoxicity when compared to the B10 (H-2^b). Inasmuch as, both strains are Ah-responsive and differ only at the MHC, our data suggested tht some MHC phenotypes may be more susceptible to TCDD-mediated immunotoxicity when compared to others. Thus, TCDD may indirectly mediate its effect through MHC. These studies are important in suggesting that not only individuals based on polymorphism in MHC, may respond in a similar fashion to TCDD.

4. TCDD was found to affect only those cells that were actively differentiating

but not those that were naive and resting. For example, thymus where the T cells differentiate actively and lymph nodes in which T cells were activated by injecting an antigen such as conalbumin, demonstrated susceptibility to TCDD-mediated toxicity, whereas, T cells from lymph nodes which had not been activated *in vivo* did not exhibit any immunological defects. This is an important finding which suggests that TCDD may not be immunotoxic to peripheral T cells unless thay are responding to an antigen *in vivo* at the same time that they are exposed to TCDD.

5. TCDD was found to directly affect the antigen-specific T cells but not the antigen-processing and presenting cells such as macrophages or B cells.

Thus the current study had identified several factors which may regulate the immunotoxicity of TCDD. Further research in this direction should shed new light in understanding the mechanism of immunotoxicity of TCDD and possible approaches to neutralize such toxicity.

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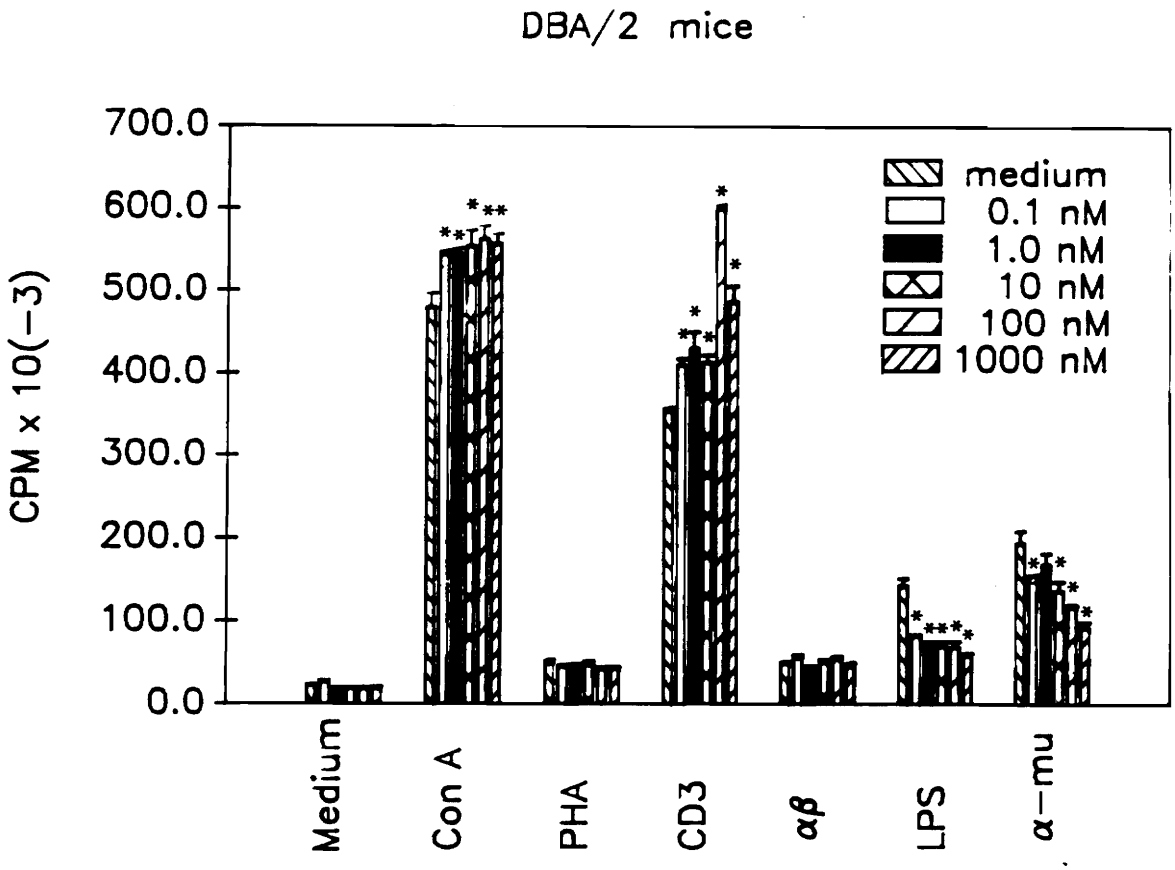

Fig. 1. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing TCDD (0.1, 1.0, 10, 100, or 1000 nM TCDD). T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

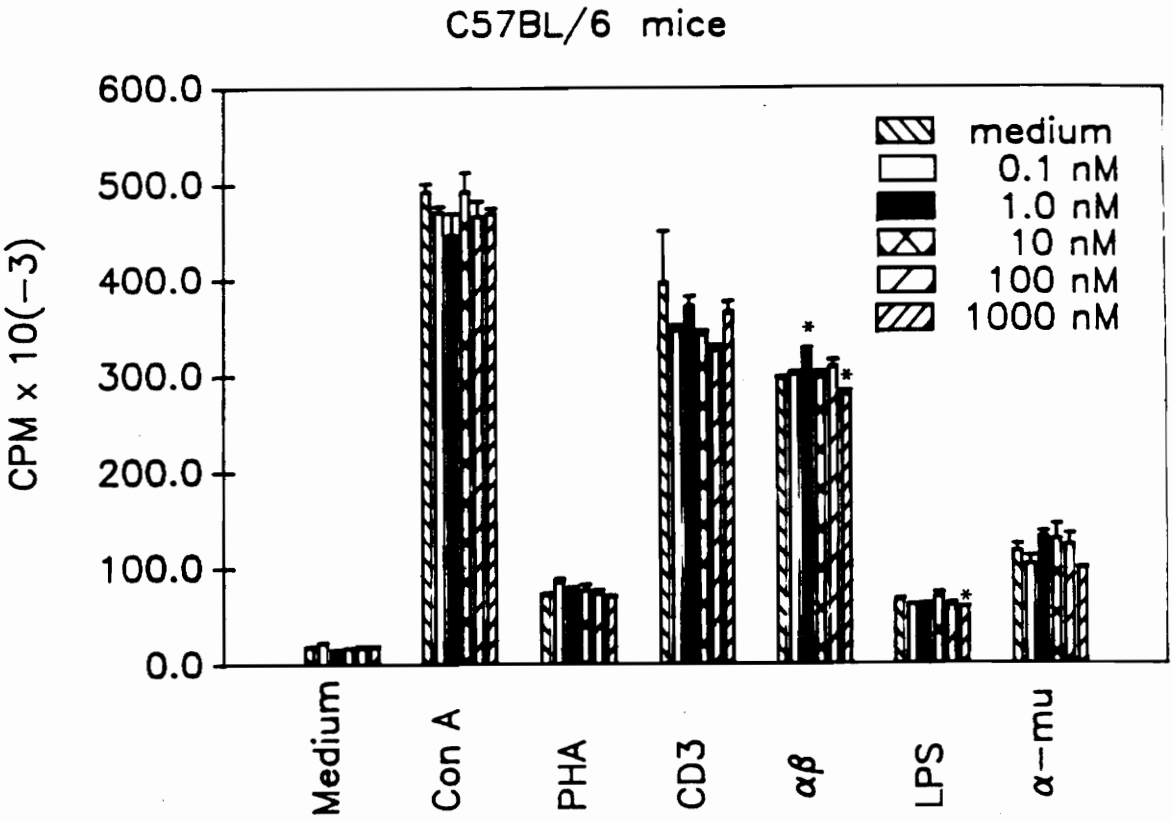

Fig. 2. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing TCDD (0.1, 1.0, 10, 100, or 1000 nM TCDD). T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

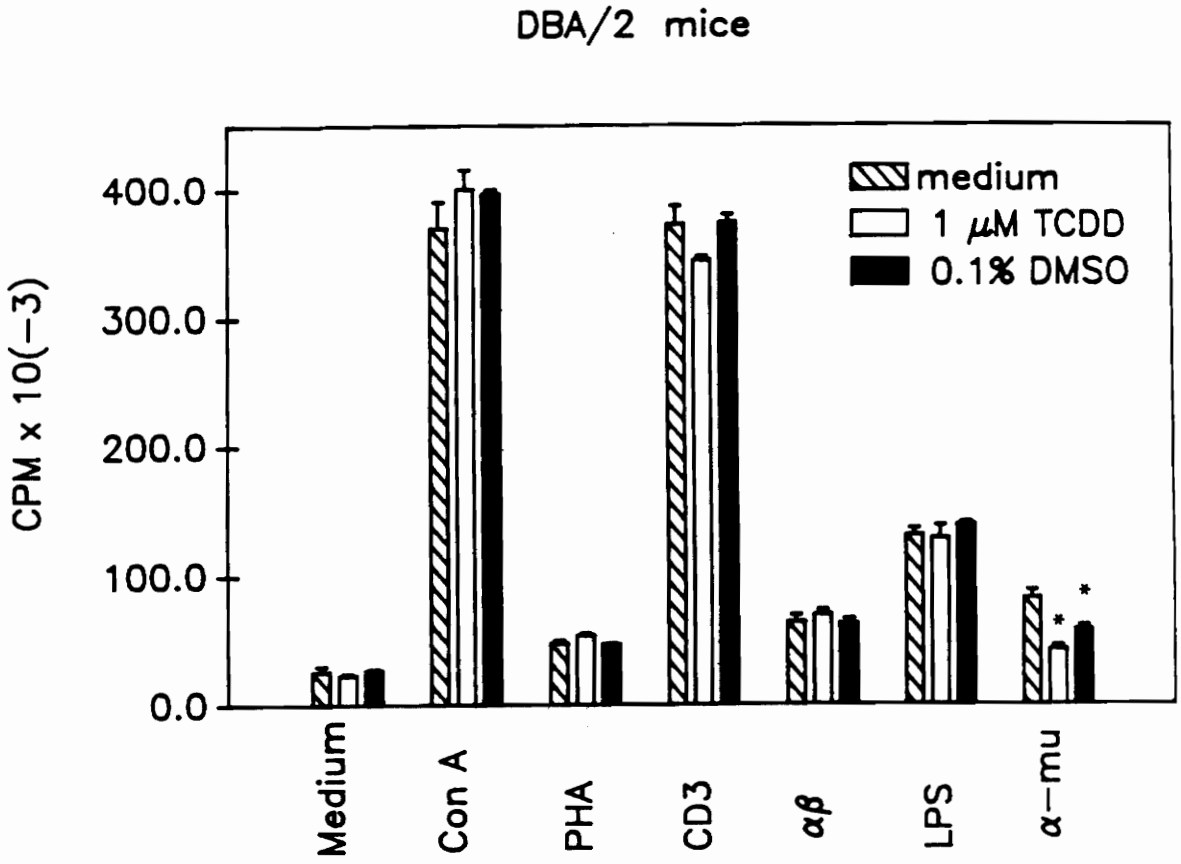

Fig. 3. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing 1 μM TCDD or control media containing 0.1% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

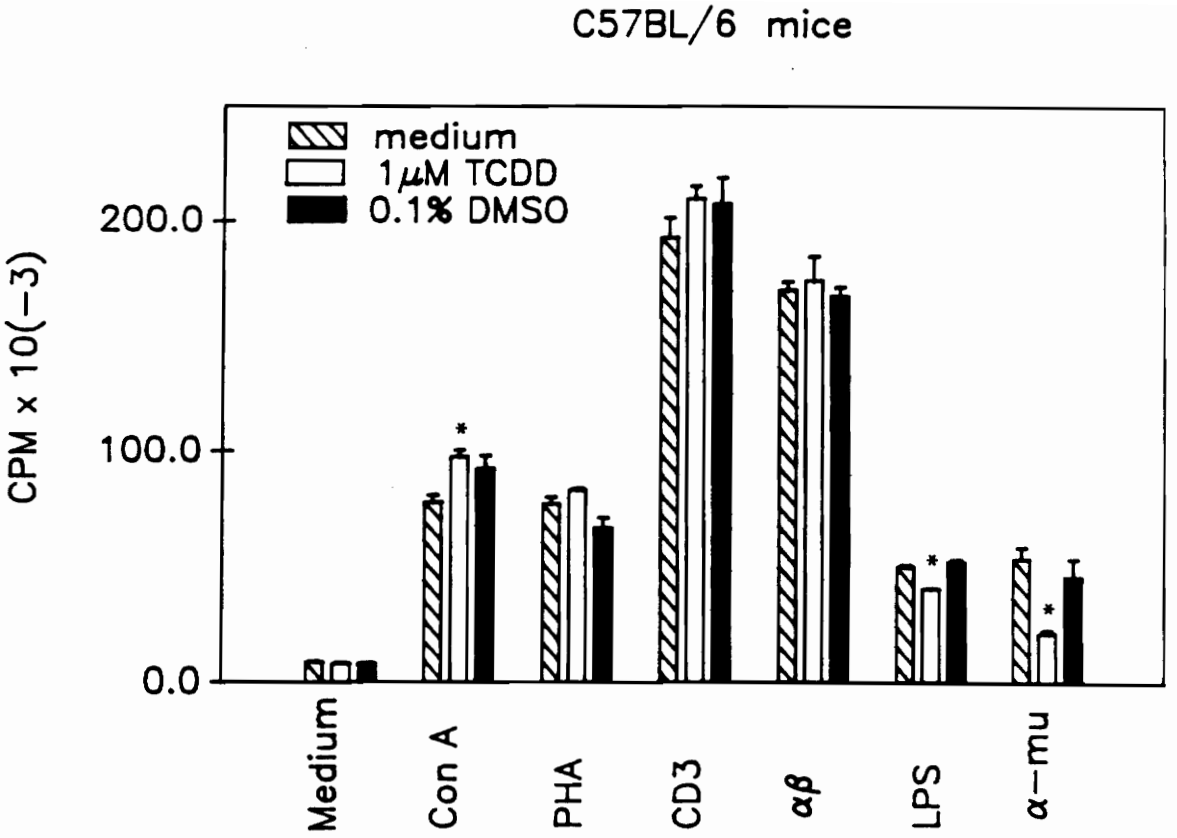

Fig. 4. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing 1 μM TCDD or control media containing 0.1% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

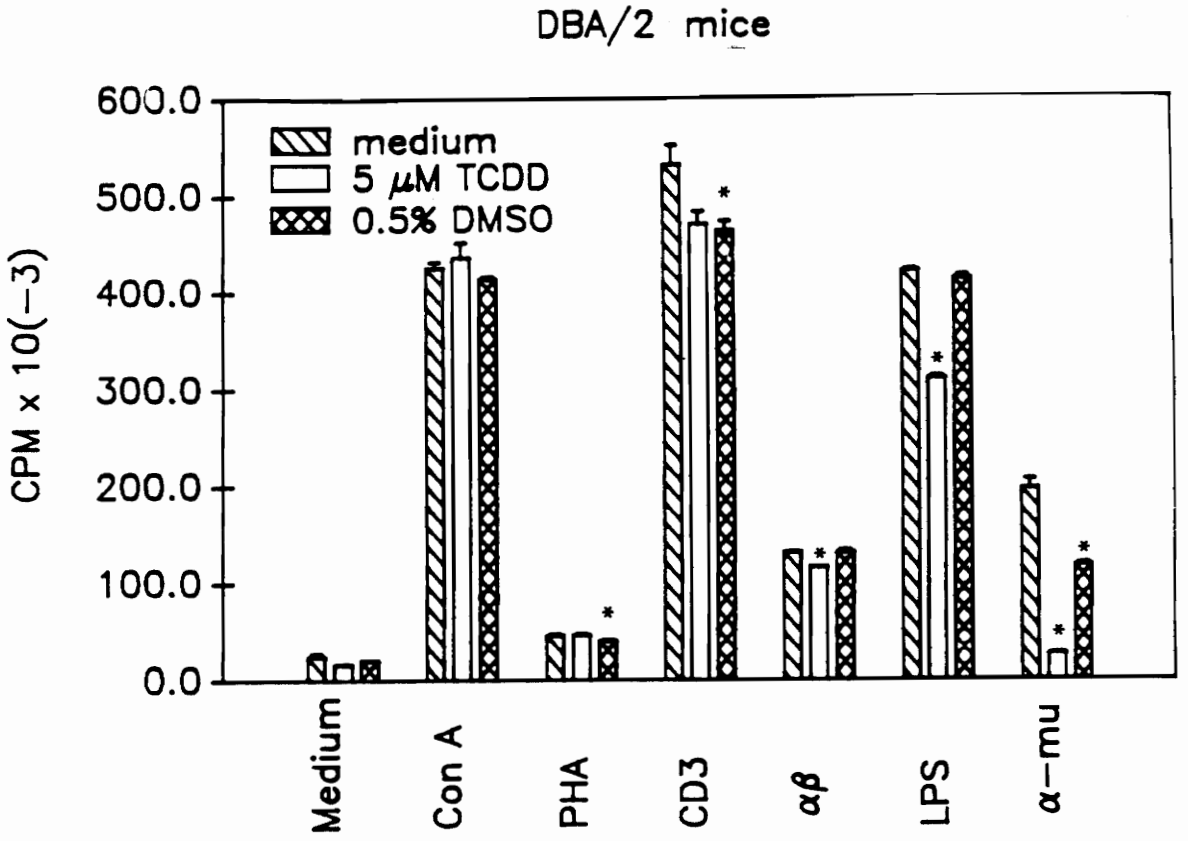

Fig. 5. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing 5 μM TCDD or control media containing 0.5% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

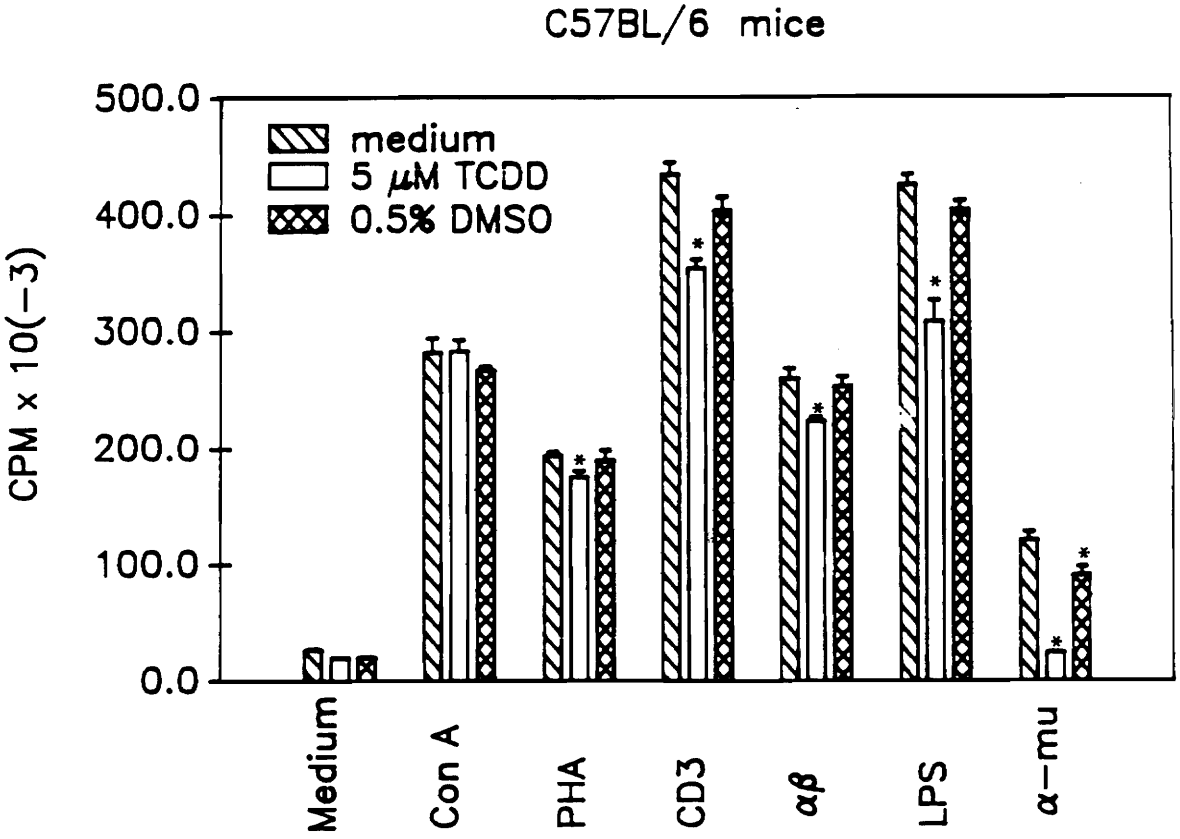

Fig. 6. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing 5 μM TCDD or control media containing 0.5% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

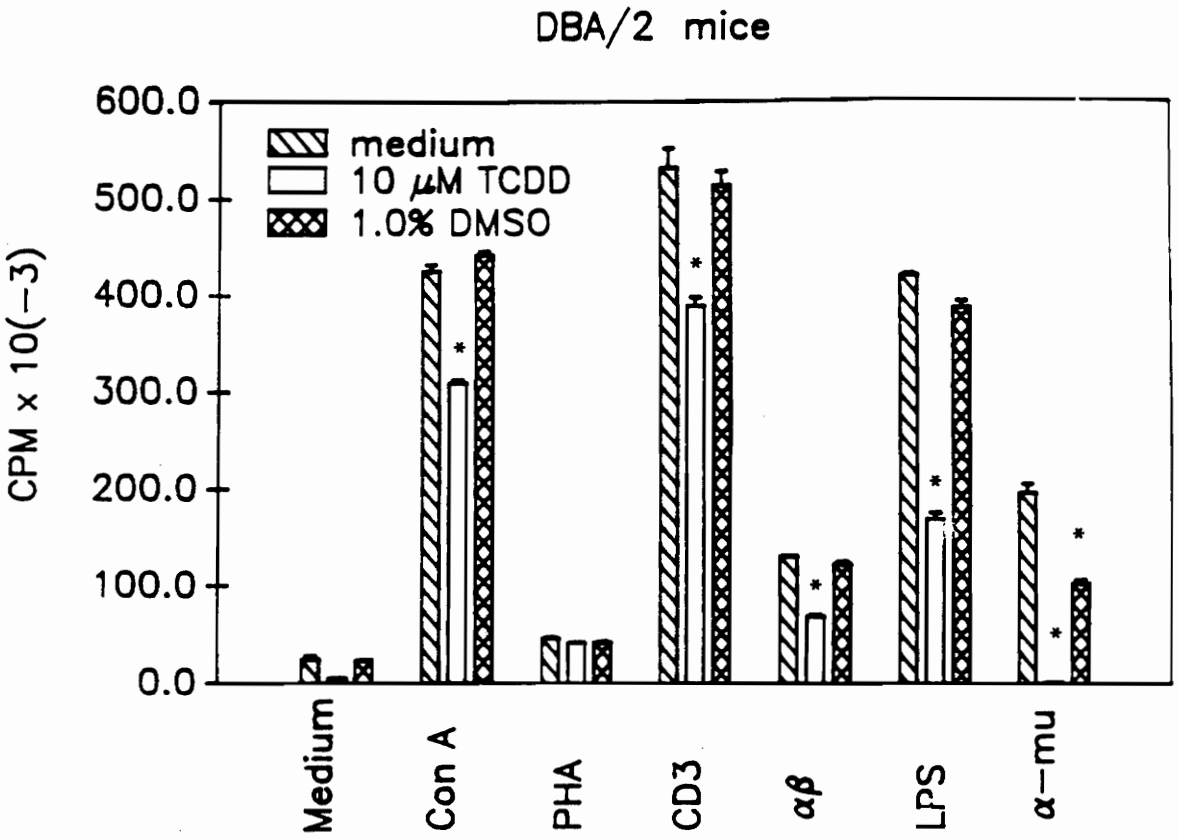

Fig. 7. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing 10 μM TCDD or control media containing 1.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

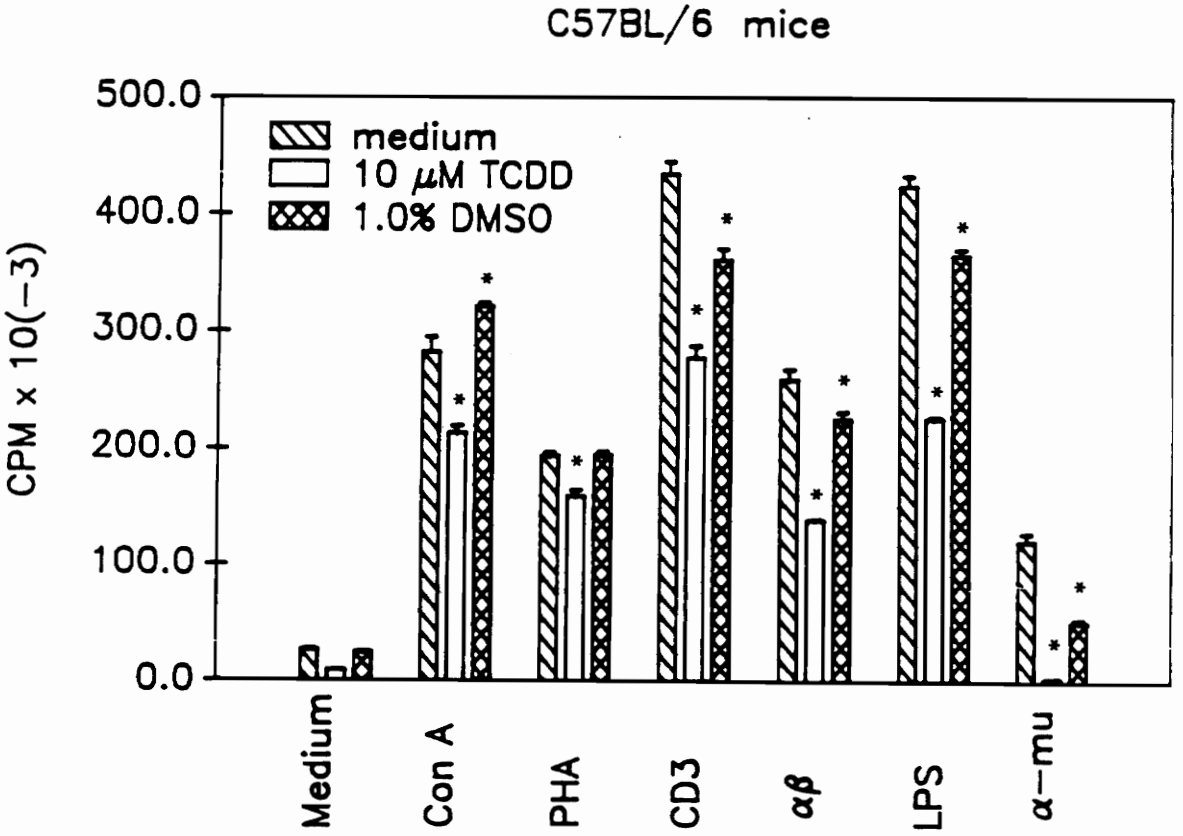

Fig. 8. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing 10 μM TCDD or control media containing 1.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

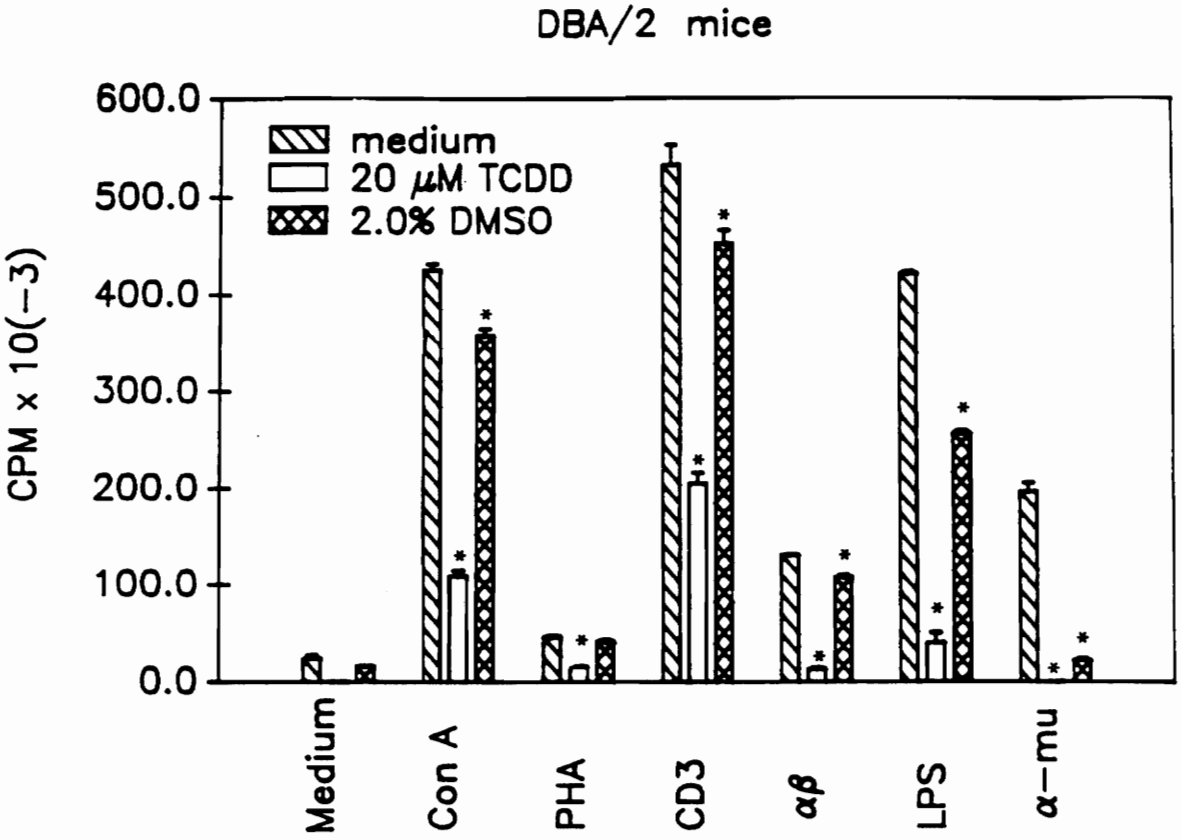

Fig. 9. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing 20 μM TCDD or control media containing 2.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

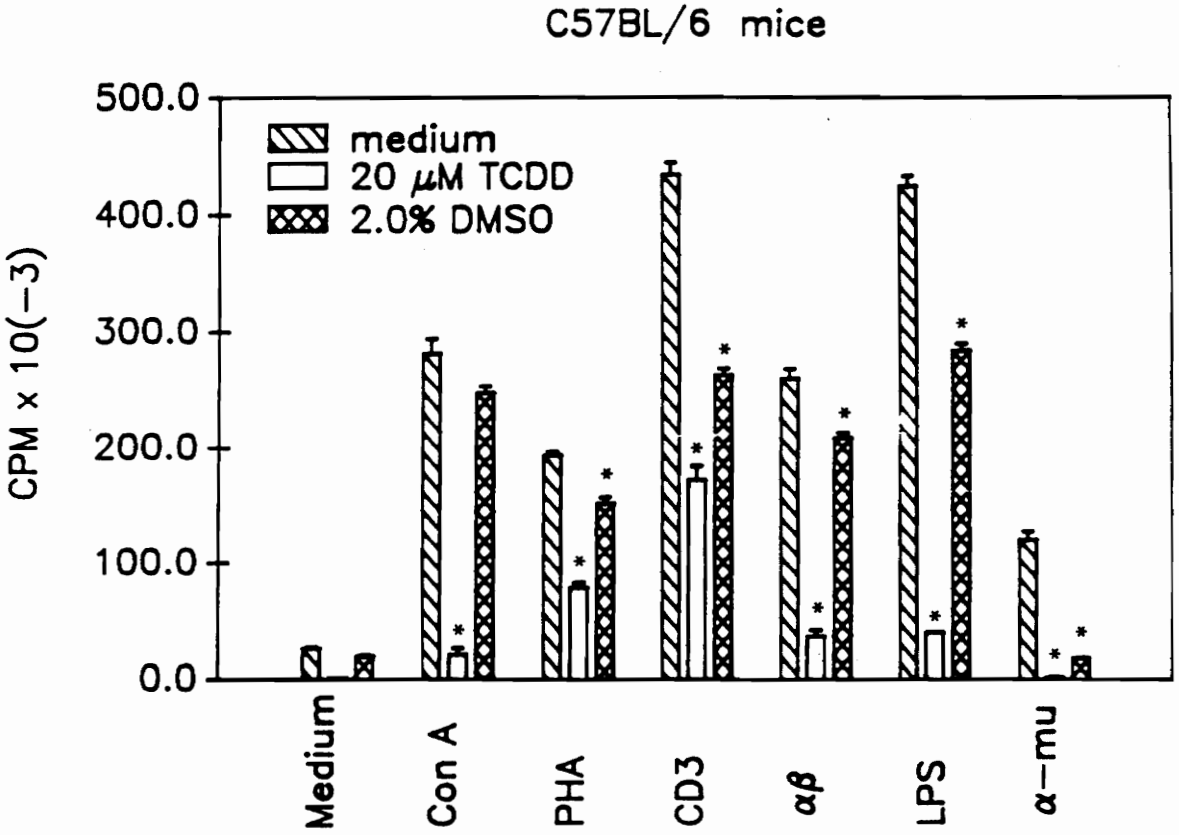

Fig. 10. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing 20 μM TCDD or control media containing 2.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

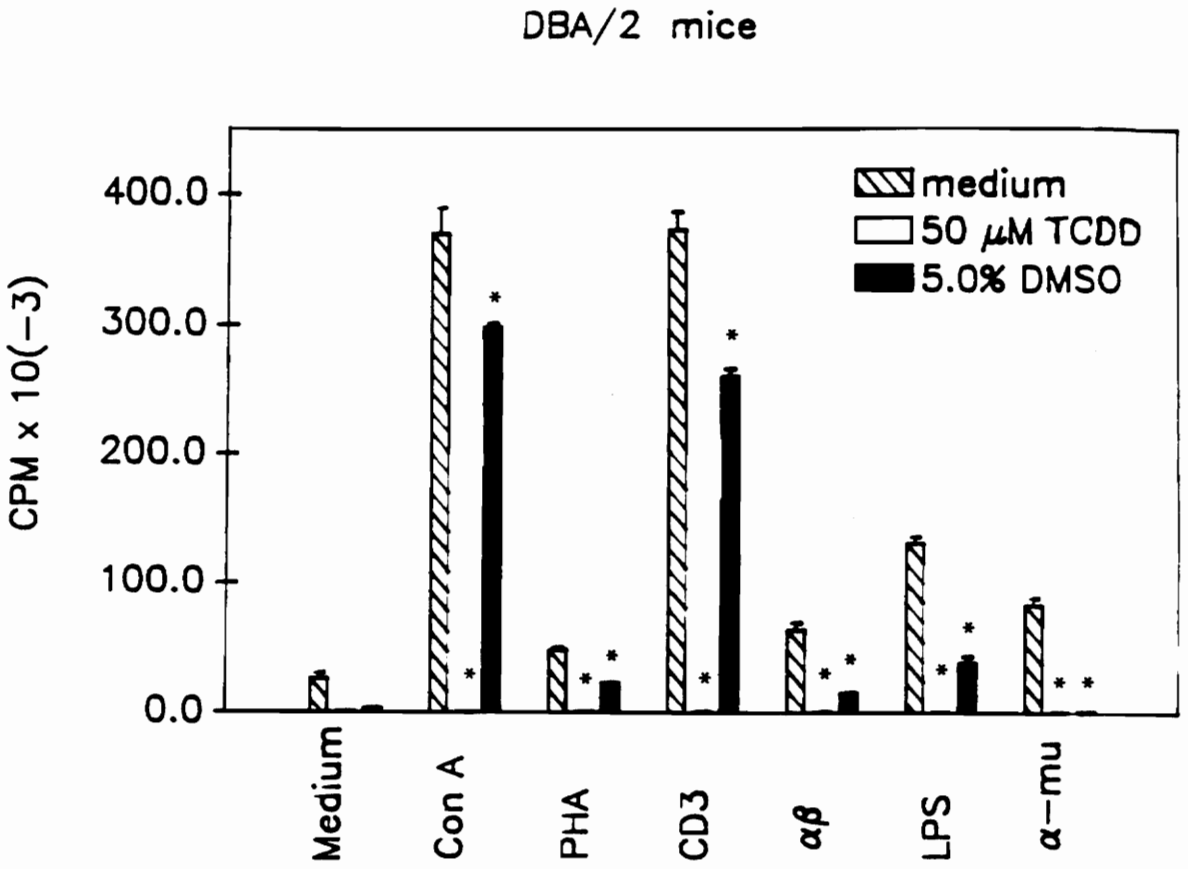

Fig. 11. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from DBA/2 mice were tested for their ability to respond to T and B cell mitogens in media containing 50 μM TCDD or control media containing 5.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

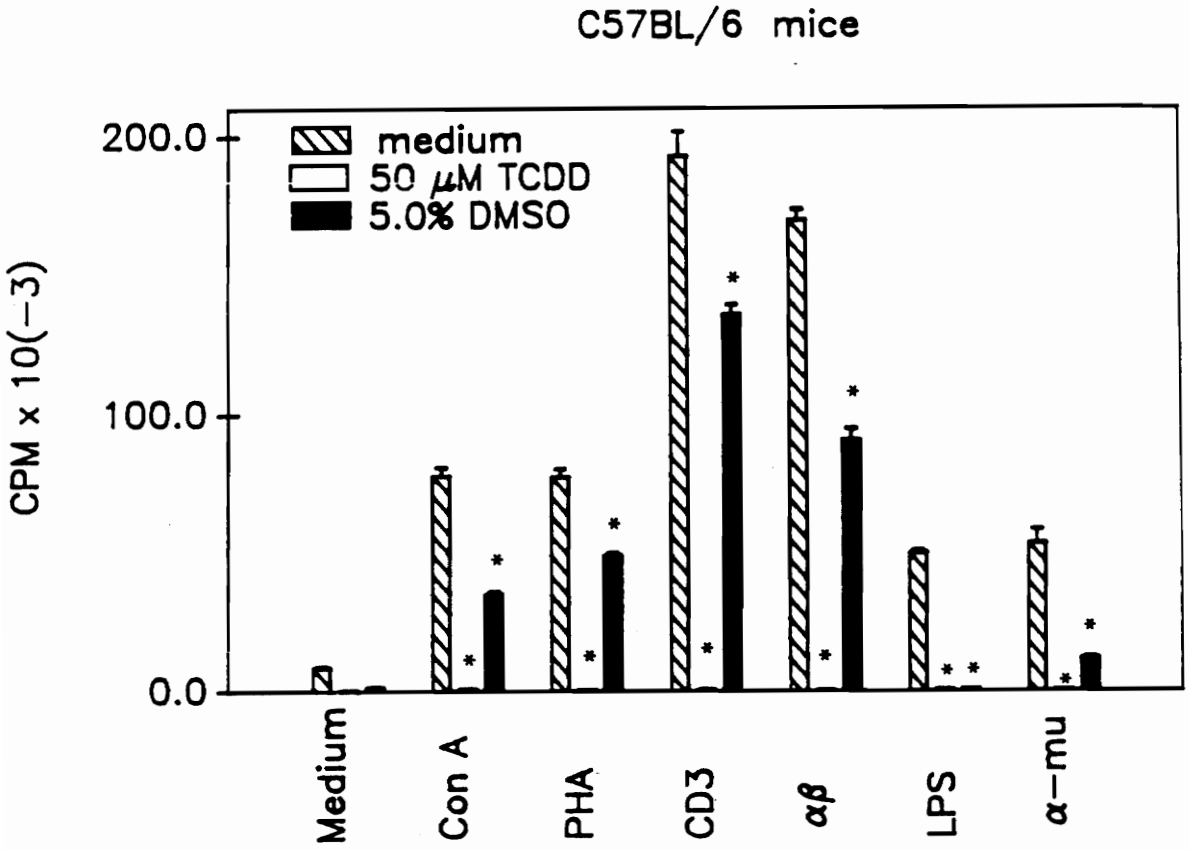

Fig. 12. In vitro effect of TCDD on the ability of spleen cells to respond to T and B cell mitogens. Whole spleen suspensions from C57BL/6 mice were tested for their ability to respond to T and B cell mitogens in media containing 50 μM TCDD or control media containing 5.0% DMSO. T cells were stimulated with Con A (5 μg/ml), PHA (25 μg/ml), anti-CD3 Ab (1:25 final dilution), and anti-αβ Ab (1:20 final dilution). B cells were stimulated with LPS (50 μg/ml) and anti-IgM Ab (25 μg/ml). Cell proliferation was measured by ^{3}H-thymidine uptake. Data with p values less than 0.05 have been depicted with an asterisk.

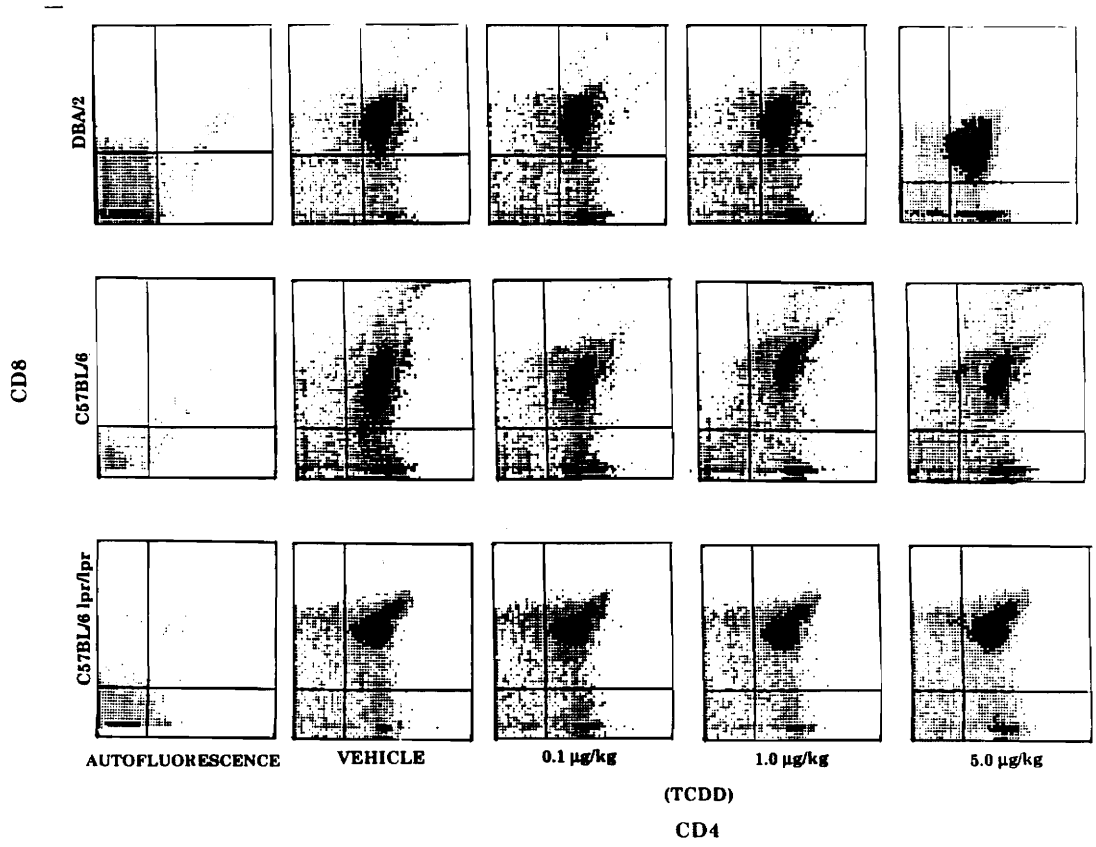

Fig. 13. Flow cytometric analysis of T cells in the thymus. Thymocytes from vehicle or TCDD-treated mice were stained with FITC-conjugated anti-CD4 mAbs and PE-conjugated anti-CD8 mAbs and the cells were analyzed using a flow cytometer. The upper left quadrant represents $CD8^+$ T cells; upper right, $CD4^+CD8^+$ T cells; lower left, $CD4^-CD8^-$ and lower right, $CD4^+$ T cells. Summary of multiple experiments depicting the percentages of various T cell subsets has been shown in Table 2.

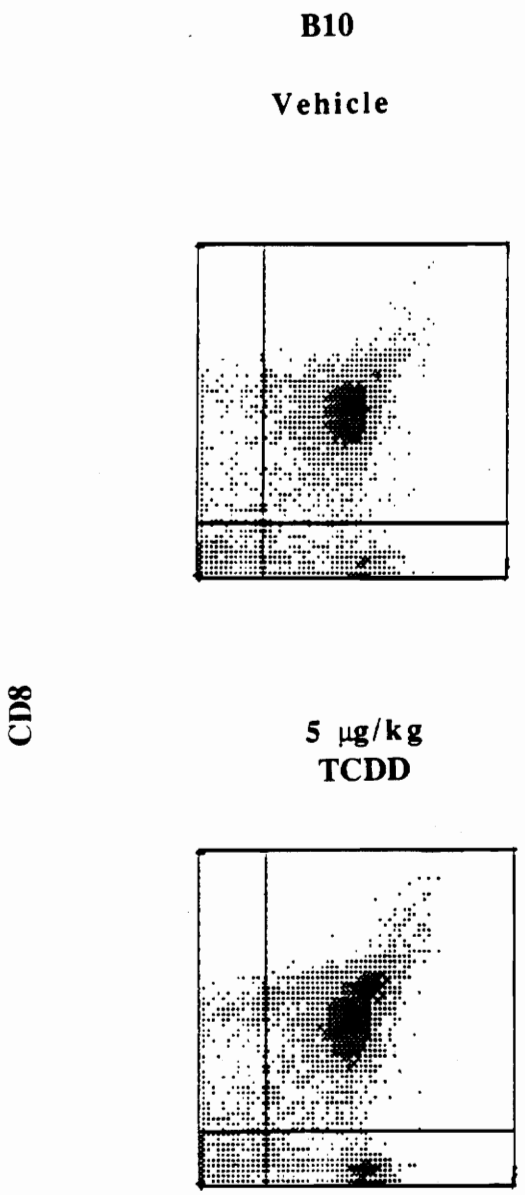

Fig. 14. Flow cytometric analysis of T cells in the thymus. Thymocytes from vehicle or TCDD-treated mice were stained with FITC-conjugated anti-CD4 mAbs and PE-conjugated anti-CD8 mAbs and the cells were analyzed using a flow cytometer. The upper left quadrant represents $CD8^+$ T cells; upper right, $CD4^+CD8^+$ T cells; lower left, $CD4^-CD8^-$ and lower right, $CD4^+$ T cells. Summary of multiple experiments depicting the percentages of various T cell subsets has been shown in Table 2.

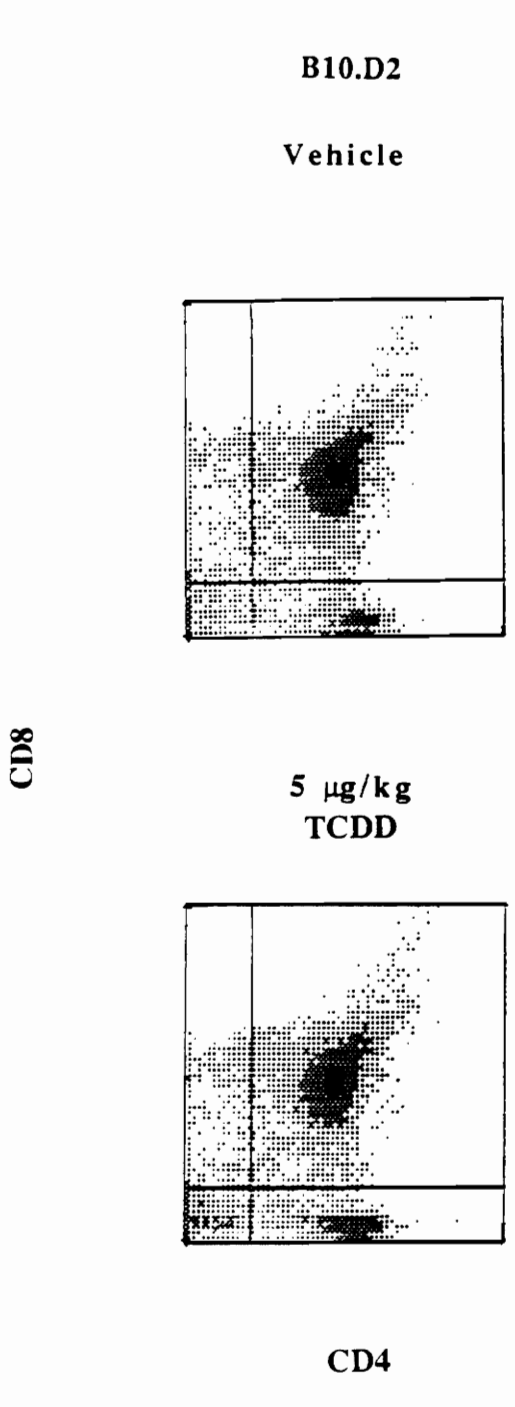

Fig. 15. Flow cytometric analysis of T cells in the thymus. Thymocytes from vehicle or TCDD-treated mice were stained with FITC-conjugated anti-CD4 mAbs and PE-conjugated anti-CD8 mAbs and the cells were analyzed using a flow cytometer. The upper left quadrant represents $CD8^+$ T cells; upper right, $CD4^+CD8^+$ T cells; lower left, $CD4^-CD8^-$ and lower right, $CD4^+$ T cells. Summary of multiple experiments depicting the percentages of various T cell subsets has been shown in Table 2.

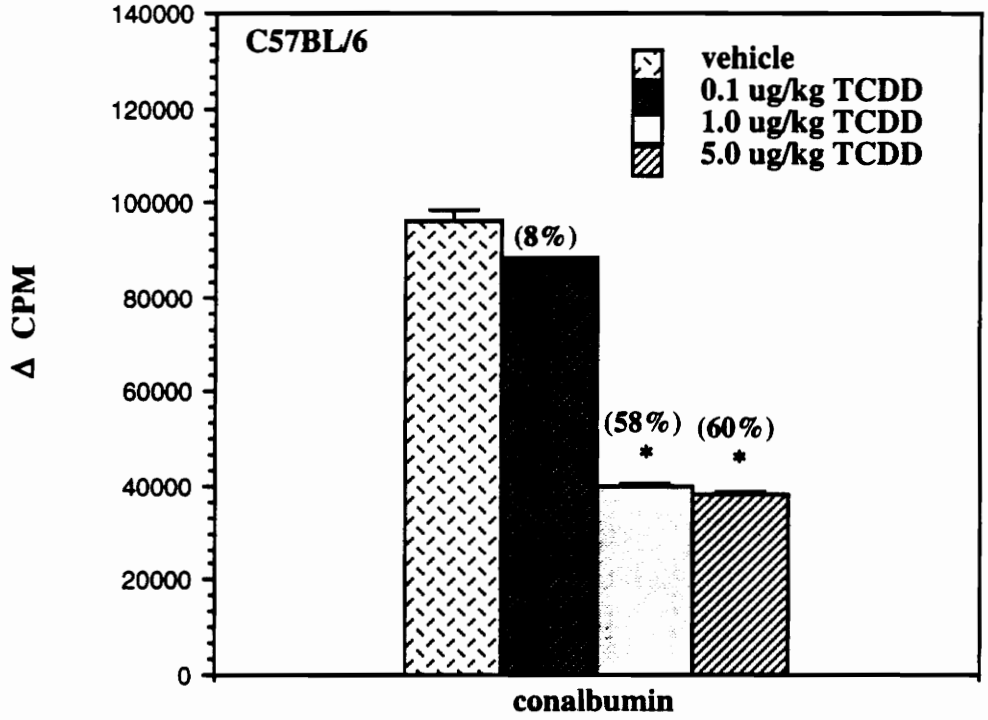

Fig. 16. Role of Fas in TCDD-mediated immunomodulation of peripheral antigen-specific T cell responsiveness to conalbumin. C57BL/6 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to conalbumin. The cell proliferation was measured by ^{3}H-thymidine incorporation assay. The vertical bars represent mean Δ cpm±S.E. of triplicate cultures. The Δ cpm was calculated by subtracting the cpm in cultures incubated with medium alone from the cpm obtained in cultures stimulated with conalbumin. Data with statistically significant differences from the controls have been depicted with an asterisk ($p<0.05$).

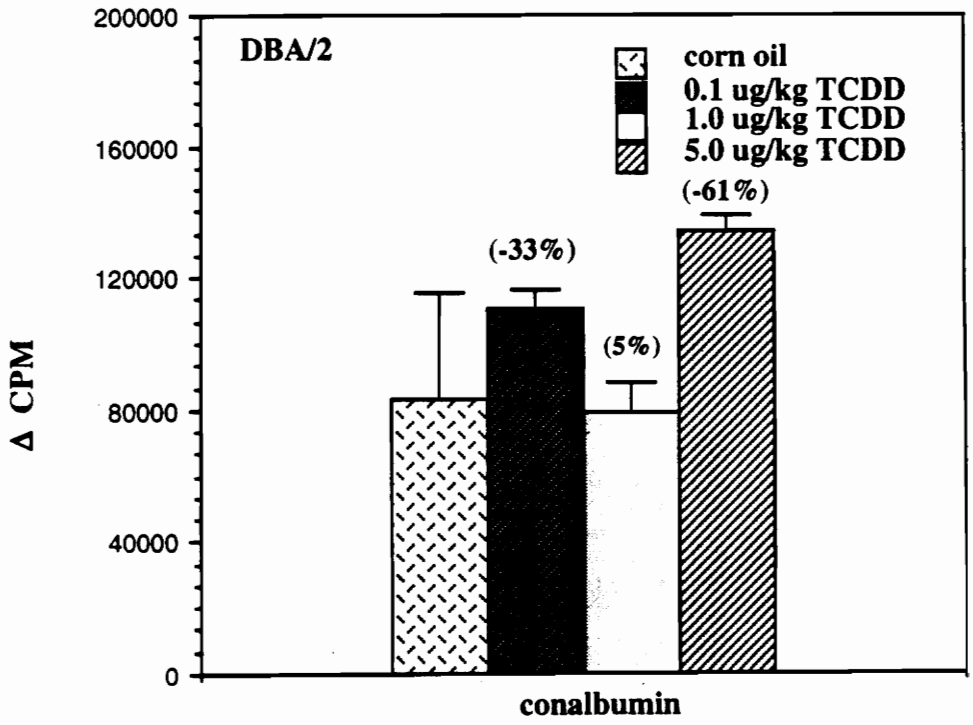

Fig. 17. Role of Fas in TCDD-mediated immunomodulation of peripheral antigen-specific T cell responsiveness to conalbumin. DBA/2 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to conalbumin as described in Fig. 16. Data were statistically insignificant at $p<0.05$.

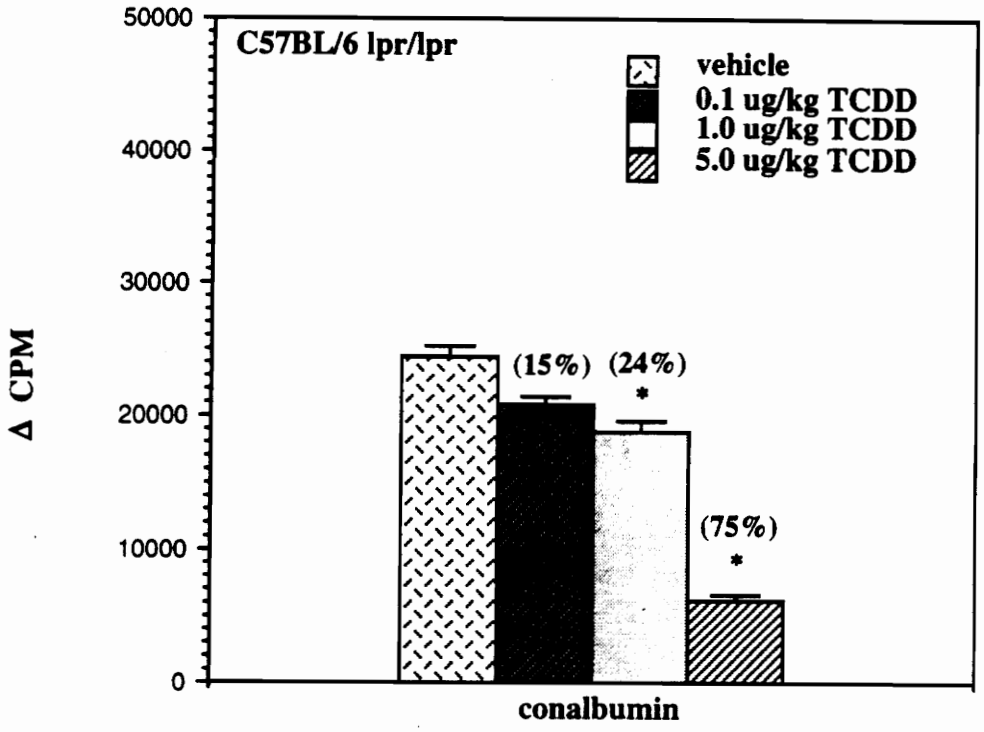

Fig. 18. Role of Fas in TCDD-mediated immunomodulation of peripheral antigen-specific T cell responsiveness to conalbumin. C57BL/6 *lpr/lpr* mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to conalbumin as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

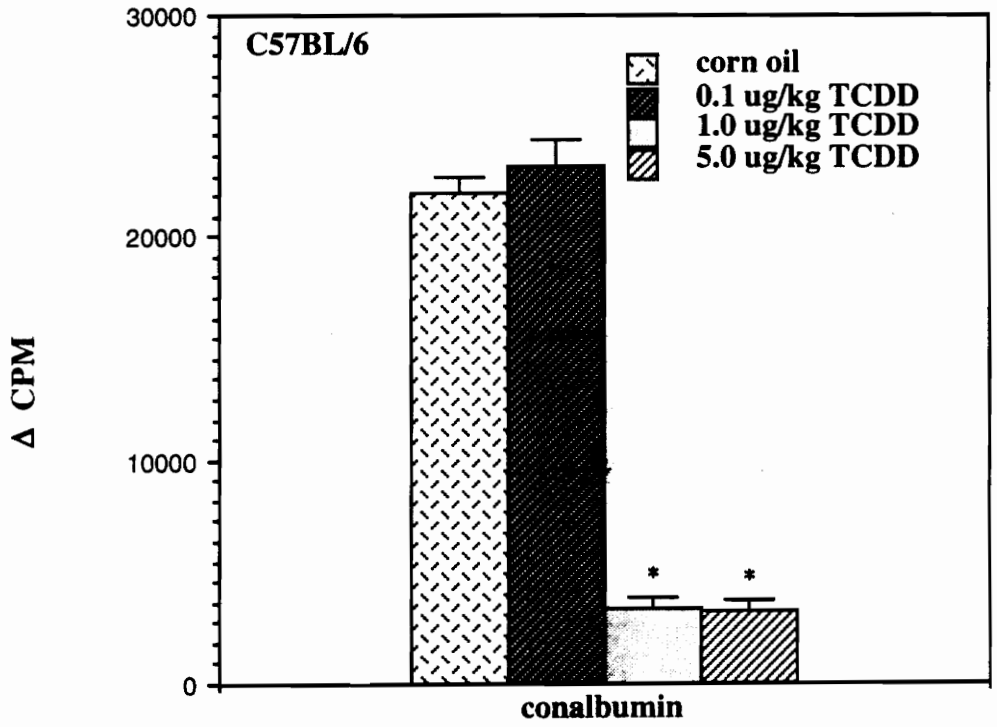

Fig. 19. TCDD-mediated immunomodulation of peripheral antigen-specific T cell responsiveness to conalbumin. C57BL/6 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the spleens were removed and the T cells were purified and tested for their ability to respond to conalbumin as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

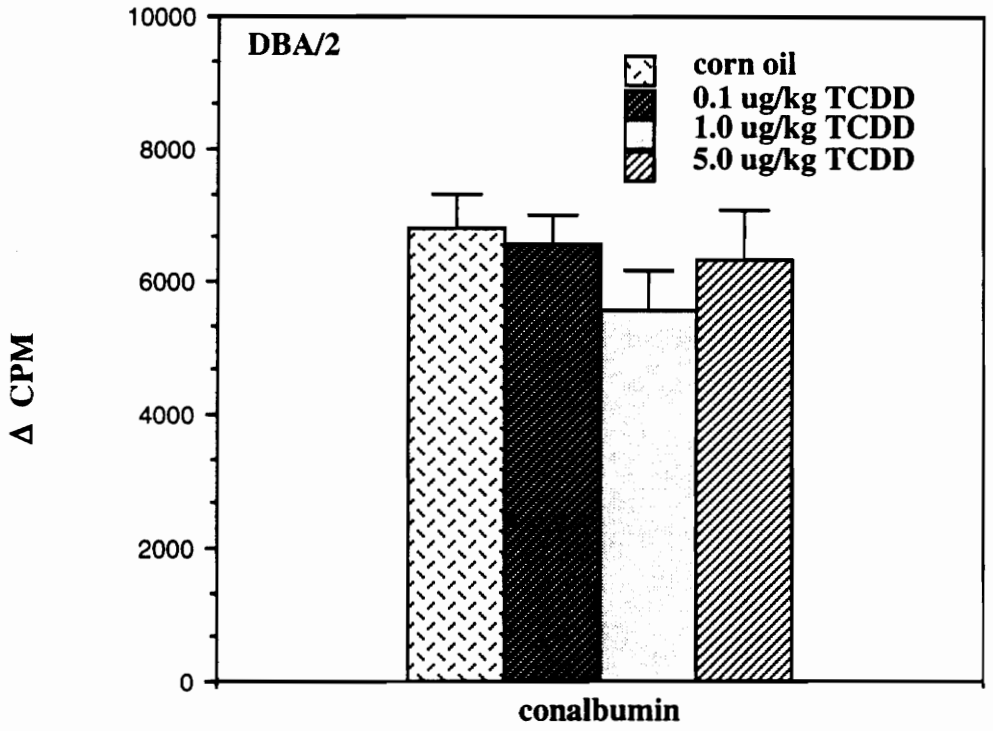

Fig. 20. TCDD-mediated immunomodulation of peripheral antigen-specific T cell responsiveness to conalbumin. DBA/2 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the spleens were removed and the T cells were purified and tested for their ability to respond to conalbumin as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

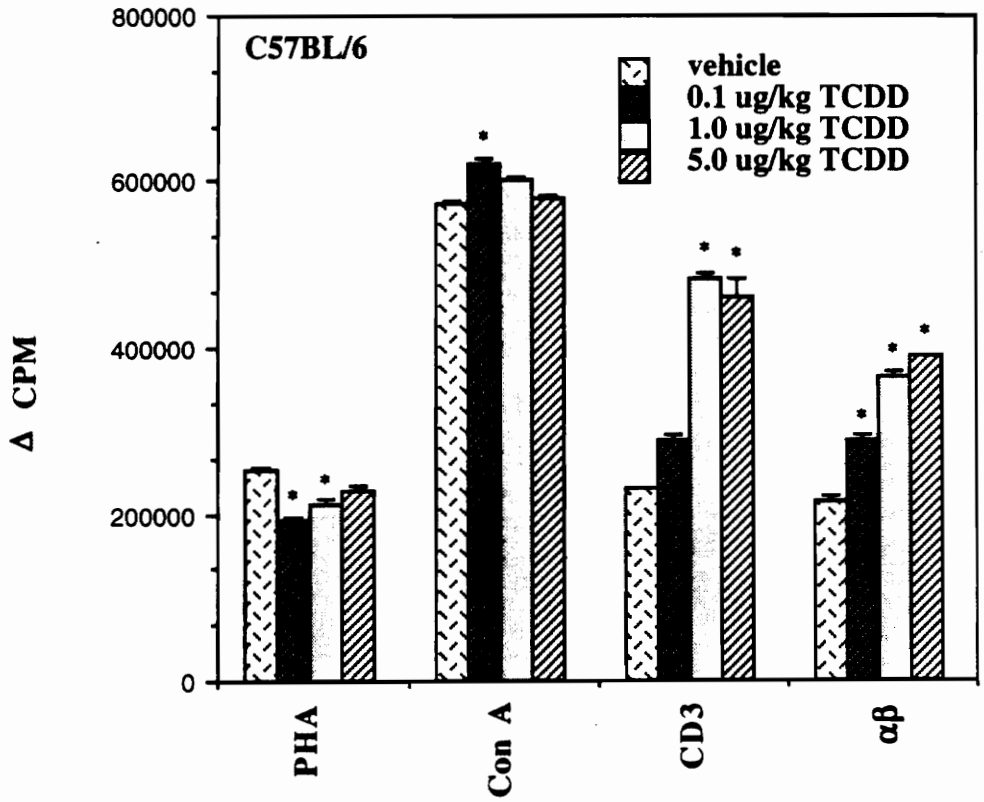

Fig. 21. Role of Fas in TCDD-mediated immunomodulation of peripheral T cell responsiveness to mitogens. C57BL/6 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to various T cell mitogens. The cell proliferation was measured by ^{3}H-thymidine incorporation assay. The vertical bars represent mean Δ cpm±S.E. of triplicate cultures. The Δ cpm was calculated by subtracting the cpm in cultures incubated with medium alone from the cpm obtained in cultures stimulated with conalbumin. Data with statistically significant differences from the controls have been depicted with an asterisk ($p<0.05$).

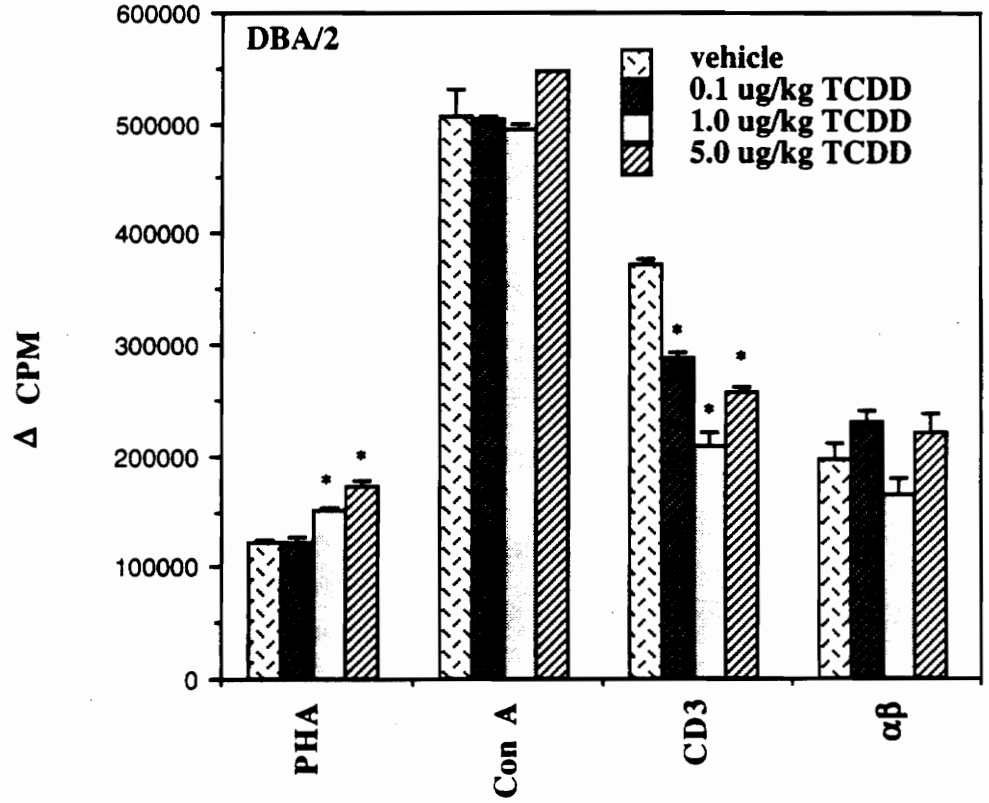

Fig. 22. Role of Fas in TCDD-mediated immunomodulation of peripheral T cell responsiveness to mitogens. DBA/2 mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to various T cell mitogens as described in Fig. 21. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

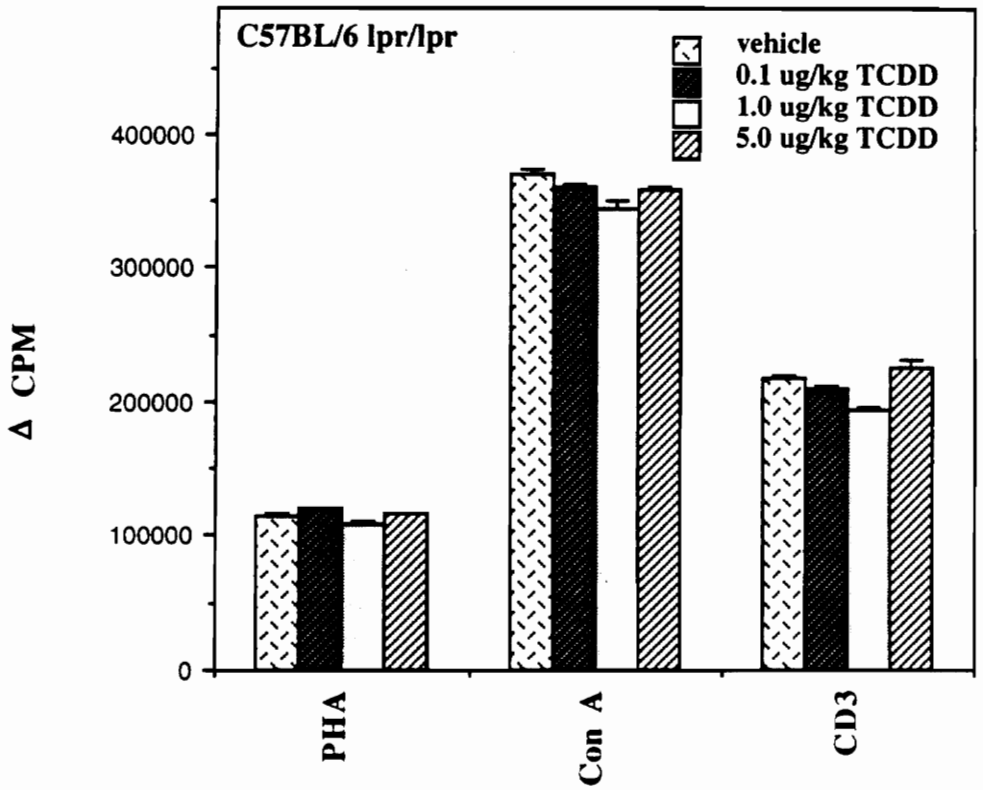

Fig. 23. Role of Fas in TCDD-mediated immunomodulation of peripheral T cell responsiveness to mitogens. C57BL/6 *lpr/lpr* mice were treated with TCDD or the vehicle for 11 days. On day 4, mice were challenged in the foot pads with conalbumin and on day 12, the draining LN cells were harvested and tested for their ability to respond to various T cell mitogens as described in Fig. 21. Data were statistically insignificant at $p<0.05$.

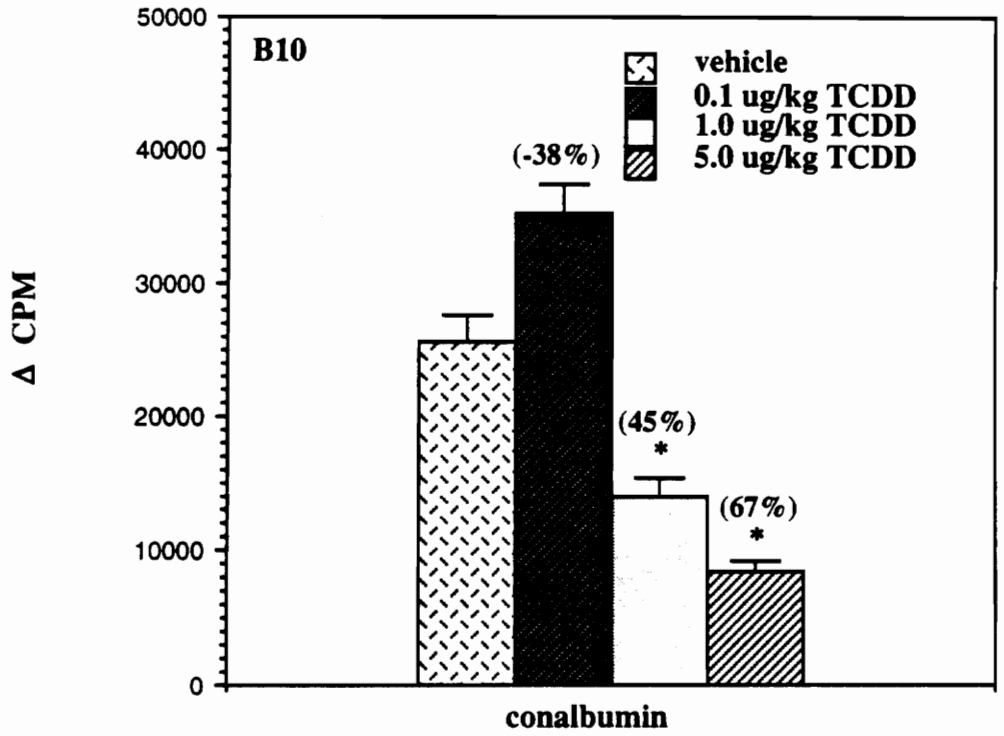

Fig. 24. Role of MHC phenotype on TCDD-induced immunotoxicity of peripheral antigen-specific responsiveness of T cells. TCDD or vehicle-treated B10 (H-2^b) mice were immunized with conalbumin and the draining lymph nodes were tested for responsiveness to conalbumin as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

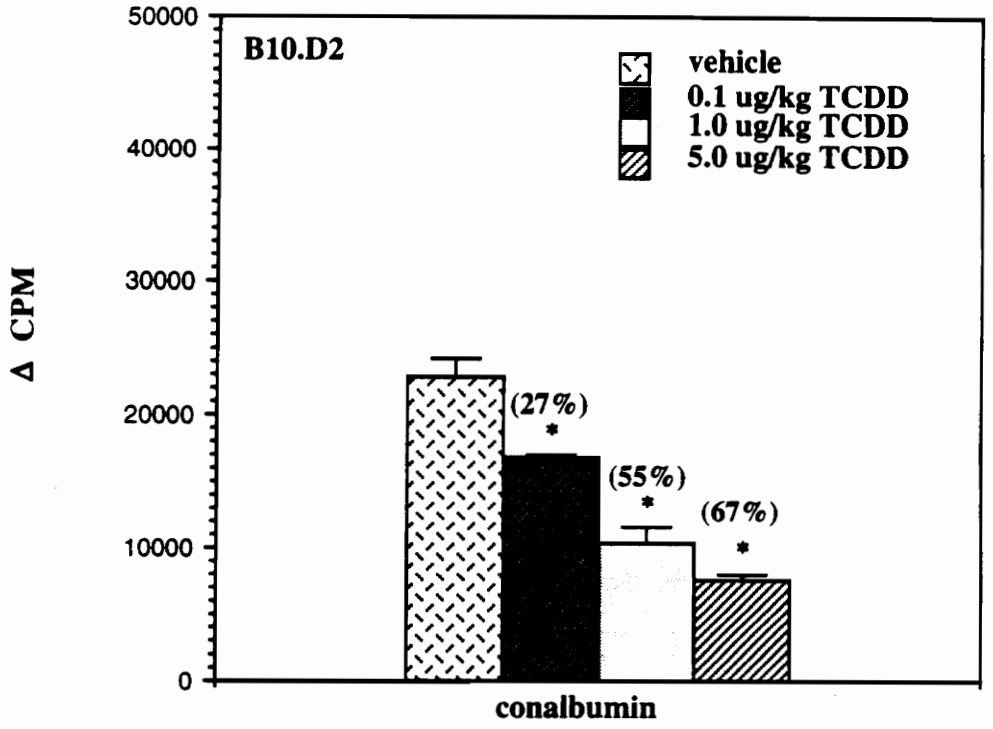

Fig. 25. Role of MHC phenotype on TCDD-induced immunotoxicity of peripheral antigen-specific responsiveness of T cells. TCDD or vehicle-treated B10.D2 (H-2^d) mice were immunized with conalbumin and the draining lymph nodes were tested for responsiveness to conalbumin as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

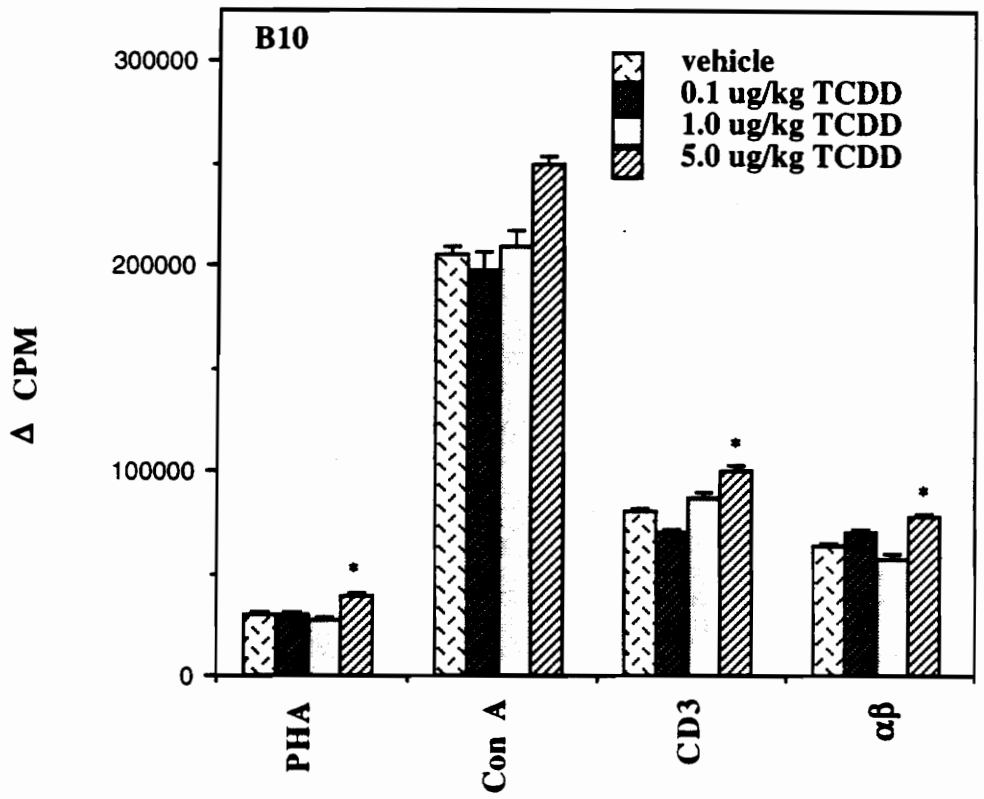

Fig. 26. Role of MHC phenotype on TCDD-induced immunotoxicity of peripheral responsiveness of T cells. TCDD or vehicle-treated B10 (H-2^b) mice were immunized with conalbumin and the draining lymph nodes were tested for responsiveness to T cell mitogens as described in Fig. 21. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

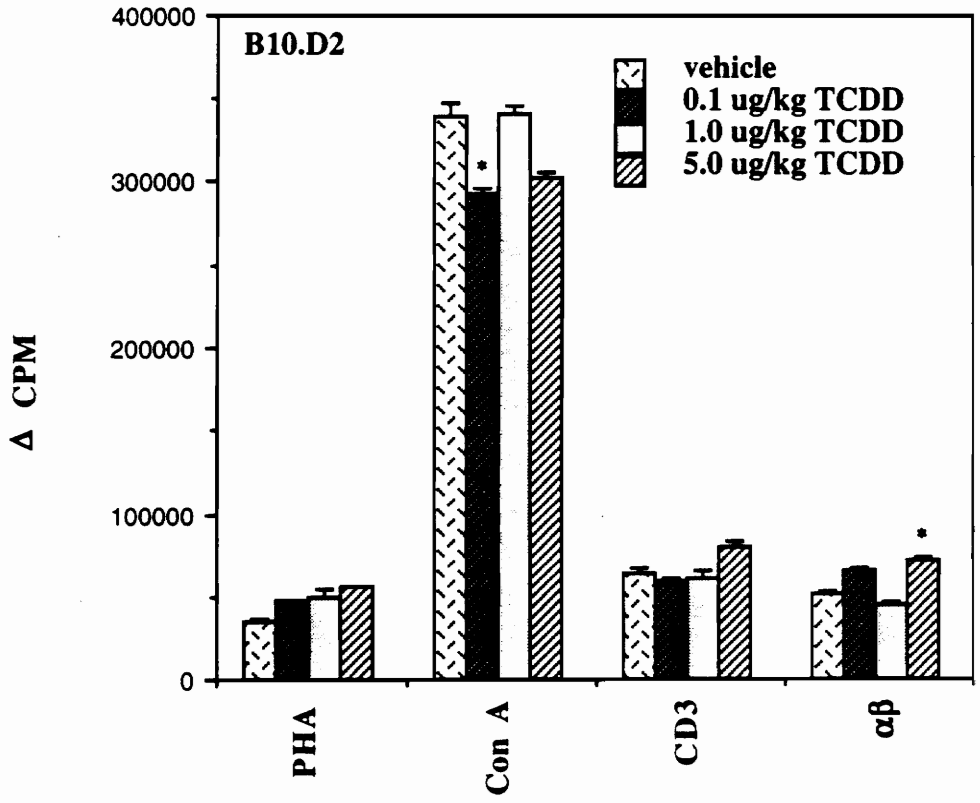

Fig. 27. Role of MHC phenotype on TCDD-induced immunotoxicity of peripheral responsiveness of T cells. TCDD or vehicle-treated B10.D2 ($H\text{-}2^d$) mice were immunized with conalbumin and the draining lymph nodes were tested for responsiveness to T cell mitogens as described in Fig. 21. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

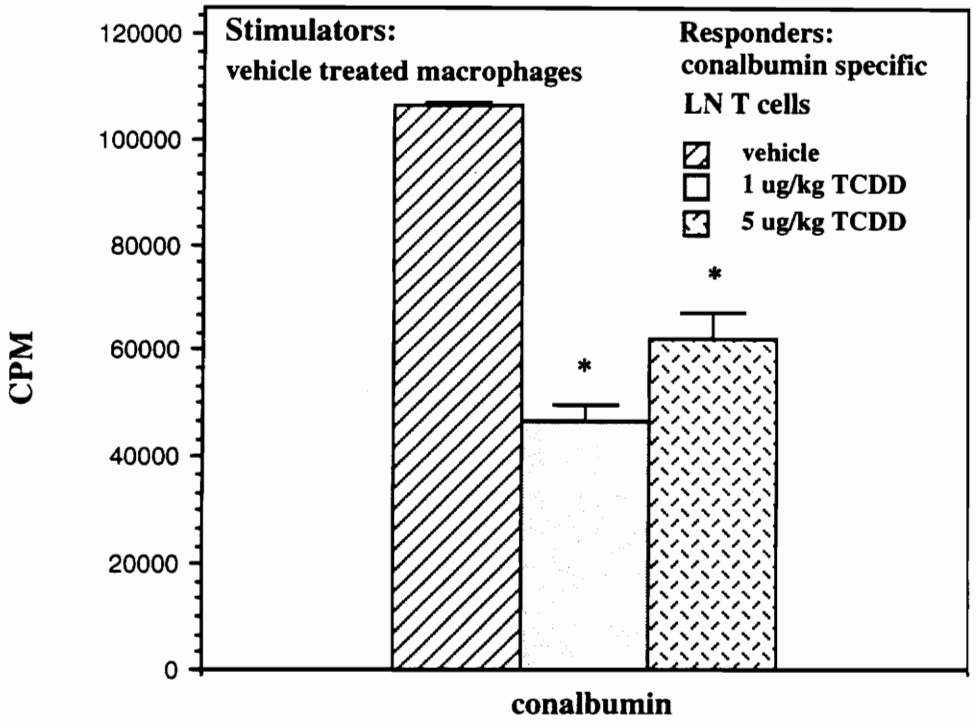

Fig. 28. TCDD affects the antigen-specific T cells but not the APC. Cell mixing experiments were carried out to investigate whether TCDD acts at the level of T cells or APC. C57BL/6 mice treated with TCDD or vehicle were immunized with conalbumin as described in Fig. 16. Next, T cells were purified from the draining lymph nodes (LN) of these mice and macrophages were isolated from the spleens. The ability of conalbumin-specific T cells from TCDD or vehicle-treated mice to respond to irradiated APC from TCDD or vehicle-treated mice in the presence of conalbumin was tested *in vitro* as described in Fig. 16. Data with statistically significant differences have been depicted with an asterisk ($p<0.05$).

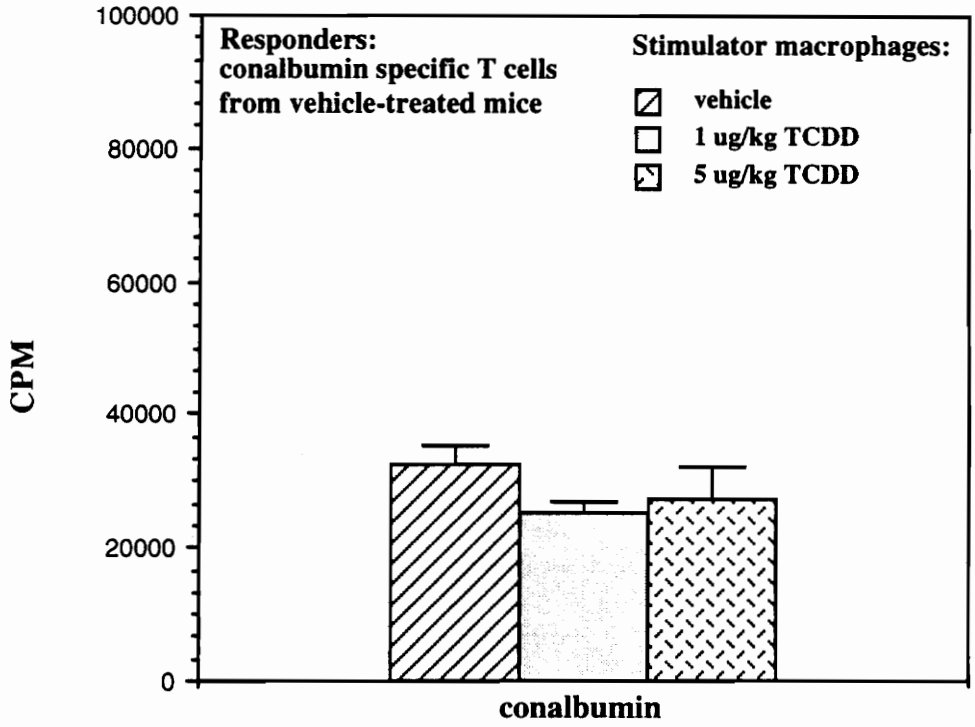

Fig. 29. TCDD affects the antigen-specific T cells but not the APC. Cell mixing experiments were carried out to investigate whether TCDD acts at the level of T cells or APC. C57BL/6 mice treated with TCDD or vehicle were immunized with conalbumin as described in Fig. 16. Next, T cells were purified from the draining lymph nodes (LN) of these mice and macrophages were isolated from the spleens. The ability of conalbumin-specific T cells from TCDD or vehicle-treated mice to respond to irradiated APC from TCDD or vehicle-treated mice in the presence of conalbumin was tested *in vitro* as described in Fig. 16. Data were statistically insignificant at $p<0.05$.

Table 1

Total cellularity and the absolute number of T cell subsets in the thymus of mice treated with TCDD.

Strain	TCDD dose (μg/kg)	Total thymic cells x 10^6/mouse [a]	Total T cells x 10^6 /mouse[b] $CD8^+$	$CD8^+CD4^+$	$CD8^-CD4^-$	$CD4^+$
DBA/2	vehicle	17.01±2..3	0.84	12.37	1.24	2.57
	0.1	21.63±2.7 (-27)[c]	1.43(-70)	13.16(-6)	2.50(-102)	4.54(-77)
	1.0	15.75±1.5 (7)	0.87(-4)	11.40(8)	1.17(6)	2.30(11)
	5.0	6.72±1.2 (60)*	0.40(52)	4.65(62)	0.65(48)	1.02(60)
C57BL/6	vehicle	147.53±20.5	5.38	110.9	8.88	21.55
	0.1	89.78±0.52 (39)*	3.69(31)	66.28(40)	6.50(27)	13.32(38)
	1.0	27.51±2.3 (81)*	0.79(85)	20.76(81)	2.14(76)	3.83(72)
	5.0	24.15±3.6 (84)*	1.27(76)	17.19(85)	2.04(77)	3.65(83)
C57BL/6 lpr/lpr	vehicle	190.19±13.4	10.75	154.15	7.55	17.76
	0.1	160.68±6.55 (16)	10.04(7)	131.93(14)	4.67(38)	14.06(21)
	1.0	110.43±3.3 (42)*	6.00(44)	93.09(40)	3.19(58)	8.16(54)
	5.0	37.7±5.15 (80)*	3.20(70)	28.35(72)	1.75(77)	4.39(75)
B10	vehicle	53.75±1.5	1.52	43.16	3.97	4.75
	0.1	39.63±6.63 (26)*	-	-	-	-
	1.0	28.63±2.13 (47)*	-	-	-	-
	5.0	2.45±0.85 (95)*	0.08(95)	1.73(96)	0.35(91)	0.25(95)
B10.D2	vehicle	67.63±3.38	3.77	48.11	7.52	9.58
	0.1	33.00±2.25 (51)*	-	-	-	-
	1.0	10.98±4.68 (84)*	-	-	-	-
	5.0	3.98±3.23 (94)*	0.33(91)	2.64(95)	0.70(91)	0.80(92)

[a] Groups of 4-5 mice were treated with TCDD or the vehicle and the mean thymic cellularity per mouse ±S.E. was calculated. Data showing statistically significant decrease ($p<0.05$) when compared to controls have been depicted with an asterisk.

[b] The total numbers of various T cell subsets was calculated based on the mean percentages of T cells as shown in Table 2 and the total cellularity of thymus as depicted above.

[c] Numbers in parenthesis represent percent decrease when compared to the vehicle control response.

Table 2

Percentages of T cell subsets in the thymus of mice treated with TCDD.

Strain	TCDD dose (μg/kg)	Percentages of T cell subsets [a] $CD8^+$	$CD8^+CD4^+$	$CD8^-CD4^-$	$CD4^+$
DBA/2	vehicle	4.91±0.83	72.71±0.13	7.3±0.79	15.08±1.75
	0.1	6.60±0.15	60.83±8.3	11.57±2.59	21.00±5.56
	1.0	5.55±1.08	72.41±4.08	7.43±1.41	14.62±1.6
	5.0	5.88±0.45	69.14±2.53	9.67±1.8	15.18±1.32
C57BL/6	vehicle	3.65±0.77	75.17±1.23	6.02±0.57	14.61±2.1
	0.1	4.11±0.79	73.82±3.51	7.24±1.19	14.84±2.13
	1.0	2.88±0.36	75.45±2.23	7.78±1.09	13.91±1.51
	5.0	5.27±0.88	71.16±2.59	8.45±1.01	15.12±1.54
C57BL/6 lpr/lpr	vehicle	5.65±0.74	81.05±0.56	3.97±0.54	9.34±0.36
	0.1	6.25±0.45	82.11±1.94	2.91±0.55	8.75±0.95
	1.0	5.43±0.02	84.3±0.32	2.89±0.18	7.39±0.13
	5.0	8.48±0.42	75.2±1.74	4.65±0.46	11.65±0.87
B10	vehicle	2.83±0.81	80.29±0.26	7.39±1.47	8.83±0.73
	5.0	3.34±0.21	70.59±10.3	14.47±7.5	10.03±1.01
B10.D2	vehicle	5.58±1.75	71.13±9.08	11.12±0.62	14.16±2.98
	5.0	8.18±3.63	66.33±6.13	17.66±9.74	20.14±4.99

[a] The percentage of T cell subsets was calculated by staining cells with mAbs against CD4 and CD8 markers followed by flow cytometric analysis as shown in Fig.1. The values represent mean ±S.E. calculated from analysis of 4-5 individual mice. The differences in the percentages of T cells in TCDD treated groups when compared to the vehicle treated controls were statistically insignificant ($p<0.05$).

Table 3

Total cellularity in the thymus of mice treated with TCDD.

Strain	TCDD dose (μg/kg)	Total thymic cells x 10^6/mouse [a]
MRL-+/+	vehicle	63.21±5.7
	5.0	77.81±12
MRL-lpr/lpr	vehicle	88.94±9.8
	5.0	74.24±1.6

[a] Groups mice were treated with TCDD or the vehicle and the mean thymic cellularity per mouse±S.E. was calculated. The differences in the thymic cellularity in TCDD-treated groups when compared to the vehicle-treated controls were statistically insignificant at $p<0.05$.

Abbreviations:

Ab, antibody; Ah, aryl hydrocarbon; AHH, aromatic hydrocarbon hydroxylase; APC, antigen presenting cell; CD, cluster of differentiation; CMI, cell mediated immunity; Con A, concanavalin A; CPM, counts per million; CSF, colony stimulating factor; CTL, cytotoxic T lymphocyte; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HAH, halogenated aromatic hydrocarbons; Ig, immunoglobulin; IFN-γ, gamma interferon; IL, interleukin; LPS, lipopolysaccharide, mAb, monoclonal antibody; MHC, major histocompatibility complex; NK, natural killer; PCB, polychlorinated biphenyl; PE, phycoerythrin; PHA, phytohemagglutin; SRBC, sheep red blood cell; T_c, cytotoxic T cell; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCR, T cell receptor; T_h, T helper cell; TNF, tumor necrosis factor.

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Virginia Polytechnic Institute and State University, January 1992 to March 1995.
M.S. Immunology/Microbiology
Dr. Prakash Nagarkatti, major advisor.

Research: Cellular and molecular mechanisms in TCDD-induced immunosuppression.

Work Experience:

Research Assistant for P.S. Nagarkatti, January 1992 to March 1995
Undergraduate Researcher for P.S. Nagarkatti, May 1991 to December 1991

Publication:

Rhile, M.J., Nagarkatti, M., and Nagarkatti, P.S. ***Role of Fas Apoptosis and MHC Genes in 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)-Induced Immunotoxicity of T Cells.*** (submitted)

Presentations:

Rhile, M.J. and Nagarkatti, P.S. ***Role of the Ah locus in immunomodulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).*** American Cancer Society 14th Annual Seminar of Cancer Researchers in Virginia, Blacksburg, VA. March 1994.

Rhile, M.J. and Nagarkatti, P.S. ***Role of Ah-locus in the Immunosuppression of Sensitized T Cells.*** American Society of Microbiologists, Lexington, VA, November 1993.

Rhile, M.J. and Nagarkatti, P.S. ***Immunotoxicity of TCDD.*** Virginia Academy of Sciences, Norfolk, VA, May 1993.

Assistantships:

Graduate Teaching Assistantship spring 1992 in the amount of $3000.
Graduate Teaching Assistantships 1992-93 academic year. ($4500/semester)
Graduate Teaching Assistantships 1993-94 academic year. ($1035/month)

Awards:

Instructional Fees Scholarships fall 1992 and spring 1993 ($1000 each).
Instructional Fees Scholarship 1993-1994 academic year ($600).
Instructional Fees Scholarship spring 1994 ($110).

Grants funded:

Sigma Xi grant, ***Cellular and Molecular Mechanisms in TCDD-induced Immunosuppression.*** $375. Funds matched by Biology Department. June, 1992.
Sigma Xi grant, ***Cellular and Molecular Mechanisms in TCDD-induced Immunosuppression.*** $355. Funds matched by Biology Department, Feb., 1994.

Professional Memberships:

American Association of Immunologists. (1992-94)
Virginia Academy of Sciences. (1993-present)
American Society of Microbiologists. (1993-present)
Sigma Xi. (1994-present)