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Nancy I. Kerkvliet

T cell-dependent immune responses are highly sensitive to suppression by exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), yet direct effects of TCDD on T cells have been difficult to demonstrate. Because the activation of naive T cells is initiated by dendritic cells (DC), the studies presented in this dissertation were designed to test the hypothesis that TCDD affects these antigen-presenting cells in a manner which ultimately results in suppressed T cell activation. The expression of numerous cell surface proteins known to be important in signaling T cells to proliferate and differentiate was evaluated on splenic DC from C57Bl/6 and Balb/c mice. The production of IL-12 and the ability of DC to activate allogeneic and antigen-specific T cells were also tested. Contrary to expectation, exposure to TCDD resulted in enhanced expression of several accessory molecules including B7-2, CD40, ICAM-1, CD24 and the major histocompatibility complex (MHCII). In contrast, expression of LFA-1 was significantly decreased on DC from TCDD-treated mice. These effects were dose-dependent, persisted for at least 14 days, and did not occur in aryl hydrocarbon receptor (AhR)-deficient mice. Interestingly, TCDD treatment also decreased the numbers of DC recovered from the spleen by day 7 following exposure in C57Bl/6 mice and by day 3 in Balb/c animals. When T cells were cultured with DC from TCDD-treated mice, the proliferative response of the T

cells and the production of IL-2, IL-4, and IFN-γ was not suppressed but instead tended to be increased. DC production of IL-12 was also enhanced. Furthermore, TCDD did not interfere with the ability of DC to internalize latex beads or to activate antigen-specific T cells, suggesting that uptake and processing of antigen by DC is not impaired by TCDD. AhR message was detected in splenic DC and AhR protein was found in two DC cell lines, indicating that DC may be directly affected by TCDD. Taken together, these results suggest that TCDD provides an activation stimulus to DC and may lead to their premature deletion. The relationship between these effects and TCDD-induced immune suppression remains to be determined.

Investigations Into the Mechanism of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-Induced Immune Suppression: Effects on Dendritic Cell Phenotype and Function

by

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

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Beth A. Vorderstrasse, Author

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analysis, and reading manuscripts. I consider myself very fortunate to have had your help over the years, and your contributions to this research are much appreciated!

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Contribution of Authors

The various co-authors of the chapters are listed in alphabetical order with their major contributions to the study.

Kerkvliet, Nancy I.	Critical review of experimental design, data, and manuscripts; financial support.
Lawrence, B. Paige.	Technical support; critical review of experimental design and data.
Silverstone, Allen E.	Critical review of experimental design.
Steppan, Linda B.	Critical review of experimental design, data, and manuscript. Technical support.

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Chapter 1

Introduction

TCDD AND HALOGENATED AROMATIC HYDROCARBONS

Overview

TCDD is the prototypic and most toxic member of a class of environmental contaminants known as halogenated aromatic hydrocarbons (HAH), a chemical group consisting of numerous congeners of halogenated dibenzodioxins, dibenzofurans, and biphenyls (Fig. 1-1) (reviewed by Webster and Commoner, 1994). TCDD and particular congeners which bind to an intracellular protein known as the aryl hydrocarbon receptor (discussed later in this chapter) are of particular concern because they cause numerous toxic effects in both humans and laboratory animals. In addition, TCDD is chemically stable, lipophilic, and has a low vapor pressure and water solubility, properties which allow for persistence in the environment and promote accumulation in animal tissues. Although the half life in rats and mice is relatively short (10-30 days), TCDD remains in human tissues for a substantially longer period (half life = 5-10 years) (Birnbaum, 1986; Pirkle *et al.*, 1989; DeVito and Birnbaum, 1994).

Sources and exposure

Unlike polychlorinated biphenyls (PCB), which were intentionally manufactured beginning in the 1920's and have been used extensively for industrial purposes in transformers, hydraulic fluids, etc., there are no intended commercial uses for the polychlorinated dioxins and furans. Instead these compounds are primarily unwanted by-products of chemical manufacture, incineration of plastic, and industrial chlorine bleaching

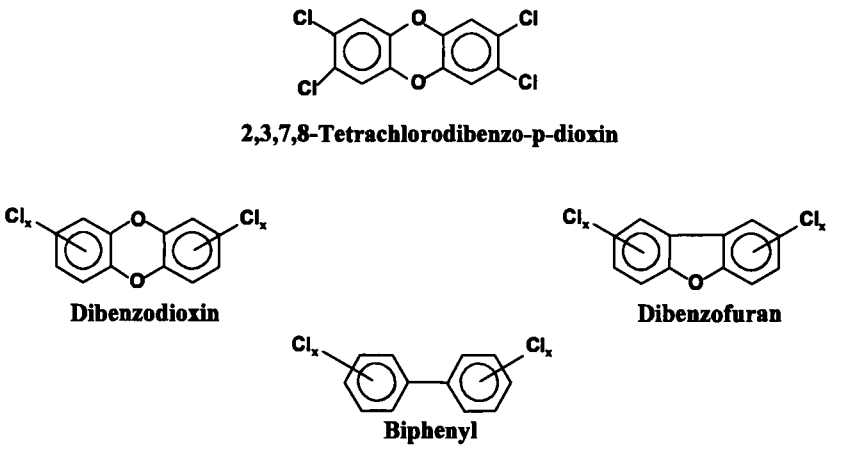

FIG. 1-1. Chemical structures of TCDD and related halogenated aromatic hydrocarbons.

processes (reviewed by Webster *et al.*, 1994, and Vanden Heuvel and Lucier, 1993). TCDD initially came to the attention of the general public when it was recognized as a contaminant in the herbicides 2,4D and 2,4,5T, the active ingredients in the Agent Orange defoliant used in the southeast Asia during the Vietnam War. Although the contamination of these herbicides has since been reduced substantially, TCDD is still released into the environment when chlorine-containing products are burned, a consequence which has raised significant concern about the incineration of garbage and especially of medical waste which has a high plastic content. In addition, TCDD is formed in the chlorine bleaching process of paper pulp, resulting in aquatic contamination.

The general population is exposed to TCDD primarily through the food chain. Due to its resistance to environmental and metabolic degradation and its lipophilicity, TCDD accumulates in fat, resulting in exposure through the consumption of meat, fish, and milk products (reviewed by Startin, 1994). It is estimated that the average daily intake of polychlorinated dioxins and furans (calculated as an equivalent toxic dose of TCDD) averages 1-3 pg/kg in industrialized countries (Webster and Commoner, 1994).

Although humans are rarely exposed to doses sufficient to cause overt health effects, a number of industrial accidents and accidental poisonings have resulted in fairly high

exposures to particular populations. For example, thousands of people in Taiwan (1979, Yu-chen incident) and Japan (1968, Yusho incident) were poisoned when they consumed PCB-contaminated rice oil. The 1979 explosion of a plant which manufactured trichlorophenol exposed the town of Seveso, Italy to TCDD. In addition, industrial workers from plants manufacturing TCDD-contaminated products represent another cohort which may have relatively high exposures.

TOXIC EFFECTS

Human toxicity

Although no immediate human deaths have resulted from these accidental exposures, numerous human health effects have been documented in highly-exposed populations. The most commonly observed acute symptom is chloracne, a severe form of cystic acne which is characterized by hyperplasia and hyperpigmentation of the skin. Chloracne may affect extensive portions of the body and can persist for decades following exposure. Skin pigmentation anomalies, as well as nail and eye effects have also been observed in children born to women poisoned with PCB-contaminated rice oil in the Yu-cheng and Yusho incidents (Masuda, 1994; Hsu *et al.*, 1994). Highly-exposed worker populations and those exposed to TCDD in the Seveso accident may have increased risk of developing diabetes and death from ischemic heart disease, and appear to have an increased risk of cancer (Vena *et al.*, 1998; Steenland *et al.*, 1999; Bertazzi *et al.*, 1998).

TCDD is characterized as a nongenotoxic carcinogen, as it is a potent tumor promoter, but is negative in most genotoxic toxicity tests and therefore is apparently not an initiator (Huff *et al.*, 1994). Numerous epidemiological studies have found a generalized excess of cancer mortality in highly-exposed populations (Huff *et al.*, 1994) and a recent cohort mortality analysis of over 5000 exposed workers found a positive trend for cancer mortality with increasing exposure (Steenland *et al.*, 1999). In this study, the authors estimated that exposures necessary to cause cancer were likely to be 100-1000 times higher than normal population exposure (Steenland *et al.*, 1999). Based on

epidemiological data and sufficient animal data, the International Agency for Research on Cancer classified TCDD as a group 1 human carcinogen in 1997.

Despite the obvious human health effects evident following high dose exposure, it is still unclear whether low dose exposure represents a significant human health hazard. Despite this uncertainty, the presence of HAH in general and TCDD in particular continues to provoke significant public concern. Last year, for example, a ban on Belgian beef, poultry, pork and milk products was in effect across Europe and internationally, when TCDD-contaminated fat was discovered to have been incorporated into animal feed. Ongoing media attention to suspected health effects resulting from exposure to TCDD in Vietnam, Love Canal and Times Beach, as well as the recent release of the popular books *Dying From Dioxin*[1] and *Our Stolen Future*[2] demonstrate that these chemicals are still very much a public policy issue. Unfortunately, an accurate assessment of the actual risk low dose exposure poses to human health will likely result only when the mechanism of toxicity is better understood.

Toxicity in animals

Although TCDD-induced toxicity has been extensively characterized in laboratory animals, it is difficult to extrapolate these toxicity data to risk assessments for human health is because of the wide range of sensitivities observed in different species of animals. The dose required to cause death, for example, differs substantially between species, ranging from a low LD50 of 2 μg/kg in the guinea pig to over 3000 μg/kg in the hamster (Pohjanvirta and Tuomisto, 1994). The variation is even more dramatic in different rat strains as the LD50 is relatively low in the Long-Evans rat (10-20 μg/kg) but extremely high in the Han/Wistar strain (over 9600 μg/kg) (Tuomisto *et al.*, 1999). The lethal effects of TCDD are unusual in that death is often delayed, in some species as long as 6 weeks after exposure (Pohjanvirta and Tuomisto, 1994). A wasting syndrome, wherein the animals slowly lose weight over an extended period, often precedes death.

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[1]Gibbs, L. M. (1995). South End Press, Boston, MA.

[2] Colborn, T., Dumanoski, D., and Meyers, J. P. (1996). Dutton, New York.

In addition to lethality, a remarkable variety of other toxic effects result from exposure to TCDD, suggesting that a number of organ systems are targeted (reviewed by Pohjanvirta *et al.*, 1994, and by DeVito *et al.*, 1994). In addition to inducing metabolic enzymes, effects on the liver effects include hepatomegaly and inflammatory cell infiltration, as well as varying degrees of necrosis observed in some species. TCDD is also a teratogen (Couture *et al.*, 1990), and a tumor promoter, and may alter growth and differentiation of cells and tissues by altering hormone levels and signaling pathways (reviewed by Pohjanvirta *et al.*, 1994). The immune system is a particularly sensitive target for TCDD, with thymic atrophy (Harris *et al.*, 1973; Vos *et al.*, 1974) and altered immune function observed in most species following exposure to low doses of TCDD (reviewed by Kerkvliet and Burleson, 1994, and discussed later in this chapter).

MECHANISM OF TOXICITY

Many, if not all, HAH-induced toxicities are initiated when the chemical binds to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor which is a member of a superfamily of basic-helix-loop-helix (bHLH) DNA binding proteins (reviewed by Landers and Bunce, 1991, and Schmidt and Bradfield, 1996). The gene locus for the receptor was originally defined approximately 30 years ago. This was based on observations that different strains of mice exhibited differential sensitivities to HAH-induced toxicities and that this differential sensitivity was associated with a genotypic polymorphism. Subsequent investigations demonstrated that the Ah locus codes for a soluble protein receptor, the AhR, which binds certain HAH with high affinity. Four different AhR alleles have been characterized in mice, and sensitivity to toxicity correlates with the affinity each receptor has for the ligand. The low-affinity receptor (MW 104,000) is encoded by AhR^{d} allele present in "resistant" mouse strains such as DBA and 129. "Sensitive" strains such as C57Bl/6 and Balb/c mice have one of three high-affinity AhR^{b} alleles, AhR^{b-1} (MW 95,000), AhR^{b-2} (MW 104,000) or AhR^{b-3} (MW 105,000) (Poland and Glover, 1990). The difference in affinities appears to result from a polymorphism in the

ligand binding domain of the receptor (Ema *et al.*, 1994). Although data regarding the human AhR is limited, studies of human cell lines have found that receptor affinity for TCDD is approximately 10-fold less than in mice (Harper *et al.*, 1991; Harper *et al.*, 1988), suggesting that humans may be slightly less sensitive to the toxic effects of TCDD than are mice.

Prior to ligation, the AhR is located in the cellular cytoplasm associated with a number of proteins including a dimer of heat shock protein 90 (hsp90), a src protein kinase (Enan and Matsumura, 1996; Blankenship and Matsumura, 1997), and an immunophilin alternately named AIP, XAP2, and ARA9 by the three laboratories which originally characterized it (Fig. 1-2) (Ma and Whitlock, 1997; Meyer and Perdew, 1999; Carver *et al.*, 1998). Following activation by ligand, the proteins dissociate and the receptor moves to the nucleus and forms a heterodimer with another bHLH protein, the AhR nuclear translocator (Arnt). This complex functions as a transcription factor, recognizing xenobiotic or dioxin response elements (DRE) which contain a core DNA sequence of 5'GCGTG (Yao and Denison, 1992).

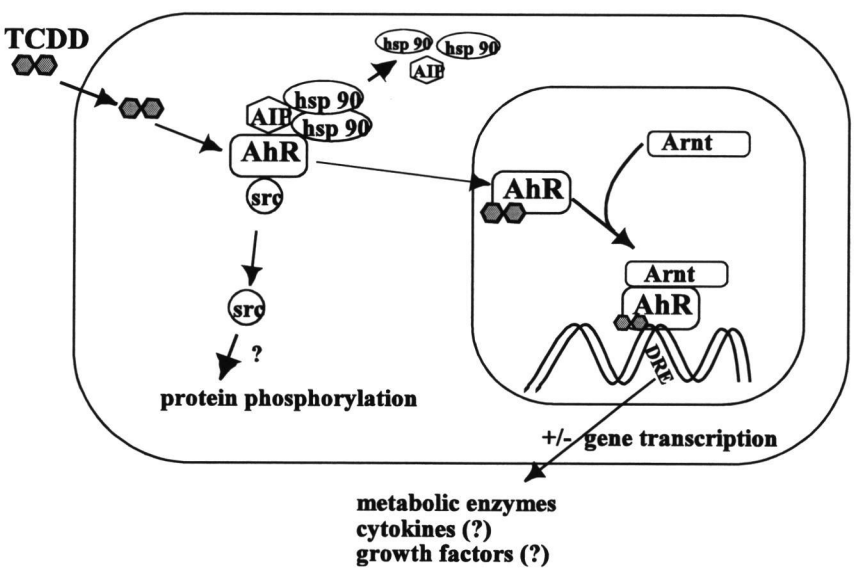

FIG. 1-2. The aryl hydrocarbon receptor. See text for details. Abbreviations include: AhR nuclear translocator (Arnt); heat shock protein 90 (hsp 90); AhR-interacting protein (AIP); src protein kinase (src); and dioxin response element (DRE).

The toxic effects induced by exposure to TCDD are generally considered to result from altered gene transcription initiated by binding of DRE. Genes which contain DRE sequences include numerous metabolic enzymes, cytokines and growth factors (Lai *et al.*, 1996), suggesting that activation of the AhR may alter cell cycle regulation or tissue growth. The most extensively-characterized transcriptional alteration is upregulation of cytochrome P4501A1, a xenobiotic-metabolizing enzyme which hydroxylates certain HAH resulting in their clearance from the body. However, AhR activation may also suppress the transcription of gene products as has been shown by the TCDD-induced decrease in estrogen receptor-mediated cathepsin D production (Kharat and Saatcioglu, 1996).

Other effects resulting from exposure to TCDD occur too rapidly to result from de novo protein synthesis, suggesting that altered gene transcription may not account for all toxicities. For example, Puga *et al*, (1992) have reported increased calcium influx in murine hepatoma cells within two minutes of TCDD exposure. Protein tyrosine kinase activity also increases rapidly after exposure (Clark *et al.*, 1991) and occurs in the presence of protein synthesis inhibitors (Enan and Matsumura, 1995). These observations, coupled with the recent discovery that src-kinase is found associated with the AhR (Enan and Matsumura, 1996; Blankenship and Matsumura, 1997), have led to speculation that certain toxic effects resulting from TCDD exposure may result from impacts on intracellular signaling pathways involving protein phosphorylation (Matsumura, 1994), calcium (Holsapple *et al.*, 1996), or transcription factors such as NF-κB and AP-1 (Tian *et al.*, 1999; Puga *et al.*, 2000; Puga *et al.*, 1992). In addition to gene transcriptional and cell signaling pathways, other mechanisms for TCDD-induced toxicity which have been proposed include sequestration of free Arnt, which may prevent its dimerization with other protein partners, saturation of coactivators which may result from sustained activation of TCDD-induced genes, and formation of reactive oxygen species which may contribute to peroxidation of lipids and DNA damage (Schmidt and Bradfield, 1996; Hassoun *et al.*, 1998).

IMMUNOTOXICITY

Overview

The immune system is one of the most sensitive targets for the toxic effects of TCDD, and immune suppression is observed at a dose which does not produce overt pathological changes (Thigpen *et al.*, 1975). The mouse has been the preferred test species, largely because murine models predominate in mechanistic immunological studies, and availability of reagents is not as great for other species. However, TCDD may cause different immune effects in other species. For example, TCDD appears to enhance the response to sheep red blood cells (SRBC) in rats whereas this response is suppressed in mice (Smialowicz *et al.*, 1994; Pazdernik and Rozman, 1985). This possible interspecies variability, as well as a lack of comparable assays available to examine human immunological parameters, make it difficult to estimate the potential for TCDD to affect immune function in humans.

Immunological effects in humans

Existing epidemiologic studies have not provided consistent evidence that TCDD and other HAH cause immune dysfunction in humans. Lu and Wu (1985) examined immune parameters in people who were poisoned with PCB-contaminated rice oil in the Yu-Cheng incident. The patients appeared to have a relatively high incidence of various infections, suggesting some degree of immunosuppression. Concentrations of B cells and total T cells in peripheral blood did not differ from control subjects, however very small, but statistically significant, differences in the concentration of CD4+ (decreased) and CD8+ (increased) T cells were detected. Interestingly, lymphocyte proliferation induced by mitogens or tuberculin were substantially enhanced in the PCB-poisoned individuals, although the potential immunological significance of this was not clear. In contrast, Mocarelli *et al.*(1991) found no evidence of altered immunity when they examined 20 people exposed to TCDD in the Seveso accident, 10 of whom developed severe chloracne. In this study, blood cell counts and differentials were done, as were various immunological function tests including mitogen-induced proliferation. Although no data were shown, the

authors stated that no differences were detected when the subjects were compared to residents of noncontaminated areas.

Two recent studies of small cohorts of highly-exposed chemical plant workers also failed to detect consistent effects of TCDD on immune parameters. Tonn *et al.* (1996) examined eleven workers from a German factory which manufactured trichlorophenol. The subjects had relatively high blood fat concentrations of TCDD (10- to 220-fold higher than the average level of the German population, 4 pg/g blood lipid), and some were affected with chloracne. No significant differences in blood lymphocyte populations were detected, nor did mitogen-induced proliferation of T or B cells differ from that of control subjects. Proliferation of blood mononuclear cells induced by IL-2 or by culture with allogeneic cells was suppressed, although the difference was significant only when one highly-responsive individual was removed from the analysis. Jung *et al.* (1998) also examined German pesticide plant employees (n=192), including a subgroup of 29 individuals with particularly high blood lipid concentrations of TCDD (33.6 - 2252 pg/g blood lipid). The incidence of infectious diseases (14 types examined) was similar in exposed and nonexposed groups, as were serum antibody levels and blood cell counts. A slight decrease in CD8+ T cells was detected in the exposed population, however, mitogen-induced proliferation of blood cells was normal.

Although none of these studies provide compelling evidence that exposure to TCDD or PCB significantly alters human immunological function, it is possible that significant effects are missed when such small cohorts are examined. In addition, techniques currently used for assessing immune function in humans are largely confined to analysis of peripheral blood cells, particularly the enumeration of leukocyte subsets and mitogen-induced proliferation. Since these parameters are generally not studied in mice and/or are resistant to suppression by TCDD, their relationship, if any, to immune dysfunction is unclear (Oughton *et al.*, 1995; Lawrence *et al.*, 1996; Lundberg *et al.*, 1991; Dooley *et al.*, 1990). Thus, in order to develop appropriate techniques for assessing immune effects in humans, a better understanding of the mechanism by which TCDD causes immunotoxicity in laboratory animals would be useful.

Effects of TCDD on host resistance in mice

Several studies have shown that host resistance to disease is compromised in TCDD-treated mice. For example, a very low dose of TCDD (0.1 μg/kg) decreased the survival of mice infected with influenza virus (House *et al.*, 1997). TCDD-treated mice also demonstrated increased susceptibility to salmonella (Thigpen *et al.*, 1975) and lethal herpes virus type II infection (Clark *et al.*, 1983). Parasitemia was also increased in TCDD-treated mice infected with *Plasmodium yoelii*, a murine model for malaria (Tucker *et al.*, 1986), as was susceptibility to challenge with syngeneic (Luster *et al.*, 1980) and allogeneic (Kerkvliet *et al.*, 1996) tumors. These results are significant because they suggest that humans exposed to sufficient levels of TCDD and related chemicals may have an increased risk of infection, or might have compromised ability to fight neoplastic disease.

Cellular targets for immune effects

Despite many years of investigation, the cellular basis for suppressed immunity following TCDD exposure has not been established. This is largely due to difficulties in replicating effects observed in whole animal studies in *in vitro* systems. For example, although *in vivo* exposure to TCDD profoundly suppressed the generation cytotoxic T cell (CTL) activity against allogeneic tumor cells (Kerkvliet *et al.*, 1996), TCDD had no effect on this response when generated *in vitro* (Dekrey and Kerkvliet, 1995). In contrast, the *in vitro* SRBC response was suppressed by TCDD (Holsapple *et al.*, 1986; Davis and Safe, 1991), however this suppression was not AhR-dependent and is therefore unlikely to accurately represent the mechanism of suppression which occurs *in vivo* (Davis and Safe, 1991). This lack of consistent effects of TCDD observed in *in vitro* systems suggests that the cellular target is an immune cell which is not present in the assays, or that culture conditions somehow compensate for effects of TCDD. Alternatively, immune dysfunction measured *in vivo* may be an indirect effect which results from a toxic insult to a non-lymphoid cell or tissue.

Elegant studies using AhR-chimeric animals and adoptive-transfer models, however, have provided evidence that the direct cellular target is an immune cell. Silkworth *et al.*

(1986) reconstituted lethally-irradiated Balb/c (AhR^{bb}) or DBA/2 (AhR^{dd}) mice with bone marrow cells from the opposite strain, thus generating chimeric animals which contained TCDD-responsive or non-responsive immune cells. When these mice were treated with an immunosuppressive dose of the AhR ligand 3,4,3',4'-tetrachlorobiphenyl and challenged with SRBC, the AhR genotype of the bone marrow cells determined whether the response was suppressed. This suggests that the immune cells are direct targets of HAH. The same conclusion was reached in studies performed by Kerkvliet *et al.* (1990a) wherein spleen cells from AhR^{bb} or AhR^{dd} congenic mice were adoptively-transferred into irradiated congenic host mice. TCDD-induced suppression of the SRBC response was equivalent when AhR^{dd} splenocytes were transferred into either AhR^{bb} or AhR^{dd} host animals. However, AhR^{dd} animals were substantially more sensitive when adoptively-transferred with AhR^{bb} splenocytes than with AhR^{dd} splenocytes. Because it was the AhR genotype of the lymphoid cells which determined the sensitivity to TCDD, these results also suggest that lymphoid cells are the direct target. Finally, we have recently performed studies using chimeric animals constructed from lethally-irradiated $AhR^{+/+}$ mice reconstituted with bone marrow from $AhR^{-/-}$ or $AhR^{+/+}$ control animals. When these animals were challenged with allogeneic tumor cells, TCDD suppressed the CTL response of control animals reconstituted with $AhR^{+/+}$ immune cells, but not the mice reconstituted with $AhR^{-/-}$ immune cells. Taken together, these results indicate that immune suppression induced by exposure to TCDD results from a direct effect on an immune cell. The specific immune cell population, however, remains to be determined.

Macrophages

The functions of macrophages have been examined in numerous studies and appear to be resistant to suppression by TCDD. House *et al.* (1997) examined peritoneal macrophages isolated from TCDD-treated mice, and found the cells had normal phagocytic and cytolytic activity, and produced normal amounts of IL-1 and hydrogen peroxide. Similarly, Mantovani *et al.* (1980) were unable to find evidence that TCDD suppressed the cytolytic activity of macrophages, even when cells were isolated from C57Bl/6 mice treated with a highly immunosuppressive dose of TCDD (30 μg/kg).

Interestingly, *in vitro* exposure to TCDD enhanced TNF-α production by LPS-stimulated IC-21 macrophages and peritoneal exudate cells (Moos *et al.*, 1997). These results indicate that macrophages may be directly affected by TCDD, however the relationship between enhanced TNF-α production and the immune suppression observed in TCDD-treated mice remains unclear.

The effects of TCDD on the ability of macrophages to function as antigen-presenting cells (APC) has been examined in a limited number of studies. Kerkvliet and Oughton (1993) detected no adverse effects when they examined activation of SRBC-primed T cells using peritoneal and splenic macrophages from TCDD-treated mice. Likewise, Dooley and Holsapple (1988), and Rhile *et al.* (1996) were unable to find evidence of suppressed macrophage function in *ex vivo* assays using antigen-primed or mitogen-stimulated T cells as responders. Thus, the APC activity of macrophages, at least when examined *ex vivo*, also appears to be resistant to suppression by TCDD exposure.

B cells

The production of antibodies by B cells is one of the most sensitive toxic endpoints of exposure to TCDD. For example, the ID_{50} for suppression of antibody-forming cells in mice challenged with SRBC is 0.6 μg/kg (Kerkvliet *et al.*, 1990a; Vecchi *et al.*, 1983). The SRBC response is the predominate assay used to examine B cell function *in vivo*, however Lundberg *et al.* (1991) and Shepherd *et al.* (2000) have also reported suppressed antibody production in mice challenged with ovalbumin. In addition, responses to various hapten-carrier conjugates have been tested and found to be sensitive to suppression (Dooley and Holsapple, 1988; Kerkvliet *et al.*, 1990a). Because most of these B cell responses also require the participation of T cells, it is difficult to address whether effects on antibody production measured *in vivo* result from direct effects on B cells, T cells, or antigen-presenting cells. However, there is evidence which suggests that B cells are directly affected by TCDD. B cells express AhR (Masten and Shiverick, 1996; Sulentic *et al.*, 1998) and direct effects of TCDD on the production of antibodies have been demonstrated *in vitro*, suggesting that suppressed antibody production may result at least

in part from direct effects on these cells (Tucker *et al.*, 1986; Holsapple *et al.*, 1986; Karras *et al.*, 1995; Sulentic *et al.*, 1998).

It is unlikely however, that direct effects on B cells can explain the suppression of T cell function observed in TCDD-treated mice. For example, studies by Kerkvliet *et al.* (1990a) showed that suppressed antibody production most likely resulted from effects on helper T cells. They found that the antibody response to SRBC, which requires T cell help, was highly sensitive to suppression by TCDD (ID_{50} 0.6 μg/kg) whereas suppression of the antibody response to the T-independent antigen TNP-LPS required over a ten-fold higher dose (ID_{50} 7.0 μg/kg). Similar differences in suppression of responses to T-dependent vs T-independent antigens were shown in studies by House *et al.* (1997), and in studies which examined immune suppression induced by 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (Kerkvliet and Brauner, 1987). In addition, TCDD -induced suppression of CTL activity was evident in B cell knockout mice challenged with allogeneic tumor cells (D. Shepherd, unpublished observations), providing compelling evidence that B cells do not contribute to the suppression of cell-mediated immune responses induced by TCDD.

T cells

As suggested by differential suppression of T cell-dependent antibody responses, immune responses which require T cells are highly sensitive to suppression by TCDD. For example, Vos *et al.* (1973) found that four weekly doses of 5 μg/kg to donor animals suppressed the subsequent generation of the graft vs host response. The contact hypersensitivity response is also affected in TCDD-treated mice, as oxazalone-induced changes in ear thickness were reduced by a dose of 4 μg/kg in studies performed by Clark *et al.* (1981). As suggested by the increased sensitivity to infection with influenza and herpes virus observed in TCDD-treated mice (House *et al.*, 1997; Clark *et al.*, 1983), the generation of cell-mediated immune responses is also sensitive to suppression. Splenic cytotoxic T cell (CTL) activity in mice responding to challenge with allogeneic tumor cells is profoundly suppressed in TCDD-treated animals (ID50 = 7.5 μg/kg) (Dekrey and

Kerkvliet, 1995). Additional studies performed in tumor-injected mice showed that production of IL-2 and IFN-γ by T cells was also suppressed (Kerkvliet *et al.*, 1996).

The role of the T helper cell in TCDD-induced suppression of an antibody response was directly addressed in studies performed by Tomar and Kerkvliet (1991). In these experiments, T helper cells were isolated from vehicle- or TCDD-treated mice which had been primed with antigen. These carrier-primed T cells were cultured with B cells isolated from untreated animals, and the generation of antibody-forming cells to hapten-carrier conjugate was measured. The production of anti-hapten antibodies was significantly reduced in cultures containing T cells from TCDD-treated mice as compared to those with cells from vehicle-treated mice. These results suggest that suppressed antibody production measured in TCDD-treated mice results from defective T cell functions, not from selective effects on B cells.

The impact of TCDD on T cell proliferation and cytokine production has also been addressed in mice challenged with ovalbumin (OVA) protein. In assays performed by Lundberg *et al.* (1992), lymph node cells from OVA-primed mice were restimulated with antigen in *ex vivo* cultures. Cells from mice exposed to a relatively high dose of TCDD (50 μg/kg) demonstrated reduced proliferation and IL-2 production as compared to cells from vehicle-treated animals. More recent studies performed by Shepherd *et al.* (2000) followed the expansion of T cells *in vivo* using mice adoptively-transferred with CD4+ OVA-specific T cells. In these studies, TCDD (15 μg/kg) did not impact the initial expansion of the OVA-specific T cells measured in the spleen, but decreased the number of cells present later in the response. In addition, TCDD appeared to impact the differentiation of the T cells as the amount of IL-2 measured in restimulated spleen cell cultures was significantly reduced.

Despite its profound suppression of T cell-dependent immune responses, there is little evidence to suggest that TCDD affects T cells directly. For example, when T cells were exposed to TCDD *in vitro*, no effects on mitogen-induced proliferation (Lang *et al.*, 1994; Vecchi *et al.*, 1980) or antigen-induced cytokine production (Lawrence *et al.*, 1996) were detected. Nor did T cells from TCDD-treated mice demonstrate suppressed proliferation (Lundberg *et al.*, 1992) or cytokine production (Prell *et al.*, 1995) when

stimulated *ex vivo* with anti-CD3. Furthermore, T cells were stimulated to function normally in TCDD-treated mice when provided with exogenous stimuli in the form of B7-2-transfected tumor cells or IL-2 (Prell and Kerkvliet, 1997; Prell *et al.*, 2000). Additional evidence that TCDD does not impact T cells directly is found in studies which addressed the AhR status of the cells. Specifically, Lawrence *et al.* (1996) found that T cells contained very low levels of AhR protein which was unable to bind the appropriate consensus sequence on DNA. Thus, because current evidence suggests that TCDD suppresses T cell responses indirectly it suggests that the cells responsible for activation of T cells, dendritic cells, may be the target of TCDD.

DENDRITIC CELLS

Overview

Although dendritic cells (DC) were first recognized in 1868 as Langerhans cells in the skin, their widespread distribution in the body and the important functions associated with these cells was realized less than 30 years ago (Steinman and Cohn, 1973; Steinman and Cohn, 1974). Since then, an exponential increase in the study of DC has occurred, especially within the past decade. DC are now recognized as the primary, or perhaps even the singular, cell which is responsible for the activation of naive T cells (reviewed by Banchereau and Steinman, 1998). As the role DC play in initiating immune responses has been elucidated, the potential for therapeutic use of the cells in vaccination and tumor immunity has been explored. Further interest in these cells is related to the roles they play in autoimmune disease, allergy, transplant rejection, and HIV.

DC must technically be characterized as part of the innate immune system as they do not have specific antigen receptors like T and B cells. However, the cells play little or no direct role in the innate immune response, and their primary function is to carry antigen away from a challenged site, enter the lymphoid tissues, and alert T cells. Thus DC are frequently referred to as the "sentinels" of the immune system.

Activation of DC

The morphology and specific functions of DC vary depending on their stage of activation. In their "immature" state, often represented by Langerhans cell in the skin, DC express constitutive, but relatively low levels of costimulatory molecules and are not very efficient at activating T cells (Fig. 1-3) (Inaba *et al.*, 1994; Girolomoni *et al.*, 1992). Instead these cells are specialized for internalization of antigen; they have a tremendous endocytic capacity and are efficient at phagocytosis (Sallusto *et al.*, 1995; Reis e Sousa *et al.*, 1993).

When DC become activated, an event which can be triggered by direct contact with antigen or by inflammatory mediators released from other cells, they leave the affected tissue via the lymphatics or the blood and move to the lymph nodes or the spleen. During this stage, the cells markedly downregulate their phagocytic activity, a property which originally led to their classification as a nonphagocytic cell (Sallusto *et al.*, 1995; Reis e Sousa *et al.*, 1993; Kitajima *et al.*, 1997). Concurrent with migration, DC begin to upregulate the expression specific cell surface proteins such as costimulatory and adhesion molecules which are needed for activation of T cells. DC also process antigen such that peptide fragments are presented in the groove of the major histocompatibility complex (MHC).

Once in the lymphoid tissue, the now "mature" DC can activate antigen-specific T cells which recognize the peptide presented on the MHC molecule (Fig. 1-4). Both CD4+ T cells, which recognize peptide on MHC class II molecules, and CD8+ T cells which respond to peptide presented on MHC class I, can be activated by DC. In addition to this antigenic signal, the DC provides costimulatory signals through molecules such as B7-1, B7-2, and CD24, and likewise receives signals through the proteins such as CD40. The exact molecular signals initiated by these protein:protein interactions are not fully understood, however the B7 molecules signal through CD28 expressed on T cells resulting in enhanced IL-2 production and cell survival (Sperling *et al.*, 1996; Jenkins *et al.*, 1991). CD40 engagement of CD154 on T cells promotes IL-12 production and survival of the DC (VanKooten and Banchereau, 1997; Cella *et al.*, 1996). The T cell ligand for CD24 has not been identified, however, like the B7 molecules, signals provided by CD24 promote

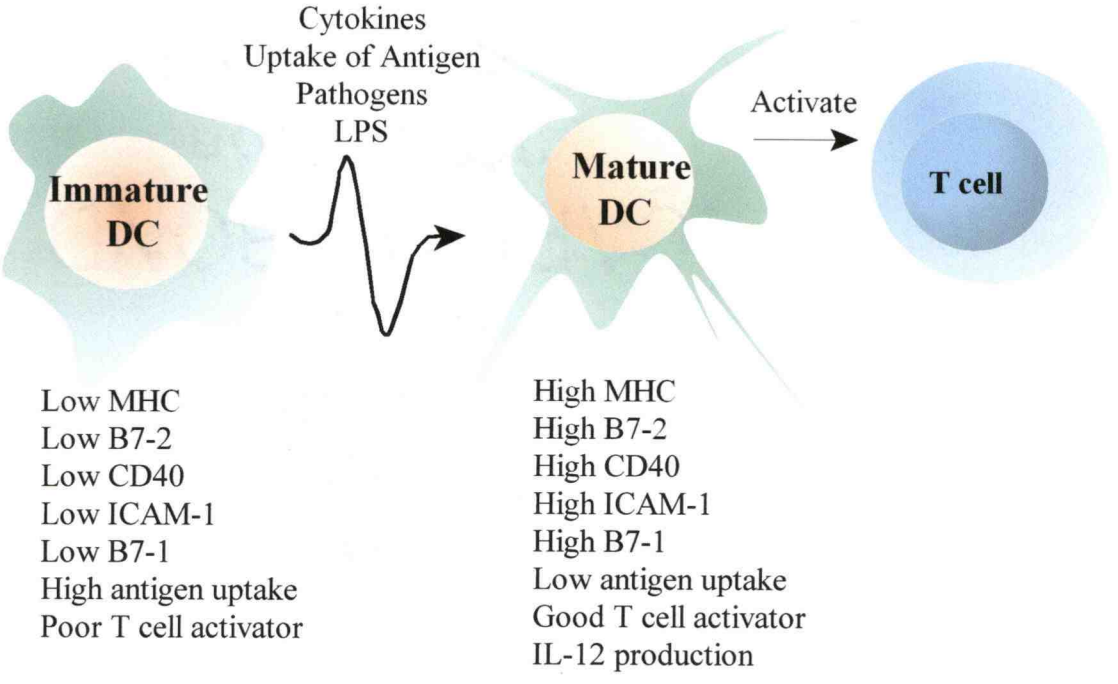

FIG. 1-3. Characteristics of immature and mature DC.

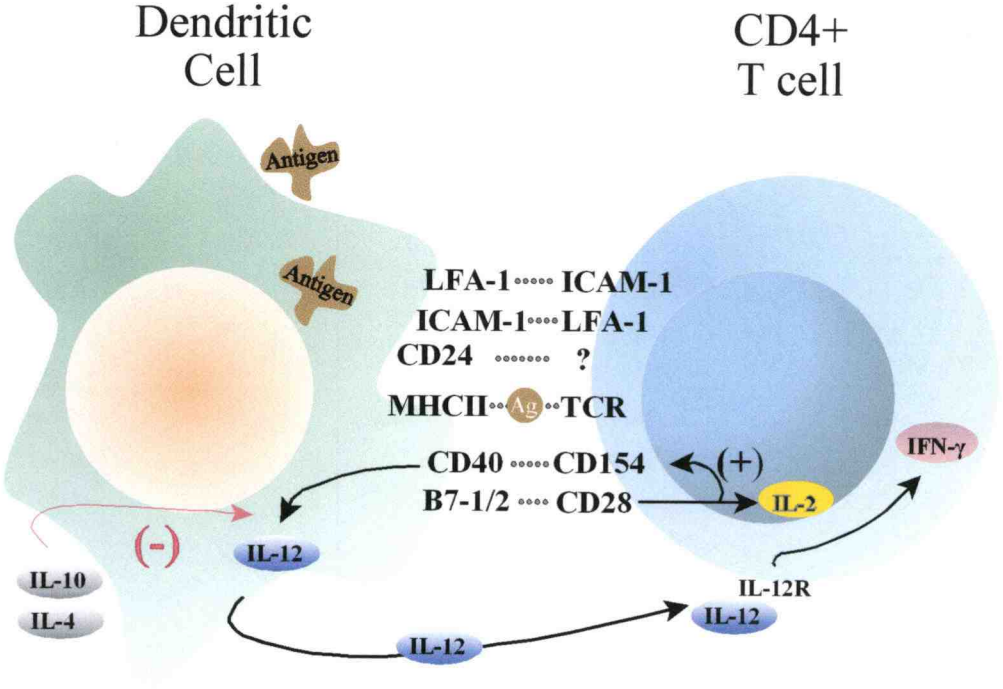

FIG. 1-4. Surface molecules and cytokines involved in activation of T cells by DC.

proliferation and IL-2 production by T cells (Enk and Katz, 1994; Liu *et al.*, 1997). Activation of the T cell also involves adhesion molecules such as LFA-1 and its ligand ICAM-1. These proteins are found on both DC and T cells and function to maintain prolonged contact during T cell activation.

Influence on the Th1/Th2 balance and tolerance

Different types of immune responses are required for different antigens, and the selection of particular effector immune functions is controlled by antigen-specific CD4+ T helper (Th) cells which secrete different cytokines. Cell-mediated immune responses which involve cytotoxic CD8+ T cells are promoted by Th1 cytokines such as IFN-γ, whereas humoral immune responses involving antibody production by B cells are promoted by Th2 cytokines such as IL-4 and IL-5. The differentiation of Th cells to the Th1 or Th2 type is thought to be regulated largely via signals provided by DC (Kalinski *et al.*, 1999; Ni and ONeill, 1997; Macatonia *et al.*, 1995).

The mechanisms by which DC promote a Th1-type immune response may include numerous signals through cell surface proteins and soluble factors, although IL-12 is currently the best characterized. DC are induced to produce IL-12 when CD40, present on the cell surface, is bound by CD154, a protein expressed on activated T cells (Fig. 1-4) (Koch *et al.*, 1996). IL-12 then binds to its receptor on the T cell, initiating IFN-γ production and promoting a Th1-type immune response (Gately *et al.*, 1998). Th1 responses are also thought to be initiated or propagated by other DC factors including TRANCE-receptor, IL-15 and IL-18 (Bachmann *et al.*, 1999; Kuniyoshi *et al.*, 1999; Stoll *et al.*, 1998).

The means by which DC contribute to the induction of Th2-type responses is less clear. It is possible that Th2 responses are initiated when IL-12 production is suppressed, as occurs when DC are exposed to IL-10 or IL-4 (Koch *et al.*, 1996; De Smedt *et al.*, 1997). It is also possible that a different population of DC, perhaps even derived from a different precursor cell, is responsible for the initiation of initiation of Th2 vs Th1 responses (Pulendran *et al.*, 1999; Maldonado-Lopez *et al.*, 1999; Reid *et al.*, 2000).

Other ways in which DC are thought to regulate immune function include deleting T cells or causing them to be nonresponsive (tolerant) to specific antigens. Thymic DC, for example, are believed to contribute to central tolerance by deleting thymic T cells which respond to self antigens (Brocker *et al.*, 1997; Ardavin, 1997). Similarly, tolerance may occur outside the thymus (peripheral tolerance) when DC activate T cells without providing costimulatory signals (Thomson and Lu, 1999; Lu *et al.*, 1995). In addition, DC may also play a role in downregulation of immune responses by directly killing T cells through FasL which is expressed on certain populations of DC (Suss and Shortman, 1996; Lu *et al.*, 1997).

In summary, DC play an important role in both the initiation of immunity and in the promotion of particular types of immune responses. In addition the cells may contribute to the downregulation of the immune response and to the induction of tolerance. Therefore, chemicals which have the ability to perturb the functions of DC could have profound effects on immunity.

OBJECTIVES AND HYPOTHESIS

The studies presented in this dissertation were designed to test the hypothesis that exposure to TCDD alters DC in a manner which ultimately results in suppressed T cell function. This hypothesis is based on observations that TCDD suppresses T cell-dependent immune responses, but does not appear to affect T cells directly. Since the activation of naive T cells is initiated by DC, these important antigen-presenting cells represent a potential alternate target for the effects of TCDD.

To address this hypothesis, the expression of cell surface proteins critical to the activation of T cells was evaluated on DC from TCDD-treated mice, and various functional activities of the cells were tested. In Chapter 2, splenic DC from vehicle- and TCDD-treated mice were evaluated in the absence of antigenic stimulation. DC from two TCDD-responsive mouse strains were examined, as were cells from AhR-deficient animals. In Chapter 3, the impact of TCDD on the activation of antigen-specific T cells by DC was

addressed, and the ability of the cells to internalize antigen was tested. Results from these studies demonstrated that TCDD affected DC phenotype and function. However, since all these studies were performed using DC from TCDD-treated mice, it was not clear whether the observed effects resulted from a direct insult to the DC or as an indirect effect on some other cell or tissue. Therefore, the expression of the aryl hydrocarbon receptor in DC was examined in Chapter 4 to determine whether it was possible for the cells to respond directly to TCDD.

In addition, because we found that TCDD appeared to activate instead of suppress DC, studies detailed in Appendix A were conducted to test the hypothesis that activation of DC *in vivo* using the bacterial antigen lippolysaccharide would suppress the generation of the cell-mediated immune response to allogeneic tumor cells. Appendix B contains experiments designed to assess the immune function of AhR-deficient mice and to verify the obligatory role of the receptor in TCDD-induced immune suppression. It was included just for fun!!

Chapter 2

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Affects the Number and Function of Murine Splenic Dendritic Cells and Their Expression of Accessory Molecules

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ABSTRACT

Primary T cell-mediated immune responses are highly susceptible to suppression by TCDD exposure, yet direct effects of TCDD on T cells have been difficult to demonstrate. Since the activation of naive T cells has been shown to be initiated primarily by dendritic cells (DC), these cells represent a potential target for TCDD immunotoxicity. These initial studies have examined the influence of TCDD exposure on splenic DC phenotype and function in the absence of antigenic stimulation. Results showed that DC from TCDD-treated mice expressed higher levels of several accessory molecules including ICAM-1, CD24, B7-2 and CD40. In contrast, the expression of LFA-1 was significantly reduced. These effects were dose-dependent, persistent, and dependent upon the AhR. Interestingly, the number of DC recovered from TCDD-treated mice was significantly decreased seven days after exposure in C57Bl/6 animals and by day three after exposure in Balb/c mice. When DC from TCDD-treated mice were cultured with allogeneic T cells, the proliferative response and production of IL-2 and IFN-γ by the T cells were increased. Production of IL-12 by the DC was likewise enhanced in comparison to cells from vehicle-treated mice. Taken together, these results suggest that in the absence of antigen, TCDD provides an activation stimulus to DC and may lead to their premature deletion. Since the survival of DC may influence the strength of the immune response, these results suggest a possible novel mechanism for TCDD-induced immune suppression.

INTRODUCTION

The immune system is recognized as one of the most sensitive targets for the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental contaminant and prototypic ligand for the aryl hydrocarbon receptor (AhR) (reviewed by Kerkvliet and Burleson, 1994). In mice, TCDD-induced immune dysfunction is characterized by a profound suppression of T and B lymphocyte effector function, as evidenced by defects in production of antibodies, generation of cytotoxic T cells, and development of delayed-

type hypersensitivity responses (House *et al.*, 1997; Kerkvliet *et al.*, 1996; Lundberg *et al.*, 1991). Interestingly, immune suppression is observed only if TCDD exposure occurs early in the generation of an immune response, suggesting that early events in T helper cell activation may be altered (Kerkvliet *et al.*, 1996). However, because T cell responses are mostly unaffected following *in vitro* exposure to TCDD, it is unlikely that TCDD affects T cells directly (Lang *et al.*, 1994; Lawrence *et al.*, 1996; Dekrey and Kerkvliet, 1995). Rather, it suggests that the cells responsible for the activation of T cells may be the target of TCDD.

Dendritic cells (DC) are the most potent antigen-presenting cells for activation of naive T cells (reviewed by Banchereau and Steinman, 1998). Located throughout the body, immature DC express constitutive levels costimulatory molecules and are specialized for capturing antigen. When exposed to antigenic stimuli, LPS or inflammatory cytokines, DC are induced to migrate to the spleen or draining lymph nodes and to undergo a maturation process. As they mature, DC downregulate phagocytic activity, increase expression of adhesion and costimulatory molecules and the major histocompatibility complex (MHC), and begin to produce cytokines. In this mature state, DC activate T cells by presenting antigen in the context of MHC and by providing necessary costimulation via accessory molecules and cytokines. Among the important accessory molecules expressed by DC are adhesion molecules such as ICAM-1(CD54) and LFA-1(CD11a), which function to maintain prolonged contact between the DC and T cell during activation, and costimulatory molecules such as B7-1(CD80), B7-2(CD86), CD24, and CD40, which signal T cells to proliferate and differentiate (Inaba *et al.*, 1994; Cella *et al.*, 1996; Enk and Katz, 1994; VanKooten and Banchereau, 1997). DC also produce important cytokines such as IL-12, a cytokine which promotes the differentiation of TH1 vs TH2 cells (Koch *et al.*, 1996; Gately *et al.*, 1998).

The potential for TCDD to influence DC in terms of their ability to activate T cells has not been previously examined. Therefore, in the studies reported here, we provide an initial characterization of the temporal and dose-related effects of TCDD on the expression of adhesion and costimulatory molecules on DC which are important in T cell

activation. In addition, the functional ability of DC from TCDD-treated mice to stimulate T cell proliferation and cytokine production *in vitro* was evaluated.

MATERIALS AND METHODS

Animal treatments

Male C57Bl/6 and female DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female Balb/c mice were purchased from B&K Universal, Inc. (Kent, WA). AhR knockout mice (Fernandez-Salguero *et al.*, 1995), which were of C57Bl/6 x 129/Sv mixed genetic background, were generously provided by Dr. A. Silverstone (State University of New York Health Science Center, Syracuse, NY). Male mice were housed singly, and female mice were housed 5-6 per cage. Animals were maintained in front of a laminar flow unit and in accordance with National Research Council guidelines. Mice were used at 7-12 weeks of age and were killed by CO_2 overdose.

TCDD exposure

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil. The vehicle control consisted of an equivalent amount of anisole in peanut oil. Mice were treated with TCDD or vehicle by gavage. Except in dose-response studies, TCDD was given at 15 μg/kg, a dose previously shown to suppress immune responses in mice (Kerkvliet *et al.*, 1996).

Reagents and antibodies

Spectral Red streptavidin was obtained from Southern Biotech (Birmingham, AL). Biotinylated-CD11c, as well as various fluorochrome-conjugated antibodies to B7-1 (CD80), B7-2 (CD86), CD8α, ICAM-1 (CD54), LFA-1 (CD11a), and MHCII (I-A^b) were obtained from PharMingen (San Diego, CA). Antibodies to CD24 was purchased from PharMingen or were produced in our laboratory using the J11d hybridoma (American Type Culture Collection, Rockville, MD). Antibodies to CD40 were purchased from

PharMingen and from Southern Biotech. For ELISA, IL-2 and IFN-γ antibody pairs were purchased from PharMingen, and antibodies to the p40 subunit of mouse IL-12 were purchased from Genzyme (Cambridge, MA).

Preparation of DC

DC were enriched from spleens using the method of Swiggard *et al.* (1992) with modifications as described in Inaba *et al.* (1997). Briefly, splenic tissue was digested with collagenase D (Boehringer Mannheim, Indianapolis, IN) at 37°C for 45-60 minutes to release DC from the capsule and increase recovery. Cell suspensions were then diluted in Ca-/Mg-free HBSS and pelleted. Recovered cells were spun over a BSA gradient (1.080 g/ml) and cells in the low density fraction were collected. These freshly isolated DC-enriched preparations were then stained for flow cytometric analysis. For mixed leukocyte reaction (MLR), the low density spleen cells were further enriched for DC by overnight culturing based on their property of transient adherence to plastic (Swiggard *et al.*, 1992). In this procedure, the low density cells were cultured in plastic dishes for 90 minutes to allow for the DC to adhere. The non-adherent contaminating cells were then washed away, and the remaining adherent cells left in culture overnight. DC become non-adherent during this culture period and were collected from media the following day. Purity after this final enrichment was greater than 80% $CD11c^{HI}$ cells.

Flow Cytometry

Cells were incubated in 96-well plates with saturating concentrations of mAb. Nonspecific mAb binding was blocked by pre-incubating cells with rat and/or hamster IgG. All cell preparations were stained with an antibody to CD11c to allow selective analysis of DC (Crowley *et al.*, 1990b) (Fig. 2-1). Typically, 10-25% of the low density spleen cells expressed high levels of CD11c. In various experiments, cells were also stained with mAb to: B7-1, B7-2, CD40, CD24, ICAM-1, LFA-1 , CD8α, or MHCII. Appropriately-labeled isotype controls were used to determine nonspecific fluorescence. A viable cell gate was established based on propidium iodide exclusion. For cell surface molecule evaluation, 10,000 viable $CD11c^{HI}$ cells were analyzed. Listmode data were collected on

a Coulter Epics XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME).

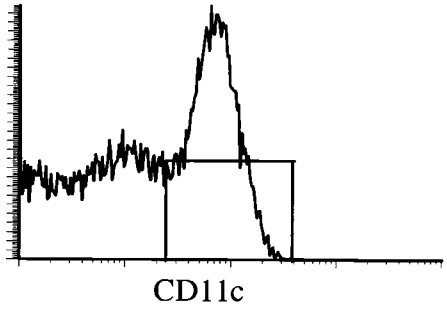


FIG. 2-1. CD11c staining on low density spleen cells. Spleens were digested with collagenase and enriched for DC by density gradient centrifugation. The low density fraction was stained for CD11c and a region was set based on high levels of staining for this DC marker. Expression of accessory molecules was subsequently evaluated by gating on these $CD11c^{HI}$ cells.

Mixed leukocyte reaction (MLR)

T cells were enriched from spleens of female DBA/2 mice by nonadherence to nylon wool (Hathcock, 1991). Resulting cell suspensions typically contained 50% CD4+ cells and 20% CD8+ cells. T cells (3×10^5) were plated in triplicate in 96-well tissue culture plates with various numbers of DC as stimulators. T cell proliferation was measured by incorporation of ^{3}H-TdR which was added (1 μCi/well) during the last 20 hours of culture.

Cytokine analysis

Culture supernatants were analyzed for cytokines using antibody sandwich ELISA techniques. The secondary biotinylated antibodies were complexed with avidin-peroxidase and visualized with 2,2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] as substrate. Absorbance was read at 405 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). For IL-2 analysis, 60 μl supernatant was removed from each well of MLR cultures just prior to addition of 60 μl ^{3}H-TdR for proliferation assay.

Statistical analysis

For most experiments, a Student's *t* test was used to compare means of the vehicle-treated group to the TCDD-treated group. Where indicated, analysis of variance modeling (ANOVA) was performed using SAS statistical software (SAS Institute, Inc., Cary, NC), and comparisons between means were made using the least significant difference (LSD) multiple comparison *t* test. ANOVA was also used for analysis of IL-2 production across multiple experiments. Due to non-constant variance, the analysis on the 28 hour data was performed on ln-transformed data.

RESULTS

Effect of TCDD exposure on DC expression of accessory molecules

A number of cell surface proteins play important roles in the function of DC. We initially examined the expression of several of these accessory molecules on splenic DC from C57Bl/6 mice three days after treatment with an immunosuppressive dose (15 μg/kg) of TCDD. As shown in Fig. 2-2, TCDD exposure significantly increased the expression of ICAM-1, CD24, CD40, B7-2, and MHC class II (MHCII) as measured by the percent of DC staining positive for the marker and/or the median channel fluorescence (MCF) of the staining on all DC. Although the degree of change was small for some of the markers, the results were confirmed in several independent studies. In contrast to other accessory molecules, LFA-1 expression was consistently decreased on DC isolated from TCDD-treated mice (Fig. 2-2), while B7-1 expression was not altered.

The dose-dependency of the observed effects of TCDD on the expression of accessory molecules was evaluated on splenic DC isolated from mice treated with varying doses of TCDD and sacrificed three days later. As shown in Fig. 2-3, all doses of TCDD augmented the expression of ICAM-1 on the cell surface as evidenced by the increased fluorescence intensity of the cells. Similarly, the expression of CD24 was enhanced at doses above 1.9 μg/kg TCDD as evidenced by the increased percent of DC staining positive for CD24 and the higher intensity of staining at the 15 μg/kg dose. In contrast,

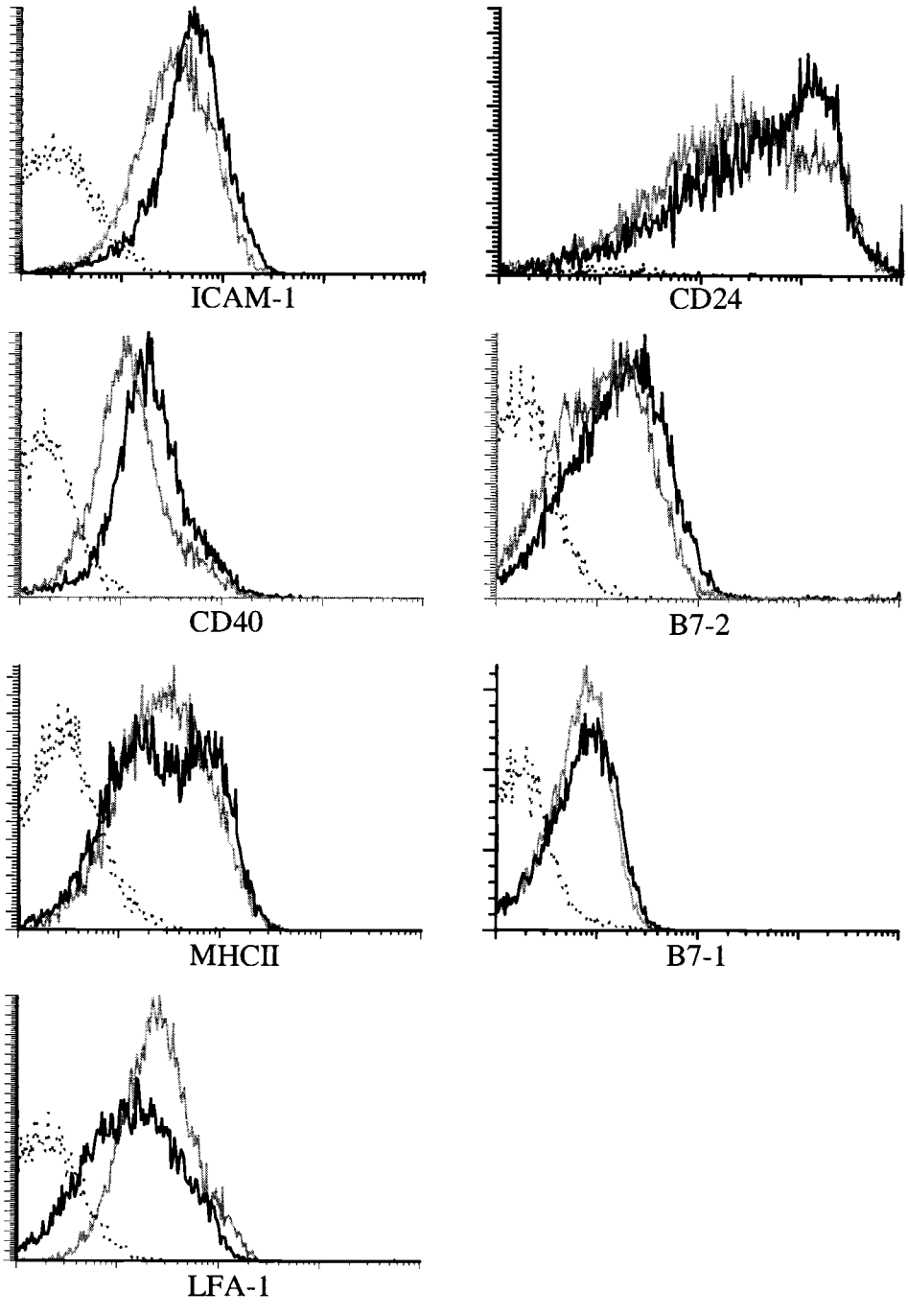


FIG. 2-2. Effect of TCDD on DC accessory molecule expression three days after exposure. C57Bl/6 mice were treated with vehicle control or 15μg/kg TCDD. Three days later, spleens were removed, digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. Representative histograms depict accessory molecule expression on $CD11c^{HI}$ cells. Solid grey line (vehicle), solid black line (TCDD), dotted line (isotype control).

both the percent of DC staining positive for LFA-1 and the fluorescence of this marker on the cells was significantly decreased at all doses of TCDD tested.

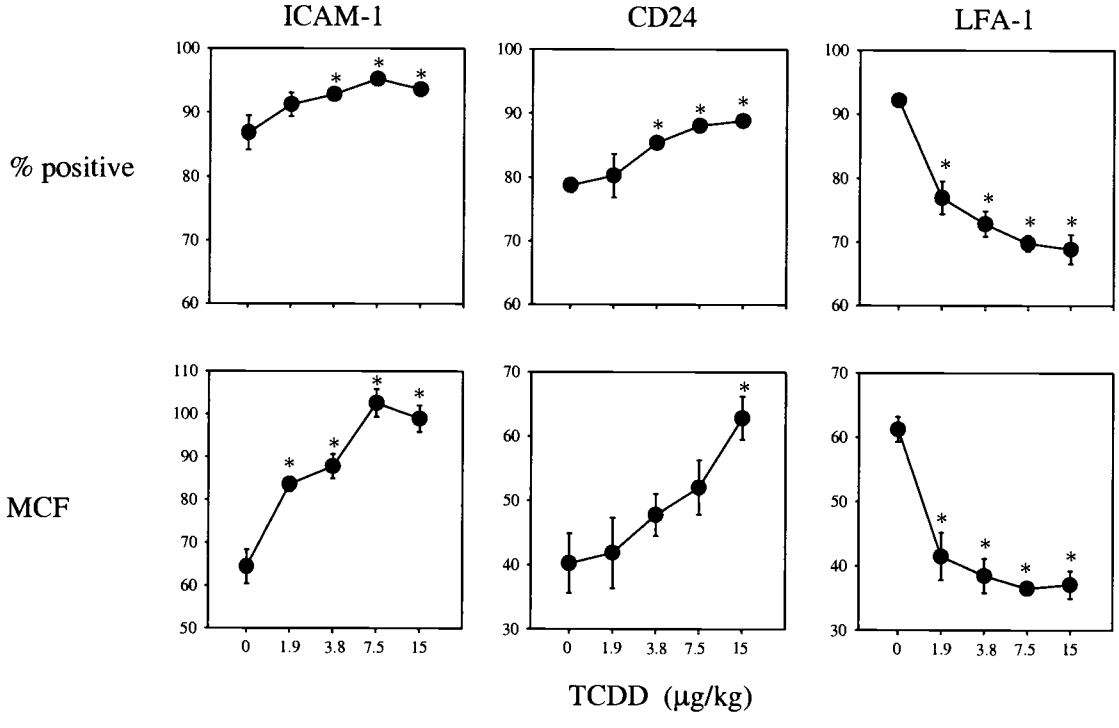


FIG. 2-3. Dose response effect of TCDD on DC accessory molecule expression. C57Bl/6 mice were treated with vehicle control or indicated dose of TCDD. Three days later, spleens were removed, digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry by gating on $CD11c^{HI}$ cells. Positive staining for each marker was determined based on isotype control antibody. Median channel fluorescence (MCF) represents the staining on all $CD11c^{HI}$ cells. Data points represent the mean ± SEM of three animals per treatment group. Data were analyzed by ANOVA and LSD. * Different from vehicle ($p \leq 0.05$).

Time course of effects of TCDD on DC phenotype and cell number

Since the immunotoxic effects of HAH are known to persist for weeks after exposure (Kerkvliet and Baecher-Steppan, 1988; Kerkvliet and Brauner, 1987), we examined DC seven and fourteen days after TCDD exposure to determine if changes in surface molecule expression were likewise persistent. The results were similar at both time points and therefore only the histograms from day seven are shown in Fig. 2-4. Consistent with changes observed three days after treatment, TCDD exposure for seven or fourteen days increased the expression of ICAM-1, CD24, CD40, and B7-2 and suppressed the expression of LFA-1. However, when compared to changes observed three days after exposure, TCDD appeared to affect expression of certain molecules more dramatically at these later time points, with particularly interesting changes occurring in the expression of CD24. CD24 expression in the vehicle-treated mice was bimodal, suggesting the presence of both a low- and a high-expressing population. In the TCDD-treated mice the peak was shifted to the right and the intensity of the staining was significantly higher (MCF 130.4 ± 21.4 on day 7) compared to the vehicle-treated mice (MCF 42.0 ± 6.2).

Interestingly, the number of DC isolated from the spleen of TCDD-treated mice was markedly reduced seven and fourteen days after exposure (Table 2-1), an effect not seen on day 3. Specifically, the total number of $CD11c^{HI}$ cells recovered in the low density spleen cell fraction was reduced by 44% on day seven and by 41% on day fourteen. To evaluate the possibility that TCDD exposure increased the density of the DC, causing them to separate into the high density cell fraction during density gradient centrifugation, we also examined the cells in the high density BSA fraction. However, no increase was seen in the percent or number $CD11c^{HI}$ cells in this fraction (data not shown). Additionally, TCDD treatment did not alter the total number of spleen cells recovered prior to density separation, suggesting a true loss of DC from the spleen.

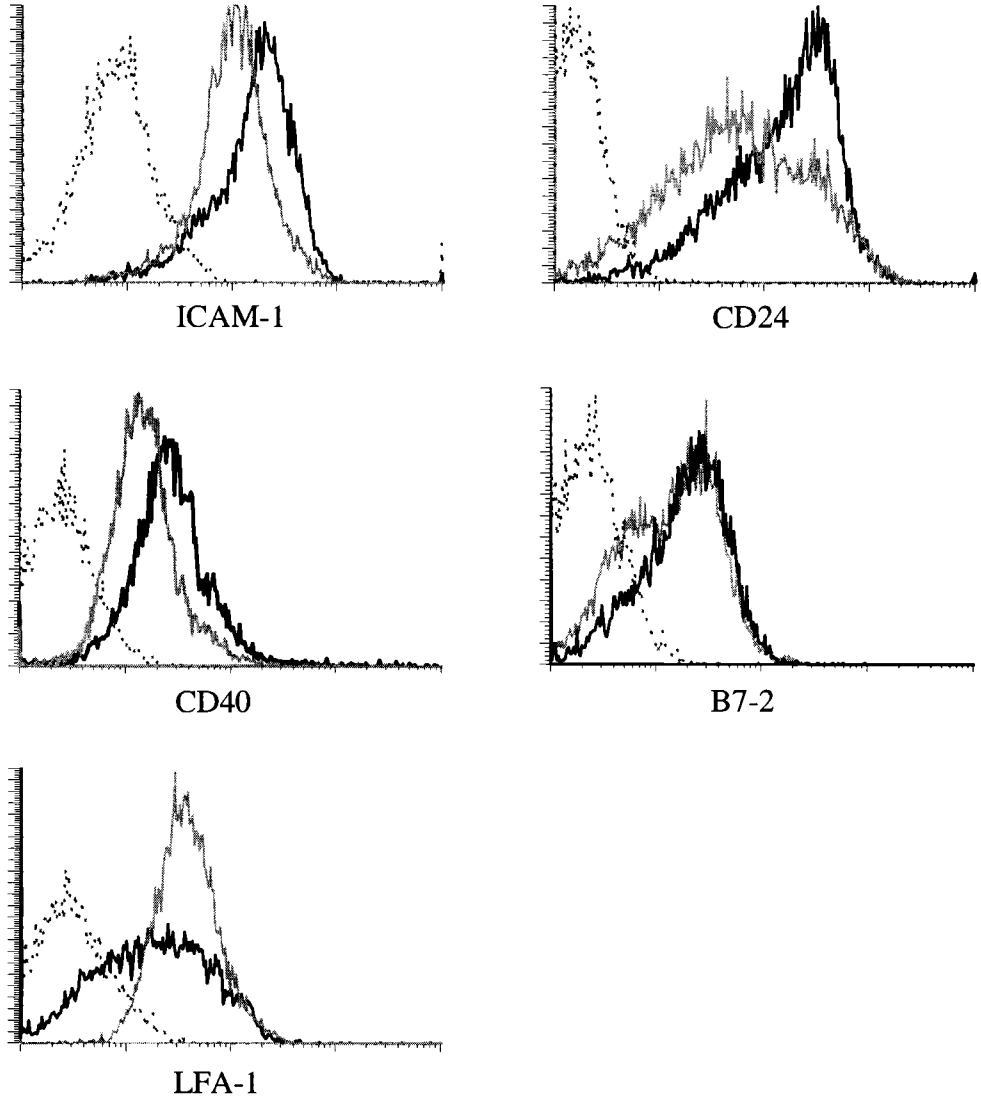


FIG. 2-4. Effect of TCDD on DC accessory molecule expression seven days after exposure. C57Bl/6 mice were treated with vehicle control or 15μg/kg TCDD. Seven days later, spleens were digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. Representative histograms depict accessory molecule expression on CD11c^{HI} cells. Solid grey line (vehicle), solid black line (TCDD), dotted line (isotype control).

TABLE 2-1
Number of Splenic DC in C57Bl/6 Mice on Days 7 and 14 after TCDD Exposure [a]

	$CD11c^{HI}$ ($x10^5$)
Vehicle	7.8 (0.2)
TCDD - Day 7	4.4 (0.1) *
TCDD - Day 14	4.6 (0.2) *

[a] Data represent the average number ± (SEM) of DC defined as $CD11c^{HI}$ cells in the low density spleen cell fraction, n = 3. Data are representative of three experiments.
* Different from vehicle ($p \leq 0.05$).

DC from Balb/c mice are sensitive to TCDD

The C57Bl/6 mouse used in the previous studies is the prototypic TCDD-responsive mouse strain which possesses an AhR genotype that confers high sensitivity to the toxic effects of TCDD. In order to determine if DC would be similarly affected in other mice which possess a sensitive AhR genotype, we examined DC from Balb/c mice. As shown in Table 2-2, exposure to TCDD for three or seven days caused the same pattern of effects in the Balb/c mice as in C57Bl/6 mice, increasing the expression of ICAM-1, CD24, CD40, and B7-2, and decreasing the expression of LFA-1 on DC. Additionally, TCDD decreased the numbers of DC recovered at both three and seven days after exposure, thus affecting DC numbers earlier in the Balb/c mice as compared to the C57Bl/6 animals.

TCDD does not decrease recovery of DC nor affect accessory molecule expression in $AhR^{-/-}$ mice

Many, if not all, of the immunotoxic effects of TCDD result from activation of the AhR (Vecchi *et al.*, 1983; Kerkvliet *et al.*, 1990b). To address whether TCDD-induced changes in DC were AhR-dependent, we examined DC from $AhR^{-/-}$ mice exposed to 15 μg/kg TCDD seven days previously. As shown in Table 2-3, TCDD treatment of the AhR^{-}

$^{-/-}$ mice did not alter the number of DC recovered from the spleen nor the expression of any markers examined, including ICAM-1, CD24, CD40, B7-2, or LFA-1. In contrast, DC from TCDD-treated C57Bl/6 mice, included as study controls, were altered in a similar manner as in previous experiments. Interestingly, the absence of the AhR in the null mice did not appear to affect the overall development of splenic DC as vehicle-treated C57Bl/6 and $AhR^{-/-}$ mice had equivalent numbers of splenic DC which expressed similar levels of accessory molecules.

TABLE 2-2
Number and Phenotype of Splenic DC in Balb/c Mice on Days 3 and 7 after TCDD Exposure[a]

		Vehicle	TCDD - Day 3	TCDD - Day 7
DC	# (x 10^5)	7.1 (0.8)	4.4 (0.5)*	3.7 (0.5)*
ICAM-1	%	91.8 (0.4)	95.3 (0.3)*	95.1 (0.4)*
	MCF	80.3 (0.7)	92.7 (0.7)*	97.0 (0.6)**
CD24	%	89.9 (0.4)	93.5 (0.2)*	96.3 (0.2)**
	MCF	102.3 (2.6)	124.0 (1.0)*	131.3 (0.9)**
CD40	%	68.4 (2.6)	78.1 (1.8)*	82.2 (1.3)*
	MCF	69.0 (1.2)	76.3 (1.5)*	81.0 (1.5)*
B7-2	%	66.3 (0.8)	72.9 (1.7)*	76.6 (1.3)*
	MCF	78.7 (0.9)	83.3 (1.2)*	85.0 (1.0)*
LFA-1	%	74.0 (1.0)	63.7 (0.9)*	62.3 (2.2)*
	MCF	88.3 (0.7)	83.3 (0.3)*	82.3 (2.2)*

[a] Data represent the mean ± (SEM) of 3 animals per group. Data were analyzed by ANOVA and comparisons between group means were performed using LSD.
* Different from vehicle ($p \leq 0.05$).
** Different from vehicle and TCDD Day 3 ($p \leq 0.05$).

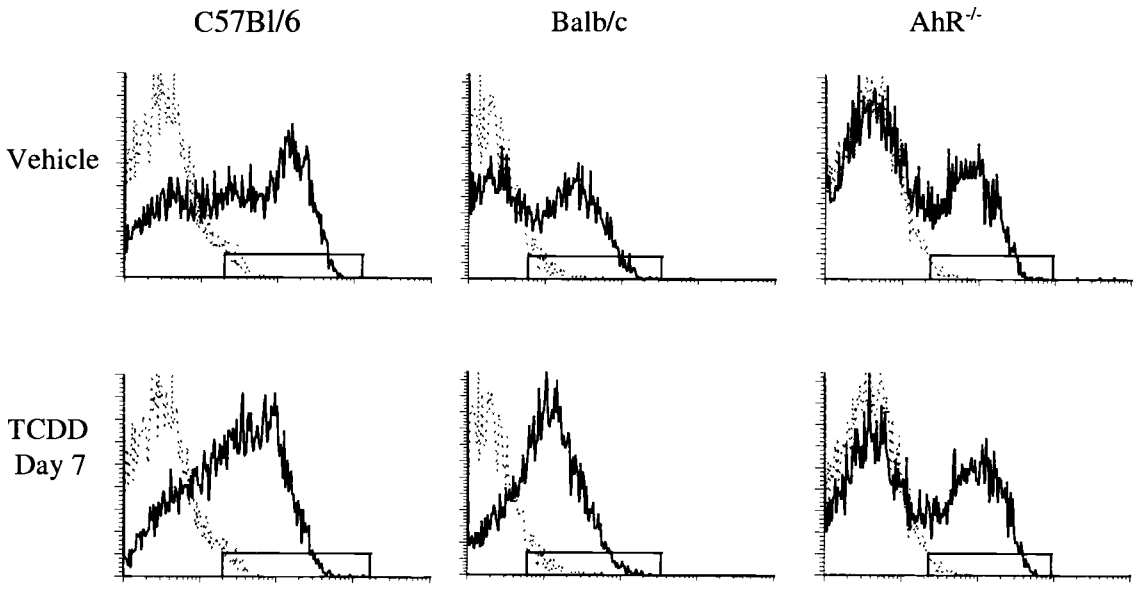
TABLE 2-3
Number and Phenotype of Splenic DC in AhR $^{-/-}$ vs C57Bl/6 Mice on Day 7 after TCDD Exposure[a]

		C57Bl/6		AhR $^{-/-}$	
		Vehicle	TCDD	Vehicle	TCDD
DC	# (x 10^5)	9.6 (0.6)	5.5 (0.6)*	11.0 (2.0)	12.0 (4.0)
ICAM-1	%	91.1 (1.4)	94.8 (0.9)	94.9 (1.5)	89.0 (4.0)
	MCF	91.0 (3.9)	150.4 (3.6)*	105.7 (5.7)	92.2 (11.6)
CD24	%	90.3 (1.0)	96.4 (0.8)*	90.7 (2.0)	92.8 (1.4)
	MCF	41.7 (4.7)	59.8 (9.1)	45.1 (17.7)	46.2 (2.5)
CD40	%	65.2 (1.3)	85.4 (0.6)*	71.9 (2.0)	64.7 (0.4)
	MCF	14.5 (0.4)	28.1 (0.9)*	18.2 (1.0)	15.5 (0.5)
B7-2	%	65.0 (0.7)	77.8 (0.7) *	69.5 (1.8)	69.5 (1.8)
	MCF	41.7 (1.3)	59.0 (1.4)*	49.0 (5.3)	44.6 (2.4)
LFA-1	%	88.7 (0.6)	73.0 (1.0)*	94.6 (1.3)	94.2 (0.8)
	MCF	47.0 (1.0)	39.3 (0.8)*	54.3 (10.6)	51.4 (0.9)

[a] Data represent the mean ± (SEM) of 2 or 3 animals per group.
* Different from vehicle ($p \leq 0.05$).

Effect of TCDD exposure on CD8α+ DC population

Current evidence suggests that two populations of DC are present in the spleen, one derived from a lymphoid precursor and one of myeloid origin (Vremec and Shortman, 1997; Shortman and Caux, 1997). The expression of CD8α has been used to identify the lymphoid-derived cells, which have also been identified by their co-expression of high levels of CD24 (Crowley *et al.*, 1990b; Vremec *et al.*, 1992). Due to the dramatically increased expression of CD24 seen following TCDD exposure, we hypothesized that TCDD may be increasing the CD8α+ lymphoid DC population in the spleen. As shown in Fig. 2-5, in C57Bl/6 and Balb/c mice, but not in AhR$^{-/-}$ mice, TCDD treatment



	C57Bl/6		Balb/c		AhR$^{-/-}$	
	%	MCF	%	MCF	%	MCF
Vehicle	48.6 (1.1)	92.2 (4.8)	43.7 (1.5)	90.0 (1.0)	46.0 (10.3)	65.0 (2.3)
Day 7	53.0 (0.6) *	59.5 (3.3)*	71.6 (2.0)*	77.0 (0.0)*	45.2 (1.5)	72.8 (7.8)

FIG. 2-5. Effect of TCDD on CD8α expression on DC seven days after exposure. Mice were treated with vehicle control or 15μg/kg TCDD. Seven days later, spleens were digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. Dotted lines show staining of isotype control antibody. Region delineates CD8α+ cells as defined by staining above control antibody. * Different from vehicle ($p \leq 0.05$).

diminished the intensity of CD8α staining (MCF) on the DC on day seven. Interestingly, although the staining intensity was diminished, the percent of CD8α+ DC was increased by TCDD treatment. In C57Bl/6 mice, this effect was seen on days seven and fourteen post TCDD exposure, but not on day three, suggesting that the increase in CD24 expression seen

at the earlier timepoint was not the result of an increase in lymphoid DC. In Balb/c animals, TCDD had similar effects on both day three and day seven (day three not shown). Since the numbers of DC in the spleen were also reduced on days seven and fourteen in the C57Bl/6 mice and on days three and seven in the Balb/c animals, the increase in the percent of CD8α+ DC at these same timepoints suggests that TCDD may be selectively reducing the myeloid DC population resulting in a relative increase in CD8α+ DC remaining in the spleen.

Effect of in vivo TCDD exposure on DC function ex vivo

The functional status of DC is often evaluated by their ability to activate allogeneic T cells in an MLR (Moser *et al.*, 1995; Girolomoni *et al.*, 1992; Metlay *et al.*, 1989). To determine if TCDD alters DC function, DC were enriched from the spleens of vehicle- or TCDD-treated C57Bl/6 mice three days after exposure and cultured with splenic T cells from DBA animals for various periods of time. As shown in Fig. 2-6, TCDD exposure tended to enhance the ability of DC to activate T cells. Specifically, cultures containing DC from TCDD-treated mice demonstrated enhanced T cell proliferation (Fig. 2-6A) and IFN-γ production (Fig. 2-6B). TCDD exposure also resulted in a slight increase in IL-2 production (representative experiment shown in Fig. 2-6C). Although not statistically significant in all experiments, the increased IL-2 in 28-hour cultures was significant when analyzed across four experiments ($p<0.01$). Similar results were obtained in a single experiment when proliferation and cytokine production were measured in cultures containing DC from animals treated with TCDD seven days previously (data not shown), suggesting that increasing the duration of TCDD exposure does not further enhance effects on DC function.

As shown in Fig. 2-7, TCDD exposure was also associated with increased production of IL-12, an APC-derived cytokine which induces IFN-γ production by T cells and thus promotes a Th1-type immune response. When DC were cultured alone, we observed low, but detectable production of IL-12 which was augmented by TCDD treatment. Likewise, when T cell-enriched allogeneic splenocytes were added to the DC cultures, IL-12 production was higher in wells containing DC from TCDD-treated mice. In the wells

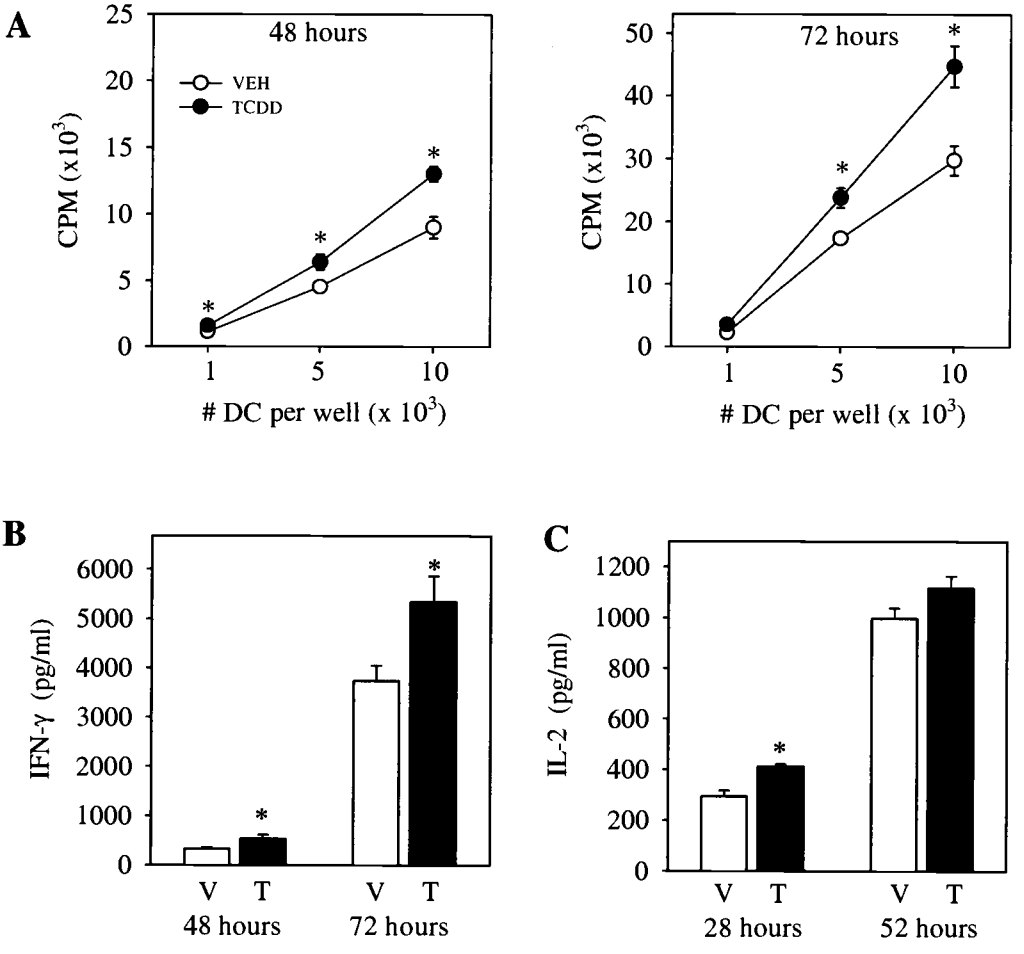


FIG. 2-6. Effect of TCDD exposure on ability of DC to stimulate T cell proliferation and cytokine production in a mixed leukocyte reaction. Spleens were removed from C57BL/6 mice three days after treatment with vehicle (open circles/bars) or 15 μg/kg TCDD (solid circles/bars). Spleens from three mice were pooled, and DC were enriched by collagenase digestion, density gradient centrifugation, and transient adherence to plastic. Indicated numbers of DC were cultured with 3 x 10^5 T cells enriched from spleens of DBA/2 mice. Proliferation (A) was measured at 48 and 72 hours by ^{3}H-TdR incorporation. Background activity was below 1000 cpm in wells containing T cells alone and below 400 cpm in wells containing DC alone. For IFN-γ (B) and IL-2 (C) analysis, culture supernatants were harvested at indicated times from wells containing T cells and 10^4 DC. Cytokine concentrations were determined by ELISA. Wells containing only DC or T cells were below the limit of detection. Data points represent the mean ± SEM (n = 4 per group). Data are representative of three (IFN-γ) or four (proliferation, IL-2) experiments. * Different from vehicle ($p \leq 0.05$).

containing both cell populations, IL-12 production was significantly enhanced relative to wells containing DC only, indicating cross-talk between the cell populations. This type of cell-cell communication likely occurs via interaction of cell surface molecules such as CD40-CD40L, or possibly as a response to production of IFN-γ, which has been shown to induce or enhance the production of IL-12 by macrophages (Ma *et al.*, 1996; Yoshida *et al.*, 1994). To address the possibility that augmented IL-12 production resulted from contaminating macrophages in the T cell preparation, we performed a separate experiment in which T cell preparations were depleted of adherent cells prior to addition to DC cultures. This depletion did not affect IL-12 production, indicating that the DC were the source of the IL-12 (data not shown).

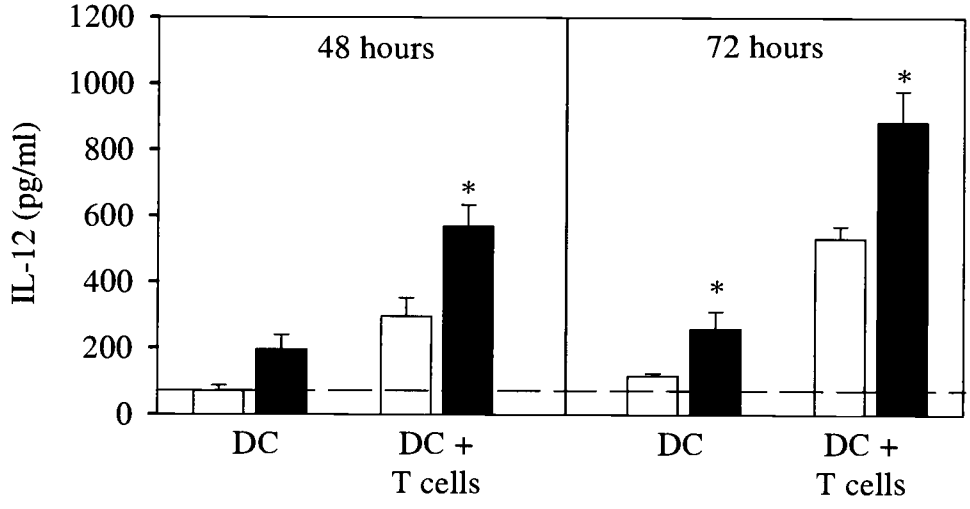


FIG. 2-7. Effect of TCDD exposure on IL-12 production by DC in a mixed leukocyte reaction. Spleens were removed from C57BL/6 mice three days after treatment with vehicle (open bars) or 15 μg/kg TCDD (solid bars). Spleens were pooled from three animals and dendritic cells were isolated by collagenase digestion, density gradient centrifugation, and transient adherence to plastic. 1 x 10^4 DC were cultured alone or with 3 x 10^5 T cells enriched from spleens of DBA/2 mice. Culture supernatants were harvested at indicated times, and cytokine concentration determined by ELISA. Data points represent the mean ± SEM (n = 4 per group). Data are representative of three experiments. Dashed line represents limit of detection for ELISA. Wells containing only T cells were below limit of detection. * Different from vehicle ($p \leq 0.05$).

DISCUSSION

The effects of TCDD exposure on DC phenotype and function have not been previously reported. Due the immunosuppressive properties of TCDD, we hypothesized that TCDD exposure would suppress the expression of key accessory molecules on DC and/or disrupt their ability to activate T cells. Contrary to expectation, we instead found that DC from TCDD-treated mice expressed higher levels of many important accessory molecules and were relatively better at stimulating basic T cell activation events. The changes induced by TCDD were dose- and time-dependent and persisted for at least fourteen days. Similar effects on DC were seen in two AhR-responsive mouse strains (C57Bl/6 and Balb/c), but not in $AhR^{-/-}$ animals, indicating that changes in DC were mediated by the AhR. The changes in DC phenotype were induced by TCDD in the absence of antigenic stimulation and suggest that TCDD may inappropriately activate DC.

We also found that TCDD exposure led to a decreased number of DC recovered from the spleen, a finding which is consistent with the apparent activation of DC by TCDD. Once a DC becomes activated, it does not revert back to a less mature state but is instead committed to ultimately undergo apoptosis (Winzler *et al.*, 1997). For example, activating DC *in vivo* by exposing mice to LPS leads to increased accessory molecule expression, causes the cells to undergo apoptosis, and results in a decreased number of DC in the spleen (De Smedt *et al.*, 1996; De Smedt *et al.*, 1998). It is therefore plausible that exposure to TCDD likewise activates DC, inducing their death and ultimate loss from the spleen. Because DC survival correlates with enhanced T cell priming *in vivo* (Josien *et al.*, 2000), a decrease in DC numbers may provide an explanation as to how TCDD could enhance the activation of DC yet paradoxically suppress the generation of an immune response to antigen.

It is also possible that because DC lose the ability to take up antigen upon activation (Kitajima *et al.*, 1997; Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1995), TCDD exposure may result in a diminished ability to internalize antigen for processing. This scenario has been observed when DC were taken from LPS-treated mice (De Smedt *et al.*, 1996). LPS-activated DC, which expressed enhanced levels of MHC class II, B7-1 and B7-2, augmented

the proliferation of SEB-activated T cells, but were unable to stimulate proliferation of a T cell hybridoma which required antigen processing. In our studies, enhanced T cell proliferation was observed in an allogeneic MLR assay where the proliferative response reflects T cell recognition of allo-MHC on the surface of the DC. Because this response is independent of antigen processing, these results do not indicate whether TCDD exposure may interfere with uptake and/or processing of antigen by DC. However, we also found enhanced T cell proliferation in other studies where DC were used as APC for antigen-specific syngeneic T cells (manuscript in preparation). Thus it appears unlikely that TCDD interferes with antigen processing.

TCDD exposure also resulted in increased IL-12 production by DC. IL-12 is produced by APC populations, including macrophages and DC, and influences the Th1/Th2 balance by promoting the secretion of IFN-γ by TH1 cells (reviewed in Gately *et al.*, 1998). As with other DC functions, production of IL-12 is dependent upon the activation state of the cell; DC produce IL-12 when exposed to activating stimuli such as uptake of antigen-coated beads and ligation of CD40 (Scheicher *et al.*, 1995; Kato *et al.*, 1997; Koch *et al.*, 1996). Thus, the observed increase in IL-12 production is consistent with TCDD causing DC activation. The increase in IL-12 production by DC observed in the absence of antigenic stimulation is intriguing in light of recent studies in our laboratory which demonstrate that exposure to TCDD during development of an immune response to allogeneic tumor or ovalbumin suppresses IL-12 production by antigen-stimulated spleen cells (Shepherd *et al.*, manuscript submitted for publication, and unpublished observations). Whether this suppression of IL-12 reflects a differential effect of TCDD on antigen-stimulated DC or an effect on other IL-12-producing cells present in the spleen remains to be determined.

The increased expression of CD24 on DC from TCDD-treated mice was an interesting finding. CD24, also known as heat-stable antigen, is a protein expressed on multiple lineages of hematopoietic cells (Bruce *et al.*, 1981; Takei *et al.*, 1981). Blocking studies have demonstrated that on APC, CD24 functions as a costimulatory molecule for T cell proliferation and cytokine production (Liu *et al.*, 1992; De Bruijn *et al.*, 1996; Enk and Katz, 1994). In the spleen, CD24 has been shown to be expressed predominantly on

interdigitating DC (IDC) in the periarteriolar region (PALS) of the white pulp, while DC in the marginal zone (MZ) are CD24 low/negative (Agger *et al.*, 1990; Crowley *et al.*, 1989). The relationship between the IDC and the MZ DC is not entirely clear. Some studies have suggested that DC initially localize in MZ where they are optimally positioned to encounter and present antigen to circulating T cells (Agger *et al.*, 1990; Kupiec-Weglinski *et al.*, 1988). Once the DC encounters the appropriate antigen-specific T cell, it moves to the PALS and develops further T cell-activating activity. Under this scenario, the enhanced expression of CD24 on DC from TCDD-treated mice is consistent with DC being activated by TCDD exposure.

Alternatively, the increased CD24 expression may suggest a selective increase of CD8α+ lymphoid DC which have been shown to co-express high levels of CD24 (Vremec *et al.*, 1992). This possibility is intriguing because current evidence suggests that lymphoid DC may play a role in downregulation of an immune response, generation and maintenance of peripheral tolerance, or in directing the development of specific T helper subsets (Maldonado-Lopez *et al.*, 1999; Suss and Shortman, 1996; Kronin *et al.*, 1996; Pulendran *et al.*, 1999). Interestingly TCDD increased the percentage of DC staining positive for CD8α on days seven and fourteen in the C57Bl/6 mice, the same times at which we observed decreased numbers of DC in the spleen. A similar effect was observed in the Balb/c mice wherein TCDD exposure decreased DC recovery but increased the percentage of CD8α+ DC as early as three days after treatment. Although TCDD exposure increased the percentage of CD8α+ DC, the fluorescence intensity of the CD8α staining was decreased on the cells. The significance of this observation is not clear since the CD8α molecule itself does not appear to play a role in DC stimulatory activity (Kronin *et al.*, 1997). Recently Vremec *et al.* (2000) described a population of cells in their DC preparations that expressed intermediate levels of CD8α . This population appeared to represent an artifact of a contaminating autofluorescent cell population. We do not believe that this explains our data as we have never seen an increase in autofluorescence with TCDD in any of our isotype controls. However, while CD8α is thought to be a stable lineage marker (Vremec and Shortman, 1997), it is possible that downregulation of CD8α

expression on the DC could reflect activation of the cell as has been shown to occur upon activation of CD8+ T cells (Oughton and Kerkvliet, 1999).

Another interesting effect of TCDD on DC was the marked reduction in LFA-1 expression. This reduced expression is unlikely to reflect activation of DC since LFA-1 is upregulated on activated macrophages and T cells (Strassmann *et al.*, 1986; Strassmann *et al.*, 1985; Maraskovsky *et al.*, 1996). Furthermore, LFA-1 expression was not reduced on splenic DC subjected to overnight culturing (Inaba *et al.*, 1994, and our unpublished observations), a stimulus which causes a substantial increase in expression of activation markers such as B7 and CD40. LFA-1 is a B-2 integrin protein which is important in cell-cell adhesion and plays a role in leukocyte trafficking and extravasation into tissue (Andrew *et al.*, 1998; Ma *et al.*, 1994). For example, studies by Ma *et al.* (1994) showed that blocking LFA-1 *in vivo* reduced the antigen-induced migration of DC to the lymph nodes and the subsequent development of a contact hypersensitivity response. Therefore, because TCDD exposure decreased LFA-1 expression on DC, it is possible that, in the presence of antigen, TCDD may interfere with the activation of T cells by inhibiting the ability of DC to carry antigen to the T cell areas of the lymphoid organs. In addition, if LFA-1 plays a role in general trafficking of DC, a decrease in expression of this molecule could affect normal repopulation of splenic DC lost to cell turnover or death, and thus could account for the loss of splenic DC we observed in TCDD-treated mice. Further studies are warranted to address the functional significance of decreased LFA-1 expression.

In conclusion, our studies have shown that splenic DC from TCDD-treated mice demonstrate enhanced expression of many accessory molecules required for activation of T cells and are relatively more efficient at stimulating T cells *ex vivo* when compared to DC from vehicle-treated mice. Although these findings were contrary to our original hypothesis, several potential mechanisms linking DC activation to suppression of the immune response have been discussed. It now remains to be directly demonstrated which, if any, alteration in DC function underlies the immunotoxicity of TCDD.

Chapter 3

Antigen-Dependent Functions of Dendritic Cells in TCDD-Treated Mice

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ABSTRACT

Previously we have shown that in the absence of antigen, exposure to TCDD results in activation of splenic dendritic cells, the antigen-presenting cell responsible for activating naive T cells. Because activation of DC is generally considered to result in enhanced T cell stimulation, these observations were difficult to reconcile with the profound suppression of T cell-dependent immune responses induced by TCDD. In the current report we have examined the effects of TCDD on DC in the presence of antigen by assessing the ability of the cells to activate antigen-specific T cells and internalize latex beads. In addition, the expression of costimulatory and adhesion molecules was examined in TCDD-treated mice challenged with allogeneic tumor cells. As in previous studies, exposure to TCDD tended to enhance the ability of DC to stimulate T cells as measured by the proliferation of 10.5.17 T cell clones and of naive DO11.10 T cells. TCDD-treated DC also enhanced IL-4 production in cultures with 10.5.17 cells, whereas IL-2 and IFN-γ production by the DO11.10 cells was not affected. Exposure to TCDD also altered the expression of MHCII, CD24, ICAM-1, CD40, and LFA-1 on splenic DC, however similar changes occurred in nonimmune mice and in tumor-injected animals. In addition, TCDD treatment did not affect the ability of splenic DC to internalize latex beads administered *in vivo*. These results suggest that TCDD is unlikely to suppress the ability of DC to internalize antigen and provide activation signals to T cells. However, it is possible that TCDD exposure causes DC to inappropriately hyperactivate T cells, resulting in activation-induced cell death.

INTRODUCTION

Dendritic cells (DC) are the most potent antigen-presenting cell of the immune system and are particularly important in initiating primary T cell mediated immune responses (Banchereau and Steinman, 1998). DC exist in different maturation stages which are characterized by the expression of key accessory molecules and the functional capacity of the cells. Unactivated, or immature DC, have a high capacity for antigen capture, but

express relatively low levels of the major histocompatibility complex (MHC) and costimulatory molecules such as B7-2 and CD40, and thus are poor stimulators of naive T cells. When DC are activated, such as by exposure to antigen, inflammatory stimuli or bacterial products, they migrate to the T cell areas of the lymphoid tissues and upregulate their expression of MHC and numerous adhesion and costimulatory molecules required for optimal T cell activation (Winzler *et al.*, 1997). In this activated state, the cells have a significantly reduced capacity for antigen capture (Sallusto *et al.*, 1995; Henderson *et al.*, 1997; Reis e Sousa *et al.*, 1993) but are instead specialized to stimulate T cells to proliferate and differentiate (Labeur *et al.*, 1999; Heufler *et al.*, 1988).

Suppression of T cell-dependent immune responses is a well-characterized effect which results from exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), however neither the mechanism of suppression nor the cellular target responsible for immune toxicity is known (reviewed by Kerkvliet, 1998). DC are attractive candidates as the target for TCDD for several reasons. First, although T cell functions are highly sensitive to suppression by TCDD, it has been difficult to demonstrate direct effects of TCDD on T cells *in vitro*, suggesting that T cells are not the direct cellular target (Lang *et al.*, 1994; Lawrence *et al.*, 1996; Dekrey and Kerkvliet, 1995). Second, delayed exposure studies have shown that TCDD treatment must occur early in the generation of an immune response (Kerkvliet *et al.*, 1996), suggesting that the initial activation of T cells is altered. Third, immune responses that require antigen processing for presentation to T cells are more sensitive to suppression than are mitogen-induced and other responses which partially or completely bypass the need for normal APC function (Neumann *et al.*, 1993; Vecchi *et al.*, 1980; Prell and Kerkvliet, 1997; Kerkvliet and Brauner, 1987). Taken together, these data support the hypothesis that DC function is altered by TCDD.

Previously we reported that in the absence of antigen, TCDD exposure caused an apparent activation of DC (see Chapter 2 of this dissertation). Specifically, splenic DC from TCDD-treated mice produced more IL-12 and showed enhanced expression of ICAM-1, B7-2, CD24, and CD40, cell surface molecules which are characteristically upregulated following activation of the cells. In addition, TCDD-treated DC enhanced T cell proliferation and cytokine production when cultured with allogeneic T cells. Due to the

immunosuppressive properties of TCDD, and in particular the sensitivity of T cell-dependent immune responses, these results were unexpected and difficult to explain.

One possible explanation is that inappropriate pre-activation of DC may negatively affect the function of the cells when processing and presentation of antigen is required. For example, activation of DC with stimuli such as TNFα, CD40L, IL-1B, and LPS diminishes their ability to internalize antigen (Sallusto *et al.*, 1995; Henderson *et al.*, 1997; Reis e Sousa *et al.*, 1993). Furthermore, DC which are activated with TNFα *in vitro* (Sallusto and Lanzavecchia, 1994) or LPS *in vivo* (De Smedt *et al.*, 1996) prior to antigenic are significantly suppressed in their ability to subsequently activate antigen-specific T cells.

In the studies presented here we have examined the effects of TCDD on DC which were exposed to antigen. We hypothesized that TCDD would suppress the ability of DC to activate T cells when processing and presentation of antigen was required. To test this hypothesis, we isolated DC from TCDD-treated mice and examined their ability stimulate antigen-specific T cells *ex vivo*. In addition, the phagocytic activity of the cells was evaluated using fluorescent beads. Finally, DC from mice injected with allogeneic tumor cells were examined to determine whether TCDD exposure affects the expression of accessory molecules differentially in mice responding to antigen.

MATERIALS AND METHODS

Animal treatments

C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Balb/c mice were purchased from B&K Universal, Inc. (Kent, WA). DO11.10 TCR-transgenic mice (Pape *et al.*, 1997) were originally provided by Dr. M. Jenkins (University of Minnesota Medical School, Minneapolis, MN) and were subsequently bred and maintained in our animal facility. The DO11.10 animals are on a Balb/c background and have a TCR which is specific for ovalbumin (OVA). Mice were housed singly, and were maintained in front of a laminar flow unit and in accordance with National Research Council guidelines. Mice were used at 7-12 weeks of age and were killed by CO_2 overdose.

TCDD exposure

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil. The vehicle control consisted of an equivalent amount of anisole in peanut oil. Mice were treated with TCDD or vehicle by gavage. TCDD was given at 15 μg/kg, a dose previously shown to suppress immune responses in mice (Kerkvliet *et al.*, 1996).

Reagents and antibodies

Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, (San Diego, CA). Ovalbumin protein (OVA) was purchased from Sigma (St. Louis, MO). Biotinylated-CD11c, as well as fluorochrome-conjugated antibodies to CD40, ICAM-1 (CD54), LFA-1 (CD11a), CD24, CD4, CD8, CD19, and I-A^b (MHCII) were obtained from PharMingen (San Diego, CA). Red670- streptavidin was obtained from Gibco (Gaithersburg, MD). For ELISA, IL-2, IFN-γ, and IL-4 antibody pairs were purchased from PharMingen, and antibodies to the p40 subunit of mouse IL-12 were purchased from Genzyme (Cambridge, MA).

Preparation of DC

DC were prepared in the following manner for use in specified assays:

For flow cytometric analysis: DC were enriched from spleens using the method of Swiggard *et al.* (1992) with modifications as described in Inaba *et al.* (1997). Briefly, splenic tissue was digested with collagenase D (Boehringer Mannheim, Indianapolis, IN) at 37°C for 45-60 minutes to release DC from the capsule and increase recovery. Cell suspensions were then diluted in collagenase or Ca-/Mg-free HBSS and pelleted. Recovered cells were spun over a BSA gradient (1.080 g/ml) and cells in the low density fraction were collected. These freshly isolated DC-enriched preparations were then stained for flow cytometric analysis. Typically, 15-25% of the low density cells expressed high levels of CD11c ($CD11c^{HI}$), a marker for splenic DC (Crowley *et al.*, 1990b) (see Fig. 2-1, pg. 26).

For use as APC for 10.5.17 cells: Low density spleen cells were obtained from C57Bl/6 mice as indicated above, and were further enriched for DC by overnight culturing based on their property of transient adherence to plastic (Swiggard *et al.*, 1992). In this procedure, the low density cells were cultured in plastic dishes for 90 minutes to allow for the DC to adhere. The non-adherent contaminating cells were then washed away, and the remaining adherent cells left in culture overnight. DC become non-adherent during this culture period and were collected from media the following day. Purity after this final enrichment was greater than 80% $CD11c^{HI}$ cells.

For use as APC for DO11.10 T cells: Spleens from Balb/c mice were digested with collagenase as indicated above, and DC were enriched using N418 microbeads (Miltenyi Biotec, Auburn, CA). The concentration of DC was determined by flow cytometry. $CD11c^{HI}$ cells comprised 13 - 47 % of the recovered cells. Cell suspensions were irradiated (1500 rads) and adjusted such that a constant number of DC were added to each well.

10.5.17 T cells

A TH2 T cell clone (10.5.17), derived from a C57Bl/6 mouse and specific for KLH, was obtained from Barbara Fox (Immunologic Pharmaceutical Corp., Waltham, MA). For normal maintenance, clones were re-stimulated every 10-14 days with irradiated C57Bl/6 spleen cells and KLH as previously described (Li and Fox, 1993). For proliferation and cytokine analysis, 10.5.17 cells were used 11 days after last activation.

Activation of 10.5.17 cells

DC (1×10^4 per well) were cultured with 10.5.17 cells (1×10^4 per well) in standard 96-well tissue culture plates. T cell proliferation was measured by incorporation of ^{3}H-TdR which was added (1 μCi/well) during the last 20 hours of culture. For IL-4 analysis, 60 μl supernatant was removed from each well just prior to addition of 60 μl ^{3}H-TdR for proliferation assay. KLH was used at 20 μg/ml when provided *in vitro*. Alternately, for *in vivo* studies, KLH (4 mg in PBS), was injected ip three hours prior to sacrifice as previously described (Crowley *et al.*, 1990a).

Activation of DO11.10 T cells

T cells were purified from spleens of DO11.10 OVA-specific T cell transgenic mice using Cellect T cell enrichment columns (Cytovax Biotechnologies Inc., Edmonton, Canada). Final T cell purity was 80% CD4+ and 11% CD8+, and no B cells or MHCII+ cells were detected. Various numbers of DC-enriched cells from Balb/c mice were cultured with T cells (3×10^5) and OVA (100 μg/ml) in 96 well Costar Ultra Low Cluster plates (Corning Inc., Corning, NY). T cell proliferation was measured by incorporation of ^{3}H-TdR which was added (1 μCi/well) during the last 20 hours of culture.

Flow Cytometry

Cells were incubated in 96-well plates with optimized concentrations of mAb. Nonspecific mAb binding was blocked by pre-incubating cells with rat and/or hamster IgG. Appropriately-labeled isotype controls were used to determine nonspecific fluorescence. A viable cell gate was established based on propidium iodide exclusion. For DC cell surface molecule evaluation, 10,000 viable CD11c^{HI} cells were analyzed. Listmode data were collected on a Coulter Epics XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME). For some markers, WinList was also used for compensation during data analysis.

P815 tumor response

C57Bl/6 mice (H-2^b) were injected ip with 1×10^7 allogeneic P815 mastocytoma cells (H-2^d). P815 cells were maintained by propagation in syngeneic DBA hosts (Kerkvliet *et al.*, 1996).

Cytokine analysis

Culture supernatants were analyzed using antibody sandwich ELISA techniques. The secondary biotinylated antibodies were complexed with avidin-peroxidase and visualized with 2,2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] as substrate. Absorbance was read at 405 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

In vivo uptake of fluorescent beads

FITC-fluorescent 2 μm latex beads (1 x 10^9) (Polysciences, Warrington, PA) were injected iv via the tail vein. Twenty-four hours later, spleens were digested with collagenase as described above, and uptake of beads by DC was analyzed by flow cytometry.

Statistical analysis

For most experiments, a Student's *t* test was used to compare means of vehicle-treated groups to TCDD-treated groups. For comparison of cell surface marker expression across multiple groups, analysis of variance modeling (ANOVA) was performed using SAS statistical software (SAS Institute, Inc., Cary, NC), and comparisons between means were made using the least significant difference (LSD) multiple comparison *t* test. IL-4 production across multiple experiments was analyzed using ANOVA. For all analyses, values of $p \leq 0.05$ were considered significant.

RESULTS

Expression of accessory molecules on DC from mice responding to allogeneic tumor cells

Previously we have shown that exposure to TCDD, in the absence of antigen, causes a phenotypic activation of splenic DC, increasing the expression of ICAM-1, CD24, CD40, and MHCII. Among the markers examined, only LFA-1 was significantly decreased by TCDD. Because the increased expression of such activation markers is generally considered to enhance the ability of DC to activate T cells, these observations were difficult to reconcile with the suppression of T cell-dependent immune responses observed in TCDD-treated mice. It was possible however, that TCDD exposure might affect DC differently when animals were also challenged with antigen. To address this possibility, we examined the effects of TCDD on DC from mice injected with allogeneic P815 tumor cells. The immune response to this antigen requires the activation of CD4+ T cells, presumably by DC, and is highly sensitive to suppression by TCDD (Dekrey and Kerkvliet, 1995; Kerkvliet *et al.*, 1996).

Accessory molecule expression was evaluated on days one through four following tumor injection, as the activation of CD4+ T cells has been shown to occur during this time (Kerkvliet *et al.*, 1996). Vehicle and TCDD-treated nonimmune animals (not injected with P815 cells) were also included as controls. Fig. 3-1 shows representative histograms of accessory molecule expression on DC evaluated three days after tumor injection. Similar results were observed on the other days examined. Injection of P815 cells alone did not significantly alter DC phenotype. Except for a small transient increase in ICAM-1 expression on day three, DC profiles from vehicle-treated nonimmune and vehicle-treated P815-injected mice were similar.

As expected, TCDD treatment of nonimmune mice (Fig. 3-1A) led to enhanced expression of ICAM-1, CD24, CD40 and MHCII, and decreased expression of LFA-1 on DC. Interestingly, nearly identical effects of TCDD were seen when DC from P815-injected animals were evaluated (Fig. 3-1B), suggesting that the presence of antigen *per se* does not modulate the effects of TCDD on DC phenotype. The only difference noted was that DC from TCDD-treated mice injected with P815 tumor cells expressed lower levels of MHCII relative to TCDD-treated nonimmune mice. However, because the expression of MHCII was still higher than in vehicle-treated animals, this difference is unlikely to be responsible for suppression of the immune response.

Effect of TCDD on ability of DC to stimulate KLH-specific 10.5.17 cells

In previous studies we showed that TCDD enhances the ability of DC to stimulate T cell proliferation and cytokine production in an allogeneic MLR, an assay which does not require antigen processing. To evaluate DC function in a response which requires presentation of antigen, we examined the ability of DC to activate KLH-specific cloned T cells (10.5.17). DC from vehicle- or TCDD-treated mice were cultured with 10.5.17 cells and KLH, and proliferation and IL-4 production were measured. As shown in Fig. 3-2, DC from TCDD-treated mice were not functionally suppressed, but instead tended to enhance both the proliferation of antigen-specific T cells and IL-4 production. Although the increases were not statistically significant in general, IL-4 production in the 28 hour cultures was significant (p=0.04) when analyzed across experiments.

FIG. 3-1. Effect of TCDD on accessory molecule expression on splenic DC from P815-injected mice. C57Bl/6 mice were treated with vehicle (grey line) or 15 μg/kg TCDD (black line) one day prior to injection of allogeneic P815 tumor cells. Vehicle- and TCDD-treated nonimmune animals were included as controls. On days one through four following P815 injection, splenic tissue was digested with collagenase and DC were enriched by density gradient centrifugation. Low density cell preparations were stained with antibodies to CD11c and indicated accessory molecules and analyzed by flow cytometry. Representative histograms show the expression of each marker on DC on day three following tumor injection, or on day four following TCDD exposure for the nonimmune mice. Dotted line shows staining of an isotype control antibody. Values on histograms indicate the staining intensity (median channel fluorescence). * indicates significantly different from vehicle; # indicates significantly different from TCDD-treated nonimmune group; % indicates significantly different from vehicle-treated nonimmune group.

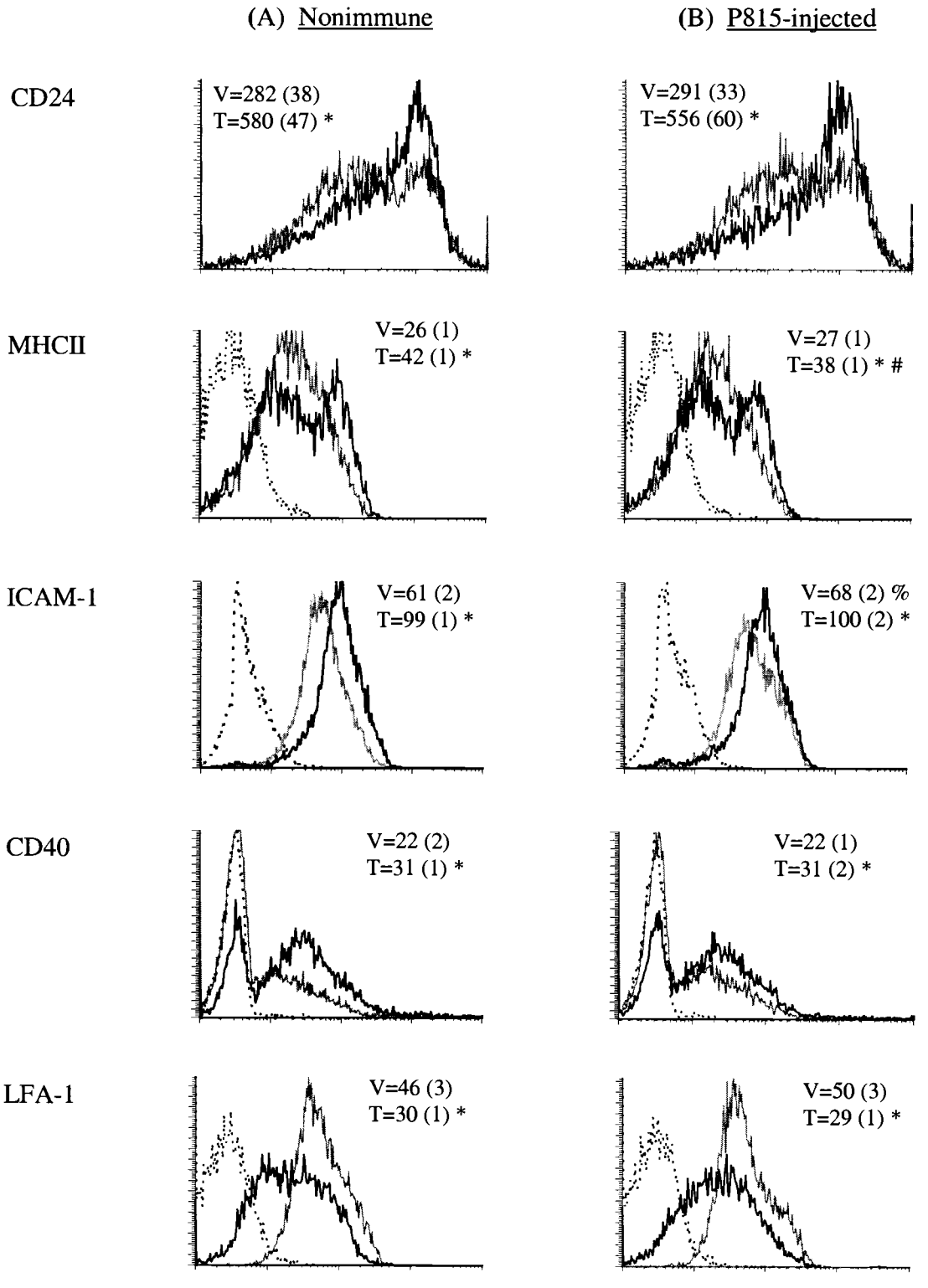

(A) Nonimmune
(B) P815-injected
CD24
V=282 (38)
T=580 (47) *
V=291 (33)
T=556 (60) *
MHCII
V=26 (1)
T=42 (1) *
V=27 (1)
T=38 (1) * #
ICAM-1
V=61 (2)
T=99 (1) *
V=68 (2) %
T=100 (2) *
CD40
V=22 (2)
T=31 (1) *
V=22 (1)
T=31 (2) *
LFA-1
V=46 (3)
T=30 (1) *
V=50 (3)
T=29 (1) *

FIG. 3-1.

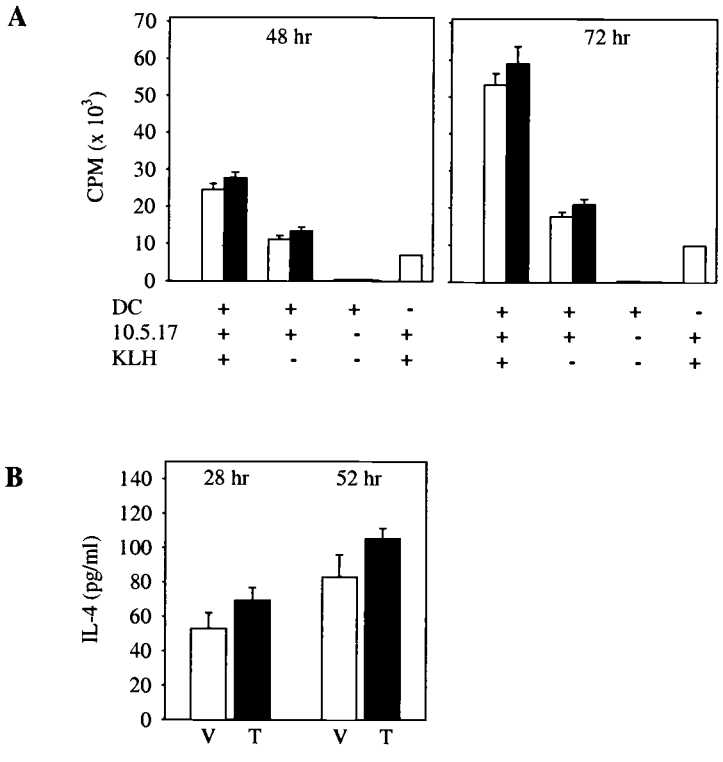


FIG. 3-2. Effect of TCDD on DC activation of 10.5.17 T cells with antigen provided *in vitro*. Spleens were removed from C57BL/6 mice three days after treatment with vehicle (open bars) or 15 μg/kg TCDD (solid bars). Spleens were pooled from three animals and dendritic cells were isolated by collagenase digestion, density gradient centrifugation, and overnight plastic adherence. DC ($1x10^4$) were cultured with10.5.17 T cell clones ($1x10^4$) and KLH (20 μg/ml). Proliferation (A) was measured at 48 and 72 hours by ^{3}H-TdR incorporation. IL-4 production (B) was measured by ELISA using supernatant removed from well immediately prior to addition of ^{3}H-TdR. No IL-4 was detected in control wells (DC + 10.5.17, no KLH), (DC alone), or (10.5.17 + KLH, no DC). Data points represent the mean ± SEM (n = 4 per group). Data are representative of three experiments.

The DC isolation procedure used in these experiments includes an overnight culturing step, a stimulus which has been shown to activate DC and reduce their ability to internalize antigen (Inaba *et al.*, 1994; Reis e Sousa *et al.*, 1993). Therefore it was possible that T cell activation measured in the previous experiment resulted from presentation of peptide fragments on surface MHC, and did not reflect the ability of DC to internalize antigen. To better assess the potential for TCDD to alter the uptake of antigen, we also tested the

function of DC which had been exposed to antigen *in vivo*. In these experiments, vehicle- and TCDD-treated mice were injected with PBS or KLH and sacrificed after three hours. Splenic DC were isolated and cultured with 10.5.17 cells without additional antigen. As shown in Fig. 3-3 , DC from KLH-, but not PBS-injected mice stimulated proliferation of 10.5.17 cells, indicating that the T cell response was antigen-dependent. As in the experiments where KLH was provided *in vitro*, TCDD did not suppress the ability of DC from KLH-injected mice to stimulate proliferation of 10.5.17 cells. In fact, in two of five experiments TCDD treatment significantly enhanced the proliferation. These results suggest that TCDD does not suppress the uptake of antigen by DC *in vivo*.

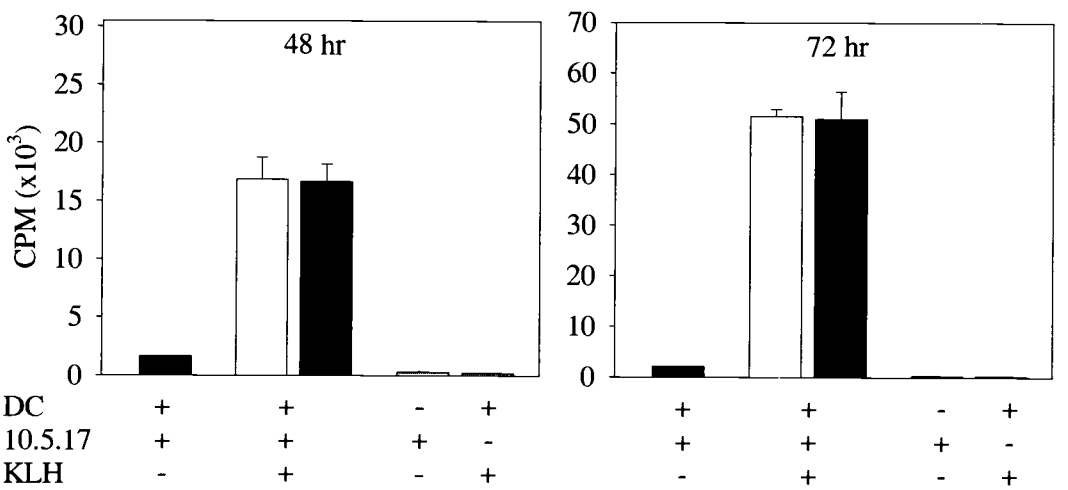


FIG. 3-3. Effect of TCDD on the ability of DC from antigen-treated mice to activate10.5.17 T cells. Spleens were removed from C57BL/6 mice one day after treatment with vehicle (open bars) or 15 μg/kg TCDD (black bars). Spleens were pooled from three animals and dendritic cells were isolated by collagenase digestion, density gradient centrifugation, and overnight plastic adherence. DC ($1x10^4$) were cultured with 10.5.17 T cell clones ($1x10^4$) without additional antigen, and proliferation was measured by ^{3}H-TdR incorporation. Data points represent the mean ± SEM (n = 4 per group). Data are representative of five experiments.

TCDD enhances the ability of DC to stimulate naive OVA-specific DO11.10 T cells

The 10.5.17 cloned T cells used in the previous experiments are maintained in long term culture by repeated stimulation with APC and antigen, and therefore are representative of memory T cells. Because the requirements for activation of memory T cells appear to be less stringent than for naive T cells (reviewed by Croft and Dubey, 1997), it is possible that activation of naive T cells might be impacted differently by TCDD-treated DC. To evaluate this possibility, we tested the ability of DC to activate naive T cells purified from spleens of unimmunized DO11.10 transgenic mice. In addition, to reduce the activation of DC caused by the isolation procedure, magnetic beads were used for DC enrichment in order to avoid the overnight adherence step, and low adherence tissue culture plates were used for the T cell stimulation assays.

As shown in Fig. 3-4, OVA-specific T cell proliferation was not suppressed, but was instead significantly enhanced in the cultures containing DC from TCDD-treated mice. Therefore, the trend for TCDD to augment DC-induced T cell proliferation was not limited to memory T cells and may even be more pronounced in naive T cells. Interestingly, although TCDD-treated DC enhanced T cell proliferation, the production of T cell cytokines IL-2 and IFN-γ was not affected (Fig. 3-5). Likewise production of IL-12, a cytokine produced by DC and other antigen-presenting cells was not altered by TCDD (Fig. 3-5)

Use of the magnetic beads for DC enrichment resulted in lower, and more variable purity of DC preparations compared to the overnight culturing method used previously. All the DC-enriched cell preparations contained B cells and an undefined population of autofluorescent cells. Although B cells have been shown to be ineffective in presenting OVA protein to DO11.10 T cells when compared to DC (Masten and Lipscomb, 1999), we wanted to assess the potential contribution of the B cells to the T cell proliferation. Therefore, the number of B cells in each preparation was determined and compared with the T cell proliferation in each culture. As expected, the number of B cells present did not correlate with proliferation and thus it is unlikely that B cells had a significant impact on the T cell response (data not shown). TCDD also tended to enhance the numbers of autofluorescent cells in the cultures, however like the B cells, the number of autofluorescent cells present in the cultures did not correlate with the amount of T cell proliferation. It was

not clear what type of cell was responsible for the autofluorescence, however they did not have typical macrophage light scatter properties, nor did they stain above isotype control levels for the macrophage marker Mac-1, suggesting they were not macrophages.

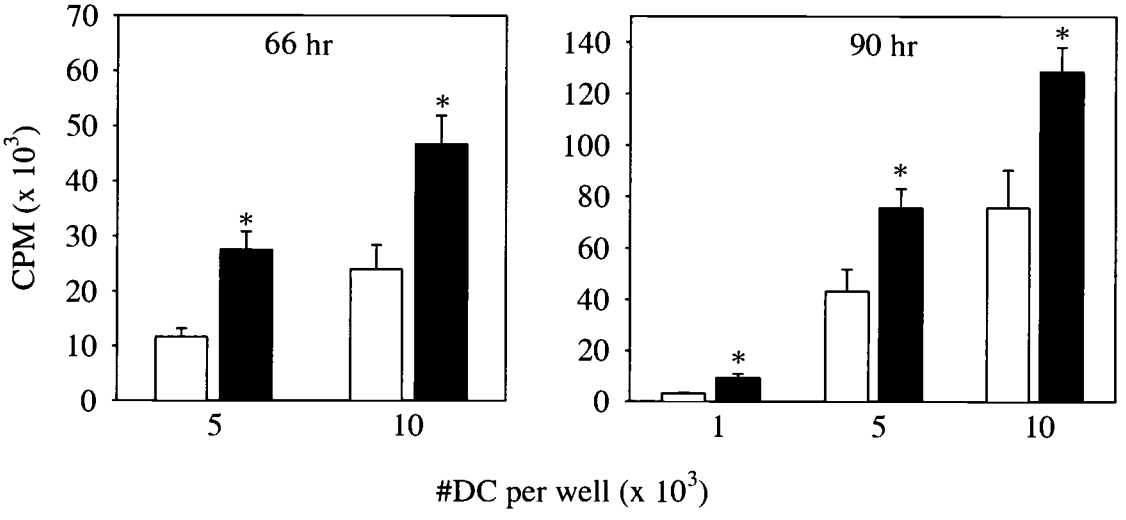


FIG. 3-4. DC from TCDD-treated mice augment the proliferation of DO11.10 T cells. Balb/c mice were treated with vehicle control (open bars) or 15 μg/kg TCDD (black bars). Three days later spleen were digested with collagenase and enriched for DC using N418-magnetic beads. Resulting cell suspensions were analyzed by flow cytometry and adjusted such that indicated numbers of DC were added to each well. DC-enriched cells were cultured with DO11.10 T cells (3 x 10^5) and OVA (100 μg/ml), and proliferation was measured by ^{3}H-TdR incorporation. Proliferation in control wells containing T cells + OVA, DC-enriched cells + OVA, and T cells + DC-enriched cells (no OVA), was below 800 cpm). Data points represent the mean ± SEM (n = 4 per group).

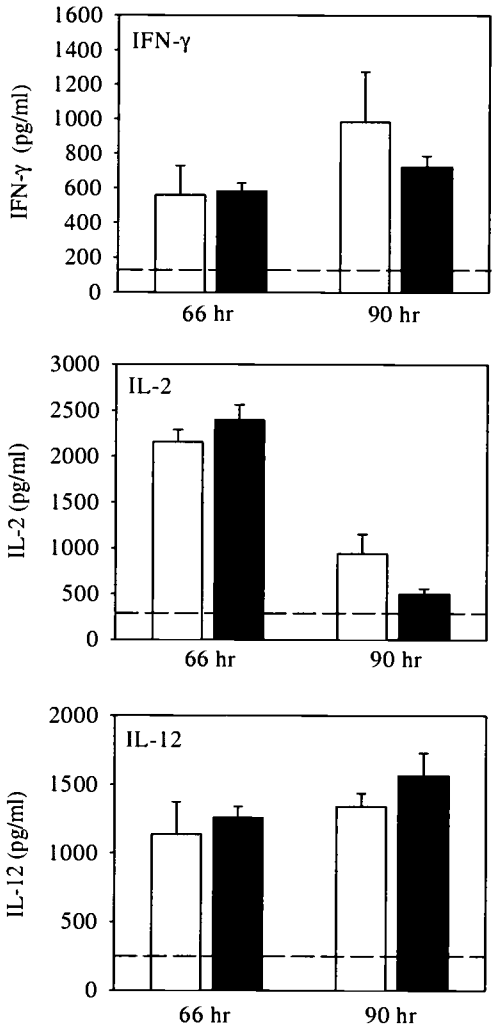


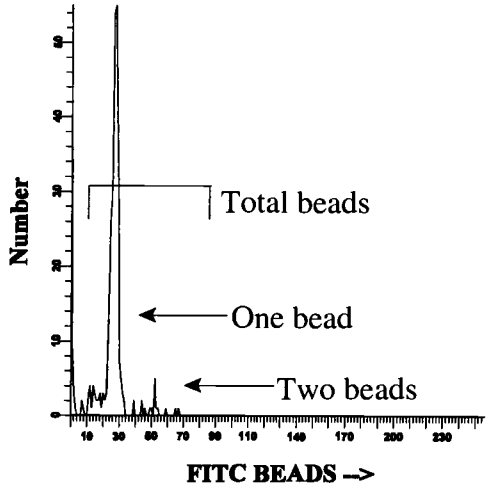
FIG. 3-5. The production of IL-2, IFN-γ and IL-12 is similar in cultures containing DO11.10 T cells and DC from vehicle- or TCDD-treated mice. Balb/c mice were treated with vehicle control (open bars) or 15 μg/kg TCDD (black bars). Three days later spleens were digested with collagenase and enriched for DC using N418-magnetic beads. Resulting cell suspensions were analyzed by flow cytometry and $1x10^4$ DC were added to wells with DO11.10 T cells (3×10^5) and OVA (100 μg/ml). Cytokine production was measured by ELISA, and the dashed line indicates the limit of detection. For IL-2 and IFN-γ, no cytokine production was detected in control cultures (DC + T cells, no OVA), (DC + OVA), or (T cells + OVA, no DC). IL-12 levels in control cultures were also at or below detection limits, except for the 90 hour cultures of DC and T cells without OVA (V= 415 ± 49, T= 662 ± 40. Data points represent the mean ± SEM (n = 4 per group).

TCDD does not suppress the ability of DC to take up latex beads in vivo

The previous studies which tested the function of DC exposed to KLH *in vivo* suggested that TCDD does not suppress the uptake and processing of antigen. However, to address the potential for TCDD to affect phagocytosis more directly, we measured the ability of DC to take up fluorescently-labeled (FITC) latex beads. In addition, to eliminate the complicating factor that phagocytic activity is reduced by the DC isolation procedure, we wanted to expose the cells to the latex beads *in vivo*. However, while latex bead uptake is frequently used in *in vitro* assays, the *in vivo* application of the beads had not been reported previously to our knowledge. Therefore, preliminary experiments were conducted to optimize the route of exposure, dose, and the ideal time for detection of the beads after injection.

In the experiment shown in Fig. 3-6, mice were injected iv with 1 x 10^9 FITC-beads in PBS, and were sacrificed after 24 hours. Flow cytometric analysis of bead uptake was performed by gating on CD11c^{HI} cells in the low density BSA fraction (DC enriched). Because it was possible that the uptake of the beads would alter the density of the cells, DC in unfractionated spleen cell preparations were also examined. The number of beads associated with each cell can be measured by flow cytometry, as there is a linear relationship between the amount of fluorescence and the number of beads present. Figure 3-6A shows a representative histogram of the FITC-bead fluorescence as gated from the CD11c^{HI} cells, and indicates that bead-positive DC were usually found associated with a single bead. In vehicle-treated animals, approximately 4% of DC in the low density BSA fraction were associated with beads (Fig. 3-6B). Unexpectedly, TCDD exposure slightly, but significantly, increased the percentage of bead-positive cells to 5.4%. However, TCDD did not alter the number of bead-positive DC in the low density BSA fraction, nor the percentage or number of bead-positive DC in the unfractionated spleen cells. Taken together with the results from studies where KLH was administered *in vivo*, these data strongly suggest that internalization of antigen is not suppressed by TCDD exposure.

A



B

	Bead-containing DC in low density cell fraction		Bead-containing DC in unfractionated spleen cells	
	# (x 10^4)	%	# (x 10^5)	%
Vehicle	3.1 (0.6)	4.0 (0.4)	1.2 (0.1)	6.3 (0.5)
TCDD	2.8 (0.2)	5.4 (0.1) *	0.8 (0.1)	5.9 (0.3)

FIG. 3-6. TCDD does not alter the ability of DC to internalize latex beads administered *in vivo*. C57Bl/6 mice were treated with vehicle or 15ug/kg TCDD three days prior to iv injection of $1x10^9$ FITC-fluorescent latex beads. 24 hours later, spleens were digested with collagenase, fractionated by density gradient centrifugation if indicated, stained with CD11c, and analyzed by flow cytometry. (A) Representative histogram showing FITC fluorescence of bead-positive DC. (B) Summary of percent and number of total bead-positive DC in low density and unfractionated spleen cells. n= 3 or 4 animals per treatment. * = $p<0.05$ when compared to vehicle.

DISCUSSION

In Chapter 2 of this dissertation we showed that in the absence of antigen, exposure to TCDD increased the expression of MHCII, the costimulatory molecules B7-2, CD40, and CD24 and the adhesion molecule ICAM-1 on DC. These phenotypic changes suggested that TCDD activates DC, a scenario which was consistent with our observations that DC from TCDD-treated mice produced more IL-12 and were better at stimulating allogeneic T cells *in vitro* than were DC from vehicle-treated animals. In the current studies we have examined the effects of TCDD on DC surface markers in mice challenged with antigen, and have tested the ability of DC to activate antigen-specific T cells.

Although we hypothesized that TCDD-induced activation of DC would inhibit the processing and presentation of antigen and suppress T cell activation, the results of the current studies do not support this conclusion. TCDD exposure did not suppress the ability of DC to present antigen to memory or naive T cells, nor was the ability of the cells to internalize latex beads affected. These observations are consistent with results from other recent studies where TCDD did not suppress the *in vivo* proliferation of OVA-specific CD4+ T cells in adoptively-transferred mice (Shepherd *et al.*, 2000). Taken together, these results suggest that TCDD does not suppress the internalization and presentation of antigen by DC and does not inhibit the ability of the cells to stimulate T cell proliferation.

Surprisingly, TCDD exposure instead tended to enhance the ability of DC to stimulate proliferation of antigen-specific T cells. Although the degree of enhancement was small when 10.5.17 cells were used as responders, similar effects were observed in numerous independent experiments. The enhanced proliferation was even more pronounced when naive DO11.10 T cells were used as responders, a result which is particularly intriguing as we have recently seen a similar phenomenon *in vivo* when proliferation of DO11.10 T cells is followed in adoptively-transferred mice. In the *in vivo* studies, TCDD exposure significantly enhanced the proliferation of antigen-specific CD4+ T cells in the spleen three days after injection of OVA. Interestingly, following this initial enhancement in proliferation, an increased percentage of OVA-specific T cells underwent apoptosis, and the number of these cells was significantly decreased by day five (ED Dearstyne, unpublished

observations). Similar results have been shown in mice treated *in vivo* with anti-CD3, where TCDD exposure resulted in enhanced T cell proliferation and also to an apparent increase in death of CD4+ T cells (Prell *et al.*, 1995). It is appealing to hypothesize that TCDD exposure may cause DC to inappropriately activate T cells, leading to activation-induced cell death. Current studies are underway in ours and others (Pryputniewicz *et al.*, 1998) laboratories to address the hypothesis that TCDD exposure is associated with enhanced T cell apoptosis.

Although DC from TCDD-treated Balb/c mice enhanced the proliferation of the DO11.10 T cells relative to DC from vehicle-treated animals, the production of cytokines was not altered. This result contrasts with our previous observations where DC from TCDD-treated C57Bl/6 mice produced more IL-12 and stimulated enhanced production of IFN-γ and IL-2 when cultured with allogeneic T cells (see Chapter 2 of this dissertation). The reasons for these conflicting results are not clear. However, C57Bl/6 and Balb/c mice may respond differently in terms of cytokine production since these animals are considered to be predisposed toward Th1 (C57Bl/6) and Th2 (Balb/c) responses (Brenner *et al.*, 1994). In addition, different methods were used to enrich DC in the current study (magnetic beads) than in the previous studies using cells from C57Bl/6 mice (adherence to plastic). Thus it is possible that the method by which the DC were isolated in the different experiments contributed to the difference in cytokine production. For example, Vremec *et al.* (2000) recently reported that particular subsets of DC were selectively lost when splenic DC were isolated by adherence to plastic. It is therefore possible that TCDD enhances IL-12 production in a sub-population of DC which was selectively enriched in the MLR experiments. Alternately, cytokine production may have been influenced by some other cell population which contaminated the DC preparations used to activate the DO11.10 T cells. However, this is unlikely as we failed to find an association between T cell activation and the other potential antigen-presenting cells present in the DC preparations.

The lack of effect of TCDD on cytokine production by DO11.10 cells also appears to conflict with the TCDD-induced suppression of IL-2 and IL-12 which we have observed when spleen cells from mice adoptively-transferred with DO11.10 T cells were cultured *in vitro* (Shepherd, *et al.*, 2000 , and manuscript in preparation). However, because these

latter experiments analyzed cytokine levels in cultures of total spleen cells, which contain relatively few DC, it is possible that other populations of cells influenced cytokine production in these studies. Alternately, because exposure to TCDD reduces the numbers of splenic DC in adoptively-transferred mice (DM Shepherd and ED Dearstyne, unpublished observations), the reduced production of IL-2 and IL-12 may reflect a relative difference in the number of DC contained in the cultures. Further study will therefore be necessary to determine which, if any, of these factors are responsible for the differences in cytokine production observed in the different studies.

It was also surprising that TCDD did not affect the expression of accessory molecules on DC from nonimmune mice compared to DC from mice responding to challenge with allogeneic tumor cells. This result was unexpected since TCDD suppresses costimulatory molecule expression on B cells and Mac-1-positive cells in mice responding to tumor (Prell *et al.*, 1997, and Shepherd *et al.*, manuscript in preparation). However, in the B cell studies, TCDD's effects were not seen until relatively late in the response, suggesting that these changes were unlikely to account for disruption of early T cell activation events. In the current studies, TCDD increased the expression of CD40, CD24, ICAM-1 and MHCII in the P815-injected mice, suggesting that suppression of key accessory molecules on DC early in the response to P815 is not responsible for immune dysfunction. Instead, these data are more consistent with enhanced ability of DC to activate T cells and provide more evidence that inappropriate hyperactivation of T cells may result from exposure to TCDD. It should also be noted that we cannot identify the specific DC which are carrying tumor antigens to the T cells, which probably represent a very small fraction of the total DC in the spleen. Therefore it is possible that TCDD does suppress accessory molecule expression on these particular DC, but we are unable to detect it.

The suppressed expression of the adhesion molecule LFA-1 on DC is of interest since it is the only marker we find consistently decreased by TCDD in both naive and P815-challenged mice. LFA-1 is an integrin protein, expressed on all leukocytes, which binds to ICAM-1, a protein expressed on numerous cells including T cells and endothelial cells. LFA-1 plays a role in the trafficking of DC from peripheral sites to the lymphoid tissues (Ma *et al.*, 1994). This suggests that suppression of LFA-1 expression by TCDD may disrupt

the generation of an immune response by preventing DC from carrying antigen to the T cell areas of the spleen or lymph nodes. However, recent studies performed in our laboratory have shown that TCDD does not suppress the proliferation of OVA-specific T cells in the spleen of antigen-challenged mice (Shepherd *et al.*, 2000). This suggests that DC are able to migrate to the spleen to activate T cells, and by extension that suppressed LFA-1 expression is unlikely to impact DC trafficking.

Alternatively, the suppression of LFA-1 expression by TCDD may disrupt LFA-1:ICAM-1 interactions which are important in maintaining cellular contact during T cell activation (Dougherty and Hogg, 1987; Simon *et al.*, 1991). However, because the suppressed LFA-1 expression observed in these studies did not correlate with reduced T cell activation, it suggests that DC:T cell interactions are not significantly disrupted. On the other hand, it is also possible that conditions which arise during the culturing of DC, such as the upregulation of costimulatory molecules and additional adhesion molecules, compensate for the suppressed LFA-1 and restore normal T cell activation. Therefore, the biological significance of decreased LFA-1 expression on DC, if any, remains unknown.

In summary, although we hypothesized that TCDD would suppress the expression of accessory molecules on DC and inhibit their ability to internalize antigen and activate T cells, results from the current studies do not support this conclusion. Instead we have found that TCDD-induced activation of DC occurs both with and without antigen and does not appear to inhibit the ability of the DC to activate T cells. Because DC from TCDD-treated mice tended to hyperactivate T cells, further studies are warranted to determine whether TCDD-induced activation of DC ultimately leads to T cell death.

Chapter 4

Expression of Aryl Hydrocarbon Receptor in Murine Dendritic Cells

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ABSTRACT

In vivo exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alters the expression of costimulatory/adhesion molecules on murine splenic dendritic cells, enhances their functional abilities and reduces their number. These effects are dependent on the aryl hydrocarbon receptor (AhR), however it is not clear whether they result from a direct toxic insult to the DC. Because the AhR status of DC had not been previously determined, the potential for TCDD to directly affect DC was not known. Therefore, in these studies we examined murine splenic DC for AhR message using RT-PCR. In addition, AhR protein was examined in the murine DC lines XS52 and DC2.4 using western blotting. Both the splenic DC and the cell lines had detectable levels of AhR, indicating that DC represent potential cellular targets for the immunosuppressive effects of TCDD.

INTRODUCTION

The immunosuppressive effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor which is a member of a superfamily of basic helix-loop-helix (bHLH) DNA binding proteins (Hankinson, 1995; Schmidt and Bradfield, 1996). Prior to activation, the cytoplasmic AhR is associated with additional proteins including heat shock protein 90, AhR-interacting protein (Ma and Whitlock, 1997), and a protein kinase which has been identified as c-src (Enan and Matsumura, 1996). When TCDD binds to the receptor, these proteins dissociate and the receptor moves to the nucleus and forms a heterodimeric complex with another bHLH protein, the AhR nuclear translocator (Arnt). This complex binds to dioxin response elements (DRE) present in the upstream regulatory region of target genes, thus altering protein expression and causing numerous toxic effects including liver hypertrophy, immune suppression, thymic atrophy, teratogenic effects and, at high doses, a wasting syndrome leading to death.

The cellular target responsible for the immune suppression induced by TCDD is not known, and current studies in our laboratory are focused on the examination of dendritic

cells (DC) as the direct target for TCDD. DC are attractive candidates for the effects of TCDD for several reasons. First, delayed exposure studies have shown that TCDD treatment must occur early in the generation of an immune response (Kerkvliet *et al.*, 1996), suggesting that the initial activation of T cells is altered. Second, previous data have shown that responses which require antigen processing for presentation to T cells are more sensitive to suppression than are mitogen-induced and other responses which partially or completely bypass the need for normal APC function (Neumann *et al.*, 1993; Vecchi *et al.*, 1980; Prell and Kerkvliet, 1997; Kerkvliet and Brauner, 1987). Finally, in this dissertation we show that DC isolated from TCDD-treated mice demonstrate various alterations such as increased expression of numerous accessory molecules including CD40, B7-2 and ICAM-1, decreased expression of LFA-1, and increased ability to stimulate T cell proliferation and cytokine production when cultured *ex vivo*. Taken together, these data support the hypothesis that DC may be the direct target of TCDD.

The AhR status of DC has not been previously investigated, however there is indirect evidence to suggest that these cells express the receptor. For example, it has recently been reported that human DC derived from peripheral blood monocytes contain message for cytochrome P450 proteins including CYP1A1 (Sieben *et al.*, 1999). Because the induction of CYP1A1 is largely, if not entirely, mediated by ligand binding to the AhR, these data suggest that DC contain the receptor. In addition Langerhans cell-like XS52 cells are able to metabolize benzo(a)pyrene (Anderson *et al.*, 1995) a polycyclic aromatic hydrocarbon whose metabolism is regulated largely through AhR-mediated induction of CYP1A1. In the studies presented here, we used RT-PCR and western blotting to examine the AhR status of murine splenic DC and of two murine DC cell lines.

MATERIALS AND METHODS

Isolation of splenic DC

C57Bl/6 male mice were treated with vehicle or TCDD (15 μg/kg) by gavage 24 hours prior to sacrifice. Spleens from 12 animals per treatment group were pooled, digested with

collagenase, and enriched by density gradient centrifugation and overnight culturing as detailed in Swiggard *et al.* (1992). Contaminating cells, primarily B cells, were removed using sheep anti-rat-conjugated magnetic beads (Dynal, Norway) and a rat antibody specific for FcγRIII (Pharmingen CD16/Cd32) which is present on B cells and macrophages. Remaining cells were ≥90% DC as determined by high level of CD11c expression.

Cell lines

The DC cell line XS52 was kindly provided by Dr. A. Takashima (University of Texas Southwestern Medical Center, Dallas, TX). XS52 is a Langerhans cell-like line established from epidermis of neonatal Balb/c mice (Xu *et al.*, 1995). The DC cell line DC2.4 was generously provided by Dr. K Rock (University of Massachusetts Medical Center, Worcester, MA). DC2.4 is derived from bone marrow of C57Bl/6 mice and has been transfected such that it produces GM-CSF(Shen *et al.*, 1997). The liver cell line Hepa 1c1c7 was provided by Dr. J. Whitlock (Stanford University, Stanford,CA).

Cell culture and TCDD exposure

Unless otherwise indicated, all media and components were purchased from Sigma (St. Louis, MO). DC2.4 cells were cultured in DMEM containing 10% FBS (Hyclone, Ogden UT), L-glutamine, Hepes, gentamicin, and sodium pyruvate. XS52 cells were cultured in RPMI media containing 10% FBS, 5% supernatant from NS47 fibroblast cells, L-glutamine, 2-mercaptoethanol, non-essential amino acids, Hepes, penicillin/streptomycin, sodium pyruvate, and GM-CSF at 2 ng/ml (PharMingen, San Diego, CA). Hepa 1c1c7 cells were grown in MEMα containing 10% FBS and penicillin/streptomycin. For AhR analysis by RT-PCR, Hepa 1c1c7 cells were cultured in $1x10^{-9}$M TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) or vehicle control (0.15% DMSO) for 24 hours prior to RNA extraction.

RT-PCR

Total RNA was extracted by lysis of cells in 4M guanidine-thiocyanate, and complementary DNA (cDNA) libraries were constructed using reverse transcriptase (Gibco)

as previously described (Kerkvliet *et al.*, 1996). PCR amplification of AhR message was performed using a DNA Engine thermocycler (MJ Research) for 35 cycles using the following primer sequences described by Yamaguchi et al. (1997): CTGGCAATGAATTTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. The cDNA product amplified by these primers is 335 bp. Message integrity was verified using β2-microglobulin primers to yield a 373 bp fragment. Products were separated on an agarose gel and visualized by ethidium bromide staining.

Whole cell lysates

Whole cell lysates were prepared by incubating cells in extraction buffer (10 mM Tris (pH 7.4), 0.5% Triton X-100, 5 mM EDTA,, 5 mM NaCl, 1 mM DTT, 3 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) for 20 minutes on ice. Samples were centrifuged at 15K rpm in a microcentrifuge, and supernatants stored at -80°C. Concentrations were determined spectrophotometrically using the colorimetric Pierce BCA protein assay (Pierce, Rockford, IL).

Protein blotting

Protein samples (100 μg/lane) from lysates of DC cell lines were denatured by boiling in SDS sample buffer and separated by SDS-PAGE according to the method of Laemmli (1970). Lysates from Hepa 1c1c7 cells were treated similarly, however the protein content of the lysate was not determined. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked overnight in TBS buffer containing 5% nonfat dry milk, and then incubated with a 1:1000 dilution of a polyclonal antibody to mouse AhR (Affinity Bioreagents, Golden, CO). An HRP-conjugated mouse anti-mouse antibody (Amersham, Piscataway, NJ) was used a secondary antibody, and antibody complexes were visualized with chemiluminescent reagents (Amersham).

RESULTS AND DISCUSSION

DC are present in small numbers in murine spleen, and we recovered only around 3 x 10^6 DC from each pool of twelve vehicle- or TCDD-treated mice. Due to this low cell recovery, we chose to examine the AhR status of these cells using RT-PCR. RNA was isolated from the DC and AhR message was examined using AhR-specific primers. RNA isolated from Hepa 1c1c7 cells, which express AhR (Lawrence *et al.*, 1996), was included as a positive control. As shown in Fig. 4-1, AhR message was detected in DC isolated from both vehicle- and TCDD-treated mice. The band from the TCDD-treated DC appears to be more intense than that from the control mice, however no conclusion regarding relative amount of product can be made as each band represents a single pool of animals.

To determine if DC contain AhR protein, we examined whole cell lysates from two DC cell lines which represent two different DC maturation stages. The XS52 line is representative of an immature, Langerhans-type DC, whereas DC2.4 cells are representative of a more mature DC expressing relatively high levels of activation markers such as MHCII and B7-2 (Shen *et al.*, 1997). As shown in Fig. 4-2, both cell lines contain AhR protein as evidenced by the approximately-100 KD band visualized by western blotting. As expected, the size of the AhR differed only slightly between the DC24.4 cells derived from C57Bl/6 mice (AhR = 95 KD) and XS52 cells derived from Balb/c (AhR = 104 KD) mice (Poland and Glover, 1990). Taken together, these data show that DC from three different sources contain detectable levels of AhR message or protein and thus these cells represent a potential direct target for TCDD.

Preliminary studies were carried out to evaluate direct effects of TCDD on splenic DC. Based on *in vivo* studies we expected that TCDD would increase the expression of the activation markers CD40, ICAM-1 and B7-2, and would suppress the expression of the adhesion molecule LFA-1 (see Chapter 2 of this dissertation). However, when splenic DC were cultured for 24 hours with 1 or 10 nM TCDD, no effect was observed on any of these markers (data not shown). This result was not entirely unexpected as the procedure used for isolation of splenic DC causes them to become highly activated and to upregulate expression of many surface markers (Inaba *et al.*, 1994). In fact, in a separate experiment,

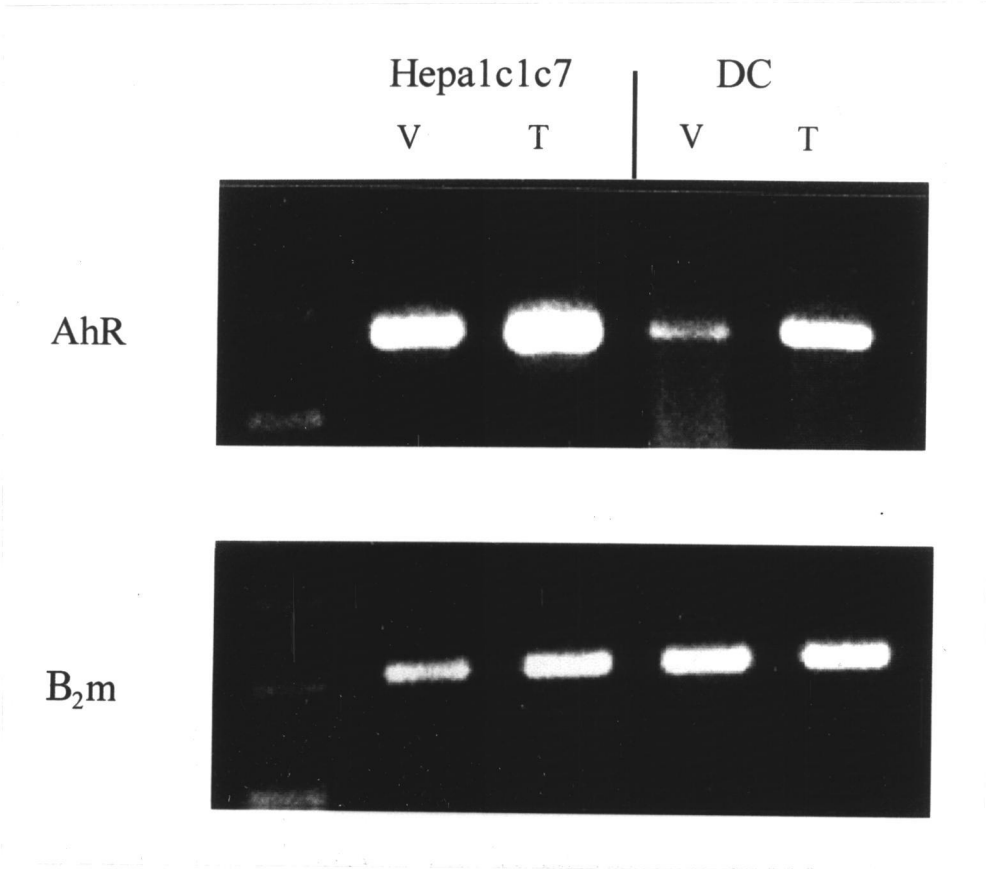

FIG. 4-1. Splenic DC contain message for AhR. Splenic DC were isolated from C57Bl/6 mice one day after exposure to vehicle (V) or 15 μg/kg TCDD (T). AhR message was amplified from DC cDNA libraries as described in Materials and Methods. As a positive control, message was likewise amplified from Hepa1c1c7 cells which had been cultured in $1x10^{-9}$M TCDD or vehicle control (0.15% DMSO) for 24 hours prior to RNA extraction. Message integrity was verified using β_2-microglobulin (β_2 m)-specific primers. The left lane shows a 123 bp ladder. cDNA products are 335 bp (AhR) or 373 bp (β_2 m).

we cultured splenic DC with TNFα, a well-characterized stimulus used for activating immature DC and cell lines. TNFα also failed to augment costimulatory molecule expression above that induced by the isolation procedure alone (data not shown). These results indicate that, at least for certain endpoints, it may not be possible to use splenic DC *in vitro* to assess direct effects of TCDD.

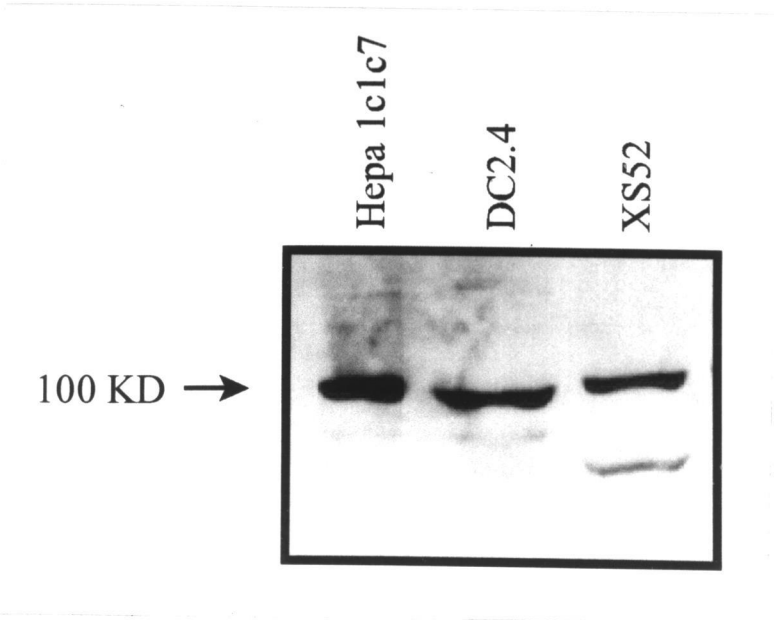

FIG. 4-2. Western blot shows presence of AhR protein in two DC lines. Protein extracts (100 μg) from the DC2.4 and XS52 murine DC lines were separated on an 8% polyacrylamide gel and transferred to nitrocellulose. Protein extract from Hepa 1c1c7 cells was included as a positive control. The AhR was detected using a polyclonal antibody as described in Materials and Methods. Arrow shows the approximate size of the protein (100 KD) as determined by comparison with molecular weight standards (not visible on film).

As an alternative approach, we have begun to characterize the effects of *in vitro* exposure to TCDD using cell lines and DC generated from bone marrow stem cells. These cells remain unactivated until given a specific activation signal, and thus provide a better model to evaluate direct effects on resting DC as well as on DC exposed to different stimuli. Studies are in progress to assess the potential for TCDD to directly affect expression of accessory molecules as well as to impact intracellular signaling through NF-κB, a transcription factor which plays an important role in DC maturation (Rescigno *et al.*, 1998; Verhasselt *et al.*, 1999). Preliminary results from these studies have indicated that TCDD negatively affects signaling through NF-κB, suggesting that TCDD may impact the

functional activity of DC (C. Ruby, unpublished observations). Thus, although the role of the DC in the immune suppression induced by TCDD is still unclear, the observed direct effects of TCDD on NF-κB signaling, combined with the finding that DC express the AhR, demonstrate that DC represent a likely cellular target for TCDD.

Chapter 5

Summary and Conclusions

The studies presented in this dissertation were designed to test the hypothesis that TCDD alters dendritic cells in a manner which ultimately results in suppressed T cell function. Although we found clear evidence that TCDD exposure alters the phenotype and functional activity of DC, the relationship between these effects and the suppression of T cell responses seen in TCDD-treated mice is still unclear. Indeed, the evidence from the majority of our studies suggested that DC function might actually be enhanced in TCDD-treated mice. Specifically, we found that TCDD caused DC to up-regulate MHCII, B7-2, CD40, CD24, and ICAM-1, proteins which are important in signaling T cells to proliferate and differentiate. Consistent with this "activated" phenotype, TCDD also enhanced the functional activity of the DC as they produced more IL-12 and were relatively better at stimulating T cell proliferation and cytokine production than DC from vehicle-treated mice. The only potentially adverse effects of TCDD exposure were decreased numbers of DC in spleen of TCDD-treated mice and suppressed expression of LFA-1, an integrin protein which is important in cell:cell adhesion and in the trafficking of DC from peripheral tissues to the spleen and lymph nodes. These results suggest a number of potential conclusions:

First, it is possible that TCDD does suppress the ability of DC to activate T cells *in vivo*, but we were unable to detect this in our studies due to complications which result from use of *ex vivo* assays to measure DC function. For example, methods used to isolate DC from tissue also cause the cells to become activated, enhancing their expression of accessory molecules, and increasing their ability to stimulate T cells. Therefore, functional defects induced by exposure to TCDD *in vivo* might be overcome when the cells are put into culture. Furthermore, by artificially combining DC with T cells and antigen in a culture well, we might circumvent a defect which is present *in vivo*, such as a disruption of cellular trafficking which prevents DC from moving to the T cell areas of the lymphoid tissue. However, as was discussed in Chapter 3 of this dissertation, other studies in our laboratory

using the DO11.10 adoptive transfer model suggest that the initial phase of T cell proliferation is normal (or even enhanced) in TCDD-treated mice. Therefore it appears unlikely that our failure to detect suppressed T cell activation in *ex vivo* experiments is simply the result of complicating factors inherent in these assays.

Second, while not preventing early stages of T cell activation, TCDD may instead suppress signals provided by DC which affect the differentiation of the T cell. This possibility is supported by results from studies also performed in the DO11.10 adoptive transfer model which indicate that TCDD does not interfere with T cell proliferation *in vivo*, but suppresses the production of certain cytokines. Although the studies in this dissertation examined numerous factors which affect activation and differentiation of T cells and found no evidence of suppression, additional cytokines and cell surface proteins continue to be described. Therefore, it remains possible that TCDD alters some other signal provided by DC which we have yet to examine.

Third, it is possible that although TCDD causes measurable changes in DC, these effects are not relevant to immune suppression. Some of the changes we observed in accessory molecule expression and T cell activation were quite small, and it is possible that they are not biologically significant.

Fourth, the reduced numbers of splenic DC observed in TCDD-treated mice may be responsible for immune suppression. Because DC survival directly correlates with T cell priming *in vivo*, the premature deletion of DC may inappropriately terminate the activation of T cells and result in decreased strength of the immune response.

Finally, because TCDD apparently caused DC to "hyper"activate T cells, enhancing their proliferation and production of certain cytokines, it is possible that such inappropriate activation causes the T cells to undergo activation-induced cell death and prematurely terminates the immune response.

In summary, these studies have shown that splenic DC from TCDD-treated mice demonstrate enhanced expression of many accessory molecules required for activation of T cells and are relatively more efficient at stimulating T cells *ex vivo* when compared to DC from vehicle-treated mice. Although these findings were contrary to our original hypothesis, several potential mechanisms linking these effects to altered immune function have been

presented. Further study will be necessary to determine if any of the observed effects on DC is responsible for the immunotoxicity of TCDD.

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Appendices

Appendix A

Exposure to the Bacterial Antigen Lipopolysaccharide Causes Activation of Dendritic Cells but Suppresses the Immune Response to Allogeneic Tumor Cells

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ABSTRACT

Previously we have shown that exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes the phenotypic and functional activation of dendritic cells (DC), the primary antigen-presenting cell responsible for initiating T cell-dependent immune responses. This result was surprising as exposure to TCDD suppresses the generation of T cell-dependent immune responses, and activation of DC is generally considered to result in enhanced T cell activation, at least *in vitro*. Interestingly, when DC are activated *in vivo* by exposing mice to the bacterial antigen lipopolysaccharide (LPS), their ability to activate antigen-specific T cells *ex vivo* is suppressed. This led us to question whether other agents which, like TCDD, activate DC, might also suppress immune function *in vivo*. Therefore, the studies presented here were performed to test the hypothesis that *in vivo* activation of DC by LPS would suppress the generation of an immune response to allogeneic P815 tumor cells. Mice were treated with vehicle (PBS) or 25 μg LPS 16 hours prior to injection of P815 cells, and the immune response was evaluated ten days later. LPS pre-treatment profoundly suppressed the generation of the cytotoxic T cell response measured in the spleen, and also suppressed the formation of alloantibodies measured in the plasma. These results suggest that activation of DC may not always result in enhanced immune function, however possible confounding factors surrounding the use of LPS in the context of a tumor response complicate data interpretation.

INTRODUCTION

Previous studies have shown that *in vivo* exposure to TCDD causes activation of dendritic cells (DC) as characterized by the enhanced expression of numerous accessory molecules including B7-2, CD40, ICAM-1, and CD24 (see chapter 2 of this dissertation). In addition, DC from TCDD-treated mice had an enhanced ability to stimulate T cell proliferation and cytokine production in a mixed leukocyte reaction (MLR), a response

which does not require antigen processing by DC. Because activation of DC is generally considered as beneficial, and characteristically results in enhanced immune function (Labeur *et al.*, 1999; Winzler *et al.*, 1997; Heufler *et al.*, 1988), the paradoxical activation of the cells was difficult to reconcile with fact that TCDD suppresses cell-mediated immune responses.

Interestingly, De Smedt et al. (1996) reported that when DC were activated by *in vivo* exposure to the bacterial antigen lipopolysaccharide (LPS), their ability to stimulate T cells *ex vivo* was either enhanced or suppressed depending on the requirement for antigen processing. Specifically, DC from LPS-treated mice expressed enhanced levels of B7-1, B7-2, and MHC molecules, indicating they were activated, but had suppressed function when used to stimulate a T cell hybridoma which required antigen processing. In contrast, the LPS-treated DC augmented the proliferation of SEB-activated T cells, a response which does not require processing of antigen by DC. These results suggest that inappropriate activation of DC early in the generation of an immune response may suppress their ability to stimulate antigen-specific T cells and thus to initiate immunity.

The immune response to allogeneic P815 tumor cell has been extensively characterized in our laboratory as a model for cell-mediated immunity (Kerkvliet *et al.*, 1996; Dekrey and Kerkvliet, 1995; Prell and Kerkvliet, 1997). In this response, C57Bl/6 mice (H-2b) are injected with P815 mastocytoma cells (H-2d), and the development of cytotoxic T cells is measured in the spleen after ten days. Cytolytic alloantibodies are also generated in response to the tumor and can be detected in the plasma. When mice are treated with TCDD prior to, or within four days following tumor injection, both of these responses are profoundly suppressed. The goal of the study presented here was to determine whether prior activation of DC by LPS is likewise associated with subsequent suppression of the immune response to P815 tumor cells.

MATERIALS AND METHODS

Animals

Male C57Bl/6 and female DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). DBA/2 mice were used for *in vivo* propagation of P815 tumor cells. Male mice were housed singly, and female mice were housed 5-6 per cage. Animals were maintained in front of a laminar flow unit and in accordance with National Research Council guidelines. Mice were used at 7-12 weeks of age and were killed by CO_2 overdose.

Reagents

LPS (serotype 055:B5) was purchased from Sigma (St. Louis, MO). Spectral Red streptavidin was obtained from Southern Biotech (Birmingham, AL). Fluorochrome-conjugated antibodies to B7-2 (CD86), CD4, CD19, CD62L, and MHCII ($I\text{-}A^b$) as well as biotinylated-CD11c were obtained from PharMingen (San Diego, CA). Antibodies to CD8, CD11b (mac-1), and CD44 were purchased from Caltag (Burlingame, CA).

Animal treatments and cell preparation

Mice were injected with vehicle (PBS) or 25 μg LPS via the tail vein 16 hours prior to the ip injection of $1x10^7$ allogeneic P815 tumor cells. Ten days after tumor injection, mice were euthanized. Blood was collected by heart puncture into heparinized syringes and plasma was separated by centrifugation. Spleens were removed aseptically and single cell suspensions were prepared by disruption of tissue between the frosted ends of microscope slides. Red blood cells were removed by hypotonic lysis.

CTL assay

The cytolytic activity of spleen cells to P815 tumor cells was measured in a standard four hour ^{51}Cr release assay as previously described (Kerkvliet *et al.*, 1996). Different ratios of effector to target cells (E:T) were tested in duplicate. The percent cytotoxicity was calculated for each ratio as: % cytotoxicity = ((experimental release - naive release)/(maximum release - spontaneous release)) x100.

Cytotoxic antibody assay

Cytotoxic antibody was evaluated using a complement-dependent ^{51}Cr release assay as previously described (Kerkvliet *et al.*, 1996). Briefly, serial dilutions of plasma samples were incubated in duplicate with ^{51}Cr-labeled P815 cells in the presence of Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada), and ^{51}Cr release into the media was measured by gamma scintillation counting. The percent cytotoxicity was calculated as: % cytotoxicity = ((experimental release - spontaneous release due to complement)/(maximum release - spontaneous release)x100.

Preparation of DC

DC were enriched from spleens using the method of Swiggard et al. (1992) with modifications as described in Inaba et al. (1997). Briefly, splenic tissue was digested with collagenase D (Boehringer Mannheim, Indianapolis, IN) at 37°C for 45-60 minutes to release DC from the capsule and increase recovery. Cell suspensions were then diluted in Ca-/Mg-free HBSS and pelleted. Recovered cells were spun over a BSA gradient (1.080 g/ml) and cells in the low density fraction were collected. These freshly isolated DC-enriched preparations were then stained for flow cytometric analysis.

Flow Cytometry

Cells were incubated in 96-well plates with optimized concentrations of mAb. Nonspecific mAb binding was blocked by pre-incubating cells with rat and/or hamster IgG, and appropriately-labeled isotype controls were used to determine nonspecific fluorescence. A viable cell gate was established based on propidium iodide exclusion. For cell surface molecule evaluation, 10,000 viable cells were analyzed. Listmode data were collected on a Coulter Epics XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME). For evaluation of cell surface markers on DC, low density spleen cells were stained with an antibody to CD11c to allow selective analysis of DC (Crowley *et al.*, 1990). Cytotoxic T effector cells (CTL_E) were defined by expression of CD8, $CD44^{HI}$, and $CD62L^{LO}$ as previously described (Oughton and Kerkvliet, 1999).

Statistics

Results are presented as the mean ± SEM of 5-6 mice per group and represent a single experiment. A Student's *t* test was used to compare means of the vehicle-treated group to the LPS-treated group. For some assays, analysis of variance modeling was performed using SAS statistical software (SAS Institute, Inc., Cary, NC). Comparisons between means were made using the least significant difference *t* test. Values of $p \leq 0.05$ were considered significant.

RESULTS

Activation of DC by LPS

Previous reports have shown that LPS treatment *in vivo* activates DC as evidenced by increased expression of surface molecules such as B7-2 and MHCII (De Smedt *et al.*, 1996, and our unpublished observations). To verify that LPS treatment activated DC in this experiment, surface marker expression was evaluated on splenic DC 16 hours after treatment. As shown in Fig. A-1, LPS activated the DC as demonstrated by the enhanced expression of MHCII and B7-2 measured by flow cytometry. Analysis of B7-2 expression in the LPS-treated animals was complicated by the increased amount of background staining seen in the isotype control (dotted lines); however 30% more DC were B7-2-positive in the LPS-treated group even when positive cells were defined using each isotype control separately. Although De Smedt et al. (1996) reported that LPS reduced the number of DC in the spleen after 48 hours, in this experiment the number of DC recovered at 16 hours was not affected (vehicle = $7.7 \pm 0.9 \times 10^5$) (LPS = $8.4 \pm 0.9 \times 10^5$).

Effect of LPS pre-treatment on body weight and spleen cell recovery in P815-injected mice

In numerous previous studies we have found that normal mice injected with P815 tumor cells have a transient gain in body weight which reflects the growth of the tumor and associated pathologic ascites in the peritoneal cavity. After ten days however, the majority

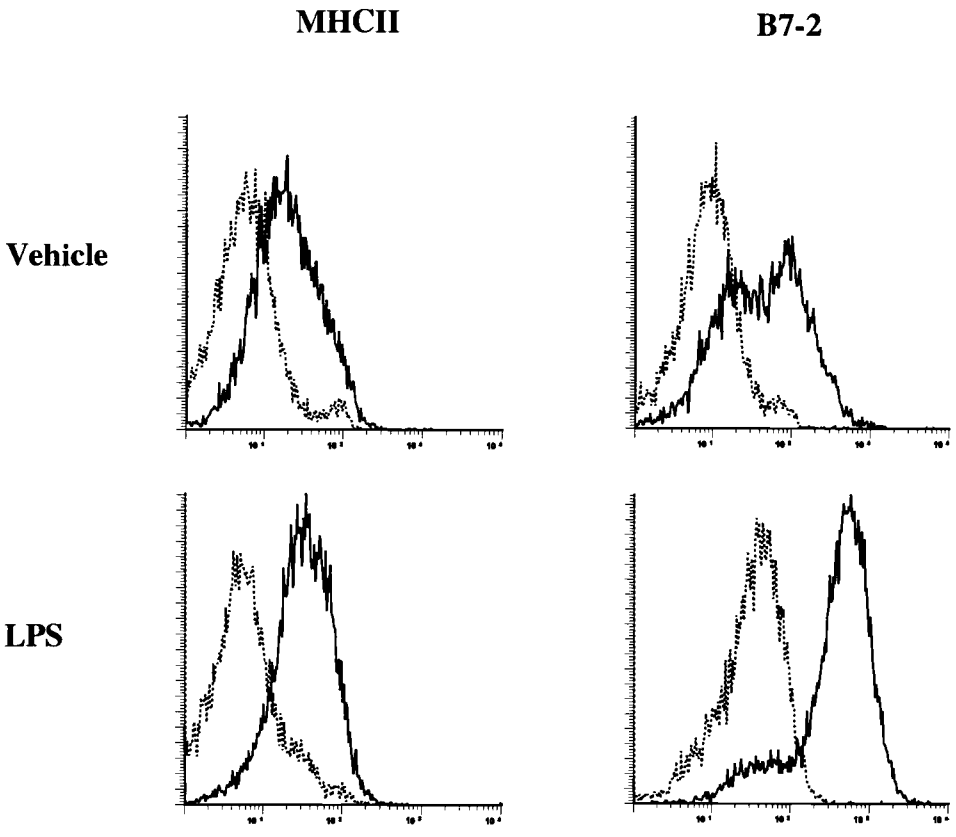

FIG. A-1. LPS treatment causes the phenotypic activation of DC. Mice were injected iv with vehicle (PBS) or 25 μg LPS and sacrificed 16 hours later. Spleens were removed, enriched for DC as detailed in Methods, and analyzed by flow cytometry. Representative histograms depict surface molecule expression on DC. Dotted lines show background staining of control antibody.

of the mice have cleared the tumor cells from the peritoneal cavity (reflected in reduced body weight), and appear healthy. Spleen cell recoveries are increased in mice responding to P815 tumor, largely due to the expansion of CD8+ cytotoxic T cells. In contrast, immune-compromised animals do not clear the tumor (reflected in increased body weight), and are usually very sick by day ten. Spleen cell numbers do not increase in such mice and CD8+ T effector cells fail to expand.

In this study, vehicle-treated animals injected with P815 cells responded as expected, appeared healthy on day ten, and had cleared most of the tumor. Relative to the nonimmune animal, the spleen cell recovery was elevated by 40% in these mice, with increases in both the percent and number of CD8+ T cells (Table A-1). In contrast, LPS-treated animals were very sick and had a great deal of ascites and tumor growth in the peritoneal cavity. This was reflected in their significantly increased body weights on day ten (Fig. A-2). The total number of spleen cells recovered was decreased, and the percent and number of CD8+ T cells were equivalent to levels seen in nonimmune mice (Table A-1), suggesting that these cells had not expanded. In addition, LPS treatment significantly reduced the percent and number of B cells and CD4+ T cells. In contrast, LPS significantly increased the CD11b+ cell population in P815-injected animals as evidenced by a three-fold increase in the percent and a 1.8-fold increase in the number of these cells relative to vehicle-treated mice.

TABLE A-1
Spleen Cell Recoveries and Subsets in Vehicle- and LPS-Treated Mice

		Vehicle	LPS	Vehicle P815	LPS P815
Spleen cell # [a]	# ($x10^8$)	1.0	1.2	1.4 (0.1)	0.8 (0.1) *
CD8+	%	9.2	9.6	18.3 (1.8)	7.0 (0.5) *
	# ($x10^7$)	0.9	1.1	2.6 (0.5)	0.6 (0.1) *
CD4+	%	19.7	14.3	9.0 (0.6)	6.3 (0.3) *
	# ($x10^7$)	2.0	1.7	1.3 (0.2)	0.5 (0.0) *
CD19+	%	62.5	63.5	54.0 (1.1)	29.1 (3.1) *
	# ($x10^7$)	6.4	7.6	7.6 (0.7)	2.6 (0.5) *
CD11b+	%	3.1	6.9	13.3 (1.5)	39.2 (1.4) *
	# ($x10^7$)	0.3	0.8	1.8 (0.1)	3.3 (0.3) *

[a] Data represent the mean ± SE of 5-6 animals per group in P815-injected mice. The vehicle and LPS alone treatments represent a single animal.

* $p \leq 0.05$ when compared to VEH/P815 group.

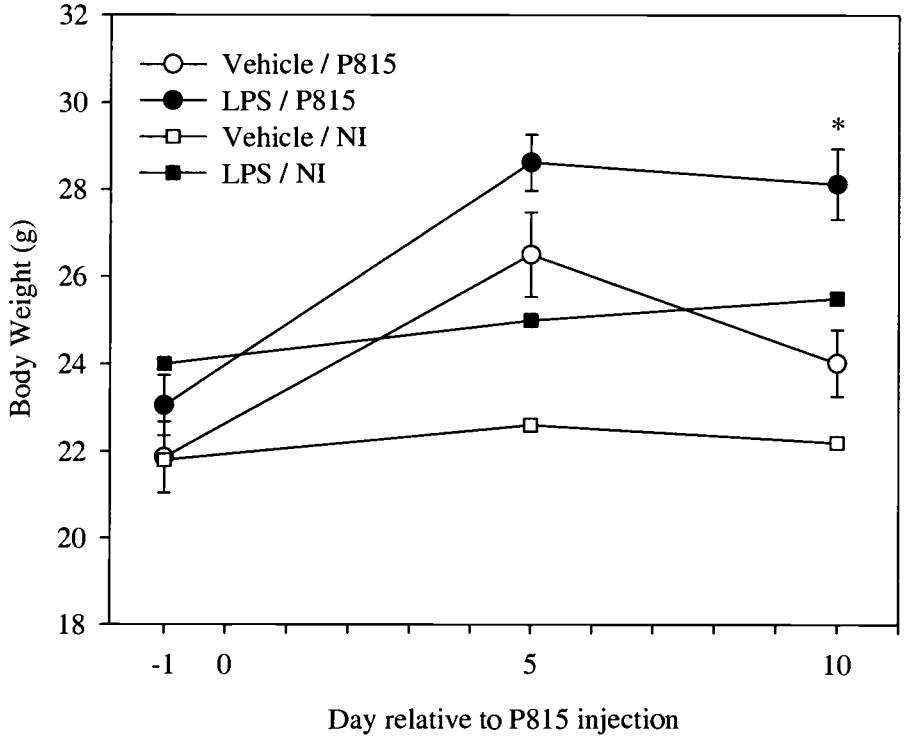

FIG. A-2. Body weight of vehicle- and LPS-treated mice responding to P815 tumor cells. Mice were injected with vehicle (open symbols) or 25 μg LPS (black symbols) sixteen hours prior to the ip injection of $1x10^7$ P815 tumor cells on day 0. Circles represent the mean ± SE of 5-6 P815-injected animals per group. Squares represent the weight of a single nonimmune (NI) vehicle- or LPS-treated animal which was not injected with P815 cells. * $p \leq 0.05$ when compared to VEH/P815 group.

It is difficult to determine with certainty whether LPS treatment alone affected the nonimmune (NI) animal, as only a single non-P815-immunized mouse from each group was included in this experiment. However, there was no evidence that LPS caused systemic toxicity as the LPS-treated animal gained a small amount of weight over the course of the study (Fig. A-2) and appeared to be healthy. As shown in Table A-1, LPS treatment did not cause overt toxic effects on the spleen as the total cell recovery and percent and number each T and B cell population were similar to the vehicle-treated non-immune mouse. LPS may have enhanced the recovery of CD11b+ cells however, as there was over a two-fold

difference in both the percent and number of these cells in the LPS-treated non-immune mouse compared to vehicle control.

Effect of LPS pre-treatment on the immune response to P815

To determine if LPS pre-treatment would suppress immune function, the splenic cytotoxic T cell response was measured ten days following injection with P815 tumor cells. As expected, a high level of cytotoxic T cell activity was detected in vehicle-treated animals (Fig. A-3A). In contrast, and as suggested by their inability to clear the tumor from the peritoneal cavity, the cytotoxic activity detected in LPS-treated mice not exceed background levels. This lack of activity in the LPS-treated mice correlated with suppression of the percent and number of cytotoxic T effector cells recovered from the spleen (Fig. A-3 B,C). In vehicle-treated mice the number of splenic CTL_E increased from 0.6 $x10^6$ in the nonimmune animal to 18.8 x 10^6 in animals responding to P815. In contrast, only 0.9 x 10^6 CTLe were recovered from the spleens of LPS-treated mice responding to P815.

The production of alloantibodies was also evaluated in plasma samples from the P815-injected mice. As shown in Fig. A-4, tumor-specific antibodies were present in vehicle-treated animals as evidenced by the complement-dependent cytotoxic activity detected in plasma. LPS-treated animals also generated cytotoxic antibodies, however the production was significantly less than in vehicle-treated mice. For example, while a 1:1280 dilution of serum resulted in 20% cytotoxicity in vehicle-treated animals, with LPS treatment the serum concentration needed to be four-fold higher, approximately 1:320, to induce the same cytotoxic effect.

DISCUSSION

Previous studies have shown that TCDD suppresses the cell-mediated immune response to P815 tumor cells, yet paradoxically appears to activate DC as evidenced by increased expression of numerous accessory molecules. The objective of the present study was to determine if LPS, a well-known activator of DC, would likewise suppress the immune

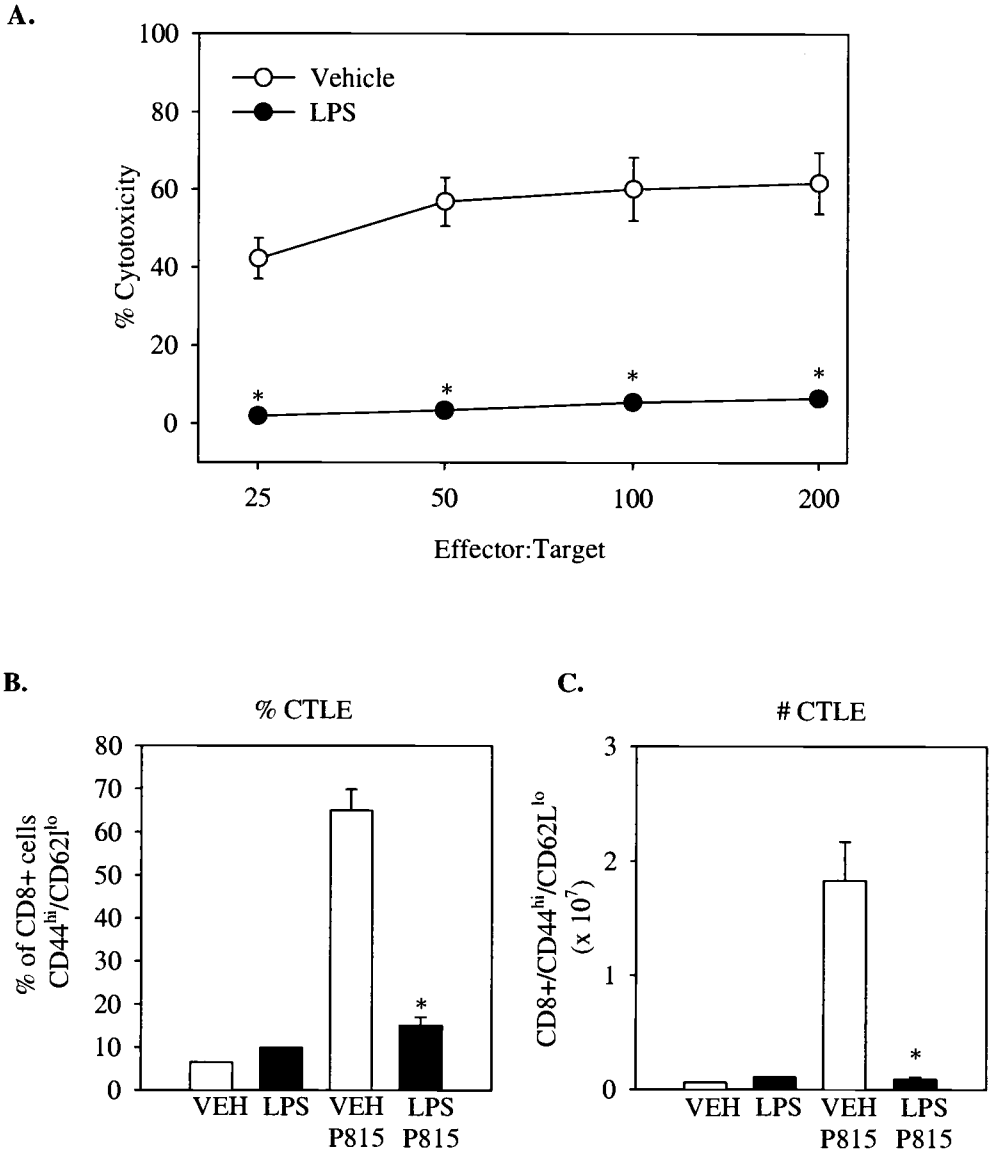

FIG. A-3. The cell-mediated immune response to P815 tumor cells is suppressed in LPS-treated mice. Mice were injected iv with vehicle (PBS) (open circles/bars) or 25 μg LPS (black circles/bars) sixteen hours prior to the ip injection of $1x10^7$ P815 tumor cells. Spleens were removed ten days after tumor injection and were assayed for CTL activity in a Cr-release assay (A) and for CTLe phenotype by flow cytometry (B, C). Data represent the mean ± SE of 5-6 animals per group in P815-injected mice. In B and C, the vehicle and LPS alone treatments represent a single animal. * $p \leq 0.05$ when compared to VEH/P815 group.

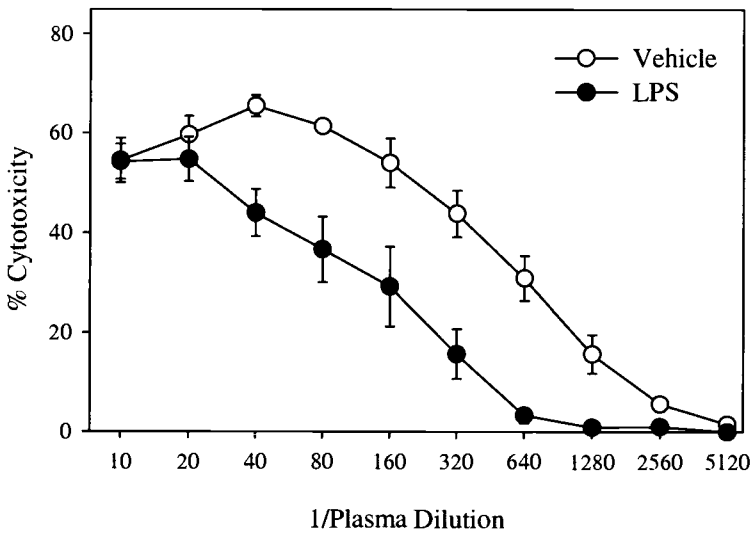

FIG. A-4. The cytotoxic alloantibody response to P815 tumor cells is suppressed in LPS-treated mice. Mice were injected iv with vehicle (PBS) (open circles) or 25 μg LPS (black circles) sixteen hours prior to ip injection of $1x10^7$ P815 tumor cells. Plasma was collected and assayed for cytotoxic activity against Cr-labeled P815 targets. Data represent the mean ± SE of 5-6 animals per group.

response to P815. The results from this study clearly demonstrate that activation of DC does not necessarily lead to enhanced immunity.

It is possible that pre-activation of DC diminishes their ability to internalize antigen and thus to activate T cells. For example, *in vitro* studies wherein DC were exposed to stimuli such as TNFα, CD40L, IL-1β, LPS, overnight culturing, or mycobacterium tuberculosis, have demonstrated that the endocytic capacity of the cells is down-regulated upon activation (Sallusto *et al.*, 1995; Henderson *et al.*, 1997; Reis e Sousa *et al.*, 1993). Because DC must internalize whole proteins and particulate antigens in order to present them to T cells, this scenario provides a potential explanation as to how exposure to LPS, and possibly TCDD, might activate DC and yet suppress the generation of an immune response.

The effects observed in this experiment were very similar to results of experiments where P815-injected mice are exposed to TCDD. Similar to LPS, TCDD treatment profoundly suppressed both the generation of cytotoxic T cells and production of cytotoxic alloantibodies (Kerkvliet *et al.*, 1996). Interestingly, like LPS, TCDD treatment also caused

a relative increase in CD11b+ cells in the spleens of P815-injected mice (Prell and Kerkvliet, 1997). The potential role of these cells in immune suppression is currently under investigation in our laboratory.

Although the immunosuppressive effects of LPS in this study were correlated with activation of DC, many potential confounding factors prohibit the conclusion that this activation caused the immune suppression. For example, while this dose of LPS did not appear to cause any overt toxicity in the non-P815-injected animal, the LPS-treated animals injected with P815 cells were sick. While it was not clear if this malaise was due to the LPS or was the result of progressive tumor growth, it is possible that toxic effects are magnified when LPS exposure occurs in combination with the tumor. This phenomenon has been reported in a study by Bartholeyns et al. (1987) where the LD_{50} for another LPS serotype (*Salmonella abortus-equi*) was reduced from 400 μg/mouse in un-immunized mice, to 50 μg/mouse when given three days after injection of carcinoma cells. Likewise, LPS treatment dramatically increases the sensitivity of mice to TNFα-induced lethal shock (Rothstein and Schreiber, 1988). Because TNFα is produced during the immune response to P815, LPS-induced immune suppression in this model could be a secondary effect of systemic TNFα toxicity. It is interesting however, that signals initiated by LPS exposure actually promote T cell survival in a superantigen model (Vella *et al.*, 1995), suggesting that immune function could be enhanced in LPS-treated mice. Further study is thus necessary to determine whether the immunosuppressive effects of LPS are seen only in particular immune responses, such as those with a significant inflammatory component, or if the effects vary based on the timing of LPS administration.

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Appendix B

Aryl Hydrocarbon Receptor-Deficient Mice Generate Normal Immune Responses to Model Particulate Antigens and are Resistant to TCDD-Induced Immune Suppression

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which mediates many of the toxic effects induced by exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a high-affinity AhR ligand which is a potent immunotoxicant. AhR-deficient mice have been constructed, and there are reports that the animals display altered splenic architecture and cellularity, and an apparent increased incidence of infection. These observations have led to speculation that the immune system of these animals might be compromised, however their functional immune response has not been directly tested. In the studies reported here, we challenged 8-10 week old AhR-deficient mice with two model antigens, allogeneic P815 tumor cells and sheep red blood cells, and examined their ability to generate cell-mediated and humoral immune responses. In addition, to address the obligatory role of the AhR in TCDD-induced immune suppression, we examined the immune response of the AhR-null animals following exposure to an immunosuppressive dose of TCDD. Results from these studies showed that AhR-deficient mice were able to mount normal, productive immune responses to both model antigens, and that neither response was suppressed by exposure to TCDD. These data suggest that the absence of the AhR does not impact development of the immune system, but confirm the findings of numerous previous studies which have indicated the AhR plays an obligatory role in TCDD-induced immune suppression.

INTRODUCTION

Many of the toxic effects resulting from exposure to halogenated aromatic hydrocarbons (HAH) such as TCDD are mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor which is a member of a superfamily of basic helix-loop-helix (bHLH) DNA binding proteins (reviewed by Hankinson, 1995, and by Schmidt and Bradfield, 1996). Prior to activation, the cytoplasmic AhR is associated with additional proteins including a dimer of heat shock protein 90, a src protein kinase (Enan and

Matsumura, 1996; Blankenship and Matsumura, 1997), and an immunophilin alternately named AIP, XAP2, and ARA9 by the three laboratories which originally characterized it (Ma and Whitlock, 1997; Meyer and Perdew, 1999; Carver *et al.*, 1998). When ligand binds, these proteins dissociate and the receptor translocates to the nucleus and forms a heterodimeric complex with another bHLH protein, the AhR nuclear translocator (Arnt). This complex then binds to dioxin response elements (DRE) present in the upstream regulatory region of target genes. Numerous DRE-containing genes have been identified, including metabolic enzymes, growth factors, and cytokines (Lai *et al.*, 1996), and it is believed that the toxic effects of TCDD and other strong AhR ligands result from altered expression of these gene products.

There is great deal of evidence which suggests that the immune suppression induced by HAH exposure is mediated by the AhR. This evidence includes the correlation between *in vivo* toxicity and chemical affinity for the receptor, including fulfillment of predicted structure/activity relationships (Kerkvliet *et al.*, 1990a; Kerkvliet *et al.*, 1985; Silkworth *et al.*, 1984; Davis and Safe, 1988) and by the differing sensitivities of mouse strains bearing the different receptor genotypes (Vecchi *et al.*, 1983; Kerkvliet *et al.*, 1990b; Silkworth *et al.*, 1986; Silkworth and Grabstein, 1982). However, there are also some reports of immune effects occurring independently of the receptor (Davis and Safe, 1991; Holsapple *et al.*, 1986a; Holsapple *et al.*, 1986b) leaving open the possibility that the AhR may not be required for all HAH-induced immune toxicity.

The recent generation of mouse strains lacking functional AhR has provided the opportunity to assess the role of the receptor in normal development as well as in a variety of TCDD-induced toxicities. In terms of development, $AhR^{-/-}$ mice have a slower growth rate than $AhR^{+/+}$ mice and also exhibit numerous organ pathologies, especially notable in liver, which increase in severity as the animals age (Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1995; Fernandez-Salguero *et al.*, 1997). In terms of sensitivity to TCDD, $AhR^{-/-}$ animals are resistant to many toxic effects such as Cyp1A1 induction (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996), hepatomegaly (Fernandez-Salguero *et al.*, 1996), thymic atrophy (Fernandez-Salguero *et al.*, 1996) and teratogenesis (Peters *et al.*,

1999; Mimura *et al.*, 1997), demonstrating that the receptor plays an obligatory role in the induction of these toxic endpoints.

Reports concerning the immune system of $AhR^{-/-}$ mice are currently limited to description of size and cellular makeup of the lymphoid organs (Fernandez-Salguero *et al.*, 1997; Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1995). Although reports of eosinophilia and alterations in splenic cellularity in these animals have led to speculation that the AhR might play a role in regulation of the immune response (Lahvis and Bradfield, 1998), the functional status of the immune system in the animals has not been characterized. Indeed, an increased incidence of Helicobacter infection in aging $AhR^{-/-}$ mice (Fernandez-Salguero *et al.*, 1997) as well as reports of lymphocyte infiltration of various organs in deceased neonates (Gonzalez *et al.*, 1995), suggest that the immune system of these animals may be compromised. Interestingly, however, a 1998 review article cited unpublished data that mice tested at various ages produced normal antibody titers when challenged with exogenous antigen (Gonzalez and Fernandez-Salguero, 1998), suggesting that certain immune functions may be intact in $AhR^{-/-}$ animals.

In the studies presented here, we examined the immune-responsiveness of two AhR-deficient mouse strains and further utilized these animals to examine the role of the AhR in TCDD-induced immune suppression. To this end, we tested the immune response to two particulate antigens which have been used extensively to address the mechanism of TCDD-induced immunotoxicity. First we examined the cell-mediated immune response to allogeneic cells in the P815 tumor model. In this response, the generation of cytotoxic T cells is highly sensitive to suppression by TCDD (ID_{50} = 7.2 μg/kg) (Dekrey and Kerkvliet, 1995; Kerkvliet *et al.*, 1996). In addition, we examined the humoral immune response to sheep red blood cells (SRBC), a response even more sensitive to suppression by TCDD (ID_{50} = 0.6 μg/kg) (Kerkvliet *et al.*, 1990b; Vecchi *et al.*, 1983), and a model which is currently recommended by the National Toxicology Program for screening immunomodulatory drugs and chemicals (Luster *et al.*, 1997).

MATERIALS AND METHODS

Animals

Ah receptor-deficient mice created by deletion of exon 1 of the AhR gene (Fernandez-Salguero *et al.*, 1995), were initially obtained from P. Fernandez-Salguero and F. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD) and were bred as homozygotes at the SUNY facility. These mice are on a mixed genetic background (129/SV x C57Bl/6N) and lack the $AhR^{b\text{-}1/b\text{-}1}$ gene. They are referred to as B6-$129^{\Delta 1\Delta 1}$ in this paper. Control animals on the same genetic background have the $AhR^{b\text{-}1/b\text{-}1}$gene and are designated B6-$129^{+/+}$.

AhR-deficient mice created by deletion of exon 2 of the AhR gene (Schmidt *et al.*, 1996) were obtained as breeders from The Jackson Laboratory (Bar Harbor, ME). These mice have been backcrossed 10 generations onto the C57Bl/6 background and are referred to as $B6^{\Delta 2\Delta 2}$ in this paper. C57Bl/6J mice used as controls are referred to as $B6^{+/+}$ and were also purchased from Jackson Labs. The $B6^{\Delta 2\Delta 2}$ mice that were used in these studies were bred as homozygotes at the OSU facility.

Currently we are producing $B6^{\Delta 2\Delta 2}$ mutant mice by breeding heterozygous ($B6^{\Delta 2/+}$) females with homozygous ($B6^{\Delta 2\Delta 2}$) males. This is because the $B6^{\Delta 2\Delta 2}$ dams had limited breeding success, an observation which has also been found in B6-$129^{\Delta 1\Delta 1}$ mice (Abbott *et al.*, 1999; Fernandez-Salguero *et al.*, 1997). For genotypic analysis of the pups, DNA was extracted from 3 μl of blood obtained from the tail vein using a Chelex (Bio-Rad, Hercules, CA) extraction procedure (Walsh *et al.*, 1991). DNA was amplified by PCR using primer sequences obtained from Jackson Labs. Primers that identify the $B6^{\Delta 2}$ gene product amplify a 280 bp DNA fragment of the KO gene whereas the wild-type primers which identify the $B6^{+}$ gene yield a 669 bp product: (KO primers: 5'-CTT GGG TGG AGA GGC TAT TC-3' and 5'-AGG TGA GAT GAC AGG AGA TC-3'). (Wild-type primers: 5'-GGA TTT GAC TTA ATT CCT TCA GCG G-3' and 5'-TCT TGG GCT CGA TCT TGT GTC AGG AAAC AGG-3'). Amplification of DNA sequences was performed with dNTP sets purchased from Boehringer Mannheim (Indianapolis, IN) and *Taq* DNA Polymerase (Promega, Madison, WI) using a thermal cycler (DNA Engine, MJ Research Inc.,

Watertown, MA) programmed to perform touchdown protocols. The protocol for the wild-type primers was set to 94°C for 3 min; 12 cycles at 94°C for 35 sec (denature), 64°C for 45 sec (anneal) decreasing by 0.5°C per cycle, 72°C for 45 sec (primer extension); 25 cycles at 94°C for 35 sec, 58°C for 30 sec, 72°C for 45 sec; and 72°C for 2 min. For the KO primers, the annealing temperatures were programed to 58°C for the initial 12 cycles and 52°C for the final 25 cycles. Gel electrophoresis of amplification products was conducted using 2% agarose (Sigma, St. Louis, MO) gels. Heterozygous $B6^{\Delta 2/+}$ mice used in these studies were identified by the presence of both 280 and 669 bp products.

Mice were maintained in front of a laminar flow unit, and were used in experiments at 7-12 weeks of age. Male mice were caged singly and females were caged in groups of 2-6. Animals were provided with food (Harlan Teklad 8604 rodent diet, Madison, WI) and water *ad libitum* and maintained in accordance with the National Research Council guidelines.

P815 and SRBC challenge, and TCDD exposure

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) obtained as certified standard with 99% purity, was dissolved in anisole and diluted in peanut oil. A vehicle control solution consisting of peanut oil and anisole was similarly-prepared. Mice were treated with vehicle or TCDD by gavage. For studies using P815 cells, mice were treated with 15 μg/kg of TCDD, a known immunosuppressive dose (Dekrey and Kerkvliet, 1995), one day prior to the ip injection of 1 x 10^7 P815 cells. P815 mastocytoma cells were propagated as an ascites by weekly passage in DBA/2J mice. For studies using SRBC, mice were treated with 5 μg/kg TCDD 7 days and 1 day prior to the ip injection of 2.5 x 10^8 washed SRBC (Colorado Serum, Denver, CO). Mice were killed by CO_2 overdose 10 days after injection of P815 tumor cells or 5 days after the injection of SRBC.

CTL Assay

The cytolytic activity of spleen cells to P815 tumor cells was measured in a standard 4-hr ^{51}Cr-release assay as previously described (Dekrey and Kerkvliet, 1995). Effector:Target (E:T) ratios ranging from 100:1 to 3.7 :1 were tested in duplicate for each sample. The amount of ^{51}Cr released into the supernatant was measured, and the percent cytotoxicity was

calculated for each E:T ratio as follows: % cytotoxicity = ((experimental release - naive release) x 100) / (maximum release - spontaneous release), where experimental release was determined using splenocytes from P815-injected mice, naive release was determined using splenocytes from nonimmune mice, maximum release was determined by incubating the ^{51}Cr-labeled tumor cells in SDS, and spontaneous release was determined by incubating tumor cells in culture medium.

Cytotoxic Antibody Assay

Cytotoxic alloantibody titers were determined using a complement-dependent ^{51}Cr-release assay as previously described (Kerkvliet *et al.*, 1996). Briefly, serial dilutions of heat-inactivated plasma samples were incubated with ^{51}Cr-labeled P815 cells and low-tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada). Specific cytotoxicity was calculated using the formula: % specific cytotoxicity = ((experimental release - complement only) x 100) / (maximum release - spontaneous release), where experimental release was determined from ^{51}Cr-labeled P815 cells incubated with plasma and complement, ^{51}Cr release from complement only was determined by incubating ^{51}Cr-labeled P815 cells with complement, maximum release was determined by incubating the ^{51}Cr-labeled P815 cells in SDS, and spontaneous release was determined by incubating the ^{51}Cr-labeled P815 cells in culture medium. All plasma samples were tested in duplicate on separate plates. Titers were transformed to $\log_2$ dilutions for statistical analysis.

Flow cytometric analysis of spleen cells

Spleen cell subsets were identified by the expression of CD4, CD8, CD19, CD11b (Mac-1), and Gr-1. CTL effector cells (CTL_E) cells were defined by the expression of CD8, $CD44^{lo}$ and $CD62L^{high}$ as previously reported (Oughton and Kerkvliet, 1999). Antibodies to CD4, CD8, CD62L, CD19 and GR-1 were purchased from Pharmingen (San Diego, CA); antibodies to CD44 and CD11b were purchased from Caltag (Burlingame, CA). Spleen cells were incubated with rat IgG (Cappel, West Chester, PA) to block nonspecific binding before addition of fluorochrome-conjugated mAb. All mAb were titrated for optimal concentration, and appropriately-labeled, isotype-matched Igs were used as controls for

nonspecific fluorescence. Listmode data were collected from 20,000-100,000 freshly-stained cells using an Epics XL flow cytometer (Beckman-Coulter Electronics, Hiahleah, FL) and analyzed using WinList software (Verity Software House, Inc., Topsham, MA).

SRBC plaque assay

The Cunningham plaque assay (Cunningham and Szenberg, 1968) as modified by Deyo and Kerkvliet (1990) was used to identify splenic plaque-forming cells (PFC). Briefly, a mixture of spleen cells, SRBC and guinea pig complement (Colorado Serum, Denver, CO) which had been absorbed with autologous SRBC, was placed as a standard volume into microscope slide chambers. The chambers were sealed with a paraffin-wax mixture and incubated for 45 min at 37°C. Plaques were enumerated using a Bellco plaque viewer (Bellco Glass Co., Vineland, NJ).

Statistics

Results are presented as the mean ± SEM of 3-8 mice per group as indicated in the figure legends. Statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC). Analysis of variance modeling was performed with a significance level of $\alpha = 0.05$. Comparisons between means were made using the least significance difference (LSD) multiple comparison *t* test with values of $p \leq 0.05$ considered to be statistically significant.

RESULTS

Immune response of AhR-deficient mice to P815 tumor cells

C57Bl/6 (H-2^b) mice develop vigorous cell-mediated and humoral immune responses when injected with allogeneic (H-2^d) P815 tumor cells. The cell-mediated response can be quantified by the cytotoxic activity of splenic lymphocytes in a ^{51}Cr-release assay (Kerkvliet *et al.*, 1996) or by enumerating splenic CTL effector cells (CTL_E) using flow cytometry (Oughton and Kerkvliet, 1999). Likewise, the production of tumor specific alloantibodies

can be measured in plasma using a complement-mediated cytotoxicity assay (Kerkvliet *et al.*, 1996).

As shown in Fig. B-1 (compare white bars), both strains of $AhR^{-/-}$ mice were able to generate a normal immune response to P815 tumor cells. Specifically, both the CTL activity and the percent of CTL_E in vehicle-treated $B6^{\Delta 2\Delta 2}$ were identical to that of vehicle-treated $B6^{+/+}$ mice. Likewise, vehicle-treated B6-$129^{\Delta 1\Delta 1}$ mice and their B6-$129^{+/+}$ counterparts had similar CTL responses. The cytotoxic antibody titers (Fig. B-2, compare white bars) were also the same in vehicle-treated $B6^{\Delta 2\Delta 2}$ and $B6^{+/+}$ mice, where plasma dilutions of approximately 1:1280 resulted in 30% lysis ($titer_{30}$) of P815 target cells. The antibody response in B6-$129^{\Delta 1\Delta 1}$ mice was slightly lower than in B6-$129^{+/+}$ mice, however the difference was not statistically significant. Taken together, these results indicate that the absence of a functional Ah receptor does not impact the ability of either $B6^{\Delta 2\Delta 2}$ or B6-$129^{\Delta 1\Delta 1}$ mice to mount a normal immune response to P815 tumor cells.

Spleen size, cell recovery and the percentage of CD4+, CD8+, CD19+, and CD11b+ subsets were similar in the $B6^{\Delta 2\Delta 2}$ and $B6^{+/+}$ mice responding to P815 cells (Table B-1, compare shaded columns). Likewise, these parameters were not different in the B6-$129^{\Delta 1\Delta 1}$ mice compared to their B6-$129^{+/+}$ controls with the exception of the total number of spleen cells which was increased by 73% in B6-$129^{\Delta 1\Delta 1}$ animals. This disparity in spleen cell recovery was not the result of selective increase in one particular cell type, however, as the percentage of each subset was equivalent in both strains.

Effect of TCDD on the immune response to P815 cells in AhR-deficient mice

The allograft response to P815 cells is dose-dependently suppressed by TCDD, with an ID_{50} of 7.2 μg/kg for the CTL response in C57Bl/6 mice (Dekrey and Kerkvliet, 1995). As expected, in the current study the CTL response of both $AhR^{+/+}$ strains ($B6^{+/+}$ and B6-$129^{+/+}$) was profoundly suppressed by TCDD as measured by CTL activity and percent CTL_E, (Fig. B-1) and antibody titers (Fig. B-2). In contrast, both $AhR^{-/-}$ strains ($B6^{\Delta 2\Delta 2}$ and B6-$129^{\Delta 1\Delta 1}$) were insensitive to the immunotoxic effects of TCDD, as demonstrated by their normal CTL and cytotoxic antibody responses to P815 tumor cells.

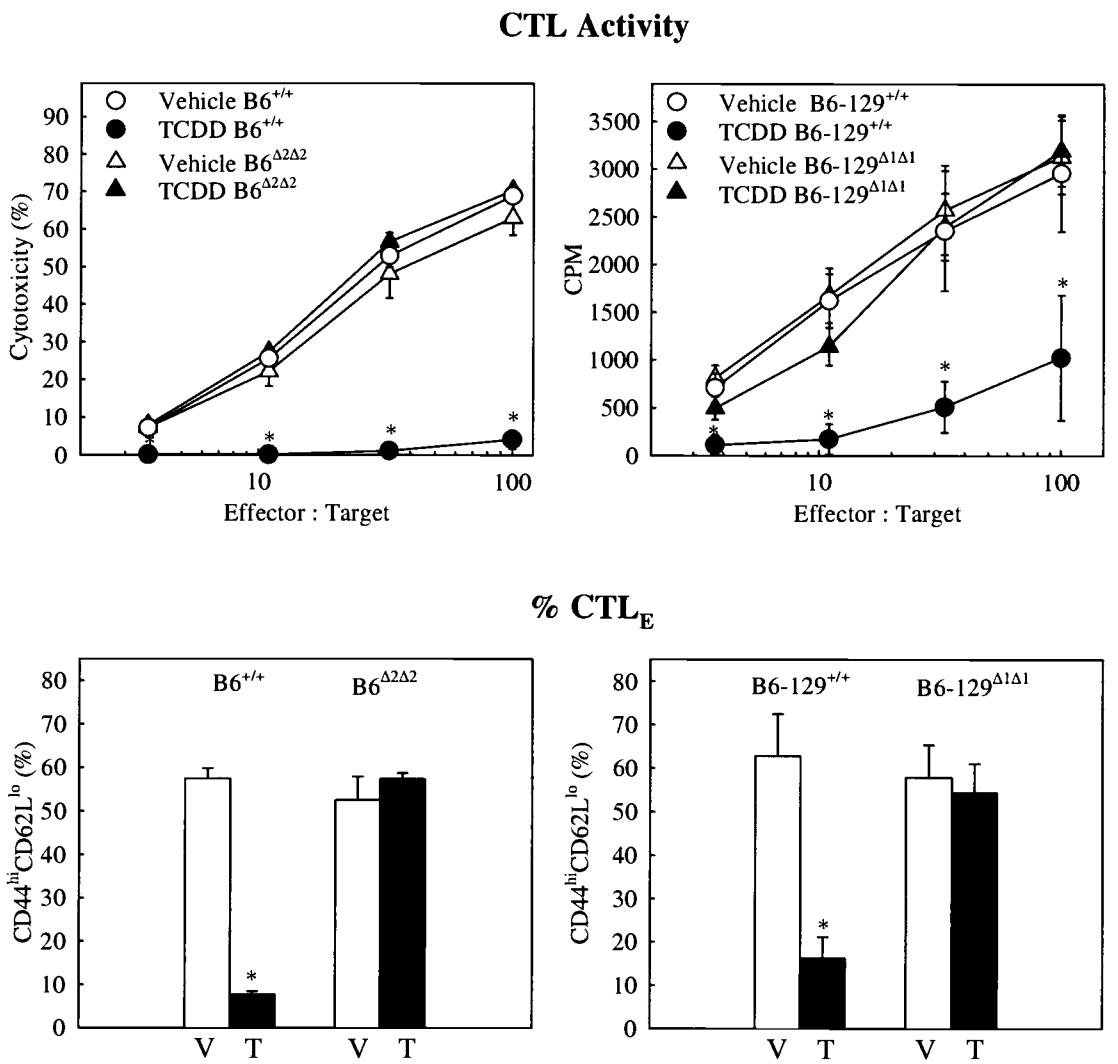

FIG. B-1. CTL response of AhR$^{-/-}$ mice challenged with P815 tumor cells. AhR$^{-/-}$ (B6$^{\Delta2\Delta2}$ and B6-129$^{\Delta1\Delta1}$) male mice and their AhR$^{+/+}$ controls (B6$^{+/+}$ and B6-129$^{+/+}$) were treated with vehicle (V) or 15 μg/kg TCDD (T) one day prior to injection of P815 cells. The CTL response was measured in the spleen on day 10, and is represented as cytotoxic activity and percent of CD8+ cells expressing the CTL$_E$ phenotype as described in *Materials and Methods*. Data points represent the mean ± SEM for 3-6 mice per treatment. * indicates $p \leq 0.05$.

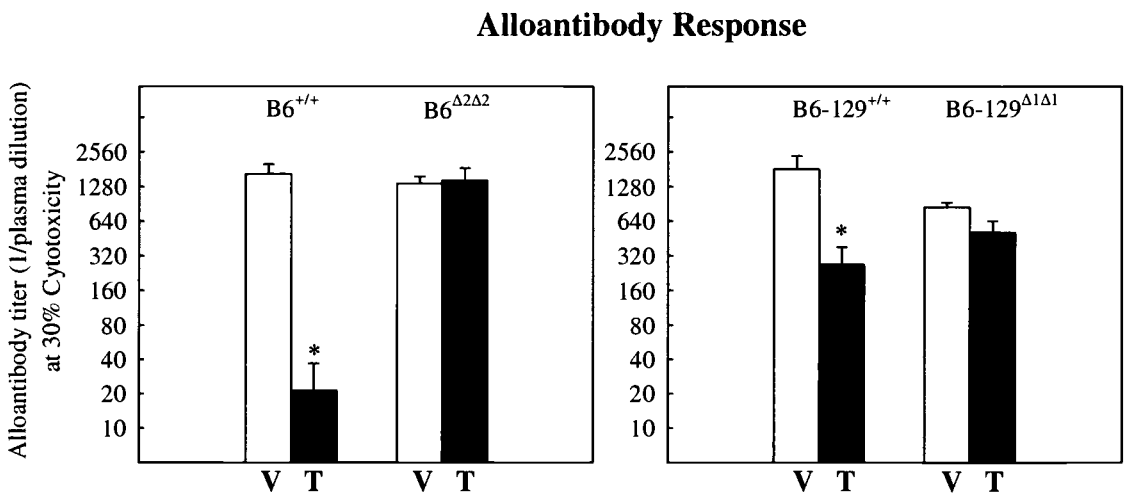

FIG. B-2. Alloantibody response of $AhR^{-/-}$ mice challenged with P815 tumor cells. $AhR^{-/-}$ ($B6^{\Delta2\Delta2}$ and B6-$129^{\Delta1\Delta1}$) male mice and their $AhR^{+/+}$ controls ($B6^{+/+}$ and B6-$129^{+/+}$) were treated with vehicle (V) or 15 μg/kg TCDD (T) one day prior to injection of P815 cells. The cytotoxic alloantibody was measured on day 10 and is represented as the plasma dilution at which 30% lysis of ^{51}Cr-labeled P815 cells occurred. Data points represent the mean ± SEM for 3-6 mice per treatment. * indicates $p \leq 0.05$.

The consequences of TCDD exposure on spleen cellularity in the mice responding to P815 cells are shown in Table B-1. As expected, TCDD decreased the spleen size and cell number of both the $B6^{+/+}$ and B6-$129^{+/+}$ mice. In contrast, the percentage of CD11b+ cells was significantly increased in these $AhR^{+/+}$ mice. These changes were not observed in either $AhR^{-/-}$ strain (B6-$129^{\Delta1\Delta1}$ or $B6^{\Delta2\Delta2}$) exposed to TCDD, indicating that the AhR plays an obligatory role in these TCDD-induced effects.

Immune response of AhR-deficient mice to SRBC

To verify that the resistance of $AhR^{-/-}$ mice to TCDD-induced immune suppression was not unique to the P815 response, we also examined the immune response to SRBC. As shown in Fig. B-3, vehicle-treated B6-$129^{\Delta1\Delta1}$ female mice mounted a normal antibody response following immunization with SRBC, as the frequency of plaque-forming cells (PFC/10^6 spleen cells) was the same as in $B6^{+/+}$ female mice. However, on a total spleen basis, the mutants had almost twice the number of PFC as the $B6^{+/+}$ mice. This difference

TABLE B-1
Spleen Cell Numbers and Subsets in Vehicle- and TCDD-Treated $AhR^{+/+}$ and $AhR^{-/-}$ Mice Injected with Allogeneic Tumor Cells

	$B6^{+/+}$		$B6^{\Delta2\Delta2}$	
	Vehicle	TCDD	Vehicle	TCDD
Spleen Wt: Body Wt	7.0 (0.4)	3.8 (0.4)*	8.9 (0.7)	9.7 (0.3)
Total #SC (x 10^7)	12.9 (0.7)	5.6 (0.9)*	13.8 (1.2)	14.4 (0.5)
%CD4	8.3 (0.5)	9.7 (1.3)	8.1 (0.8)	7.1 (0.3)
%CD8	12.0 (0.8)	11.2 (1.6)	11.9 (1.4)	11.3 (0.6)
%CD19	52.8 (2.3)	49.7 (2.4)	49.9 (1.4)	46.7 (0.4)
%CD11b-Gr1	8.6 (0.5)	17.1 (1.9)*	9.7 (0.6)	9.1 (0.5)

	B6-$129^{+/+}$		B6-$129^{\Delta1\Delta1}$	
	Vehicle	TCDD	Vehicle	TCDD
Spleen Wt: Body Wt	6.6 (0.8)	4.2 (0.2)*	6.6 (0.7)	6.6 (0.7)
Total #SC (x 10^7)	8.9 (0.4)	6.0 (1.0)	15.4 (2.0)**	14.7 (1.3)
%CD4	10.0 (0.2)	13.2 (0.6)*	10.0 (0.5)	12.3 (1.3)
%CD8	19.6 (2.9)	16.4 (1.9)	16.2 (1.8)	16.3 (1.0)
%CD19	35.6 (5.6)	32.1 (6.5)	43.7 (2.9)	50.8 (1.4)
%CD11b	14.9 (2.6)	22.1 (4.0)*	11.9 (2.4)	9.5 (1.1)

Values represent the mean ± (SEM) for 3-6 mice per treatment.
* $p \leq 0.05$ when compared to the corresponding vehicle control
** $p \leq 0.05$ when compared to vehicle-treated B6-$129^{+/+}$ mice

in total PFC number was a reflection of the substantially larger number of cells recovered from the mutant mice (30 ± 1.8 x 10^7 cells/spleen) as compared to age-matched $B6^{+/+}$ mice (17 ± 2.2 x 10^7 cells/spleen). However, the percentages of CD4+, CD8+ and CD19+ subsets were not different between the two strains (data not shown). It should be noted that $B6^{+/+}$ mice were used as controls in this study due to the unavailability of B6-$129^{+/+}$ animals.

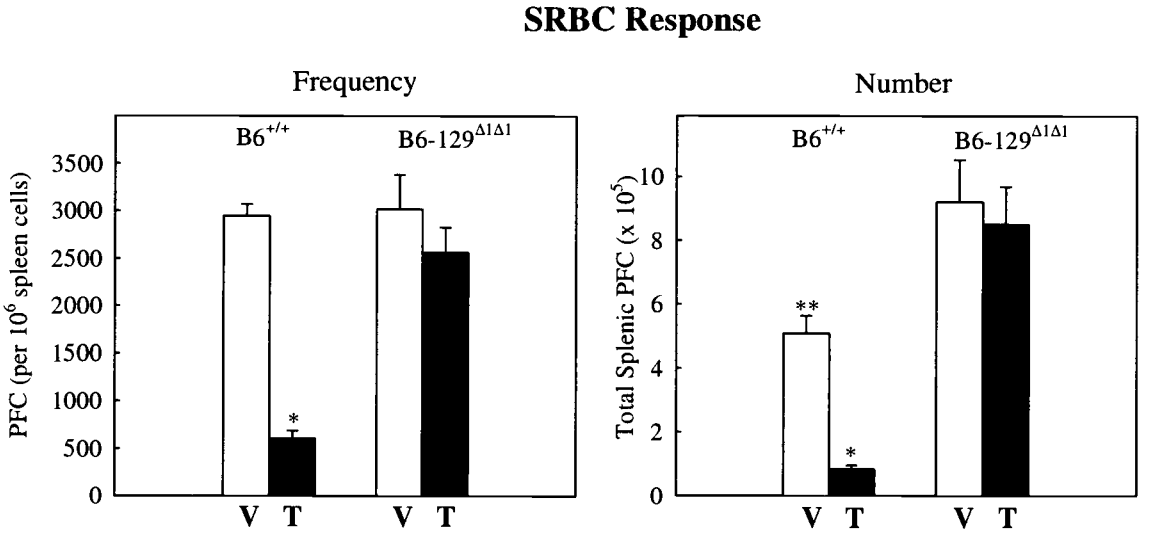

FIG. B-3. Antibody response of $AhR^{-/-}$ mice challenged with sheep red blood cells. B6-$129^{\Delta 1\Delta 1}$ and $B6^{+/+}$ female mice were treated with vehicle (V) or 10 μg/kg TCDD (T). The splenic PFC response was measured 5 days after the injection of SRBC and is represented as the frequency of PFC per million spleen cells and the total number of PFC per spleen. Data points represent the mean ± SEM for 6-8 mice per treatment. * indicates $p \leq 0.05$.

Effect of TCDD on the immune response to SRBC in AhR-deficient mice

The antibody response to SRBC is very sensitive to suppression by TCDD, with an ID_{50} of 0.6 μg/kg in C57Bl/6 mice (Kerkvliet *et al.*, 1990b). For these studies, $B6^{+/+}$ and B6-$129^{\Delta 1\Delta 1}$ mice received 10 μg/kg TCDD prior to the injection of SRBC. As expected, TCDD suppressed the response in $B6^{+/+}$ mice as evidenced by an 80% decrease in both the frequency and total number of splenic PFC (Fig. B-3). In contrast, B6-$129^{\Delta 1\Delta 1}$ mice were

unaffected by TCDD exposure as the antibody response was equivalent to that of vehicle-treated controls.

Sensitivity of heterozygous ($AhR^{+/-}$) mice to TCDD-induced immunotoxicity

Currently in our laboratory, $B6^{\Delta 2\Delta 2}$ mice are produced by breeding $B6^{\Delta 2/+}$ ($AhR^{+/-}$) females with $B6^{\Delta 2\Delta 2}$ ($AhR^{-/-}$) males, which results in $B6^{\Delta 2/+}$ and $B6^{\Delta 2\Delta 2}$ offspring. Since we anticipated using the $B6^{\Delta 2/+}$ offspring as littermate controls in future studies, their sensitivity to TCDD-induced immune suppression was evaluated using the P815 tumor response. As shown in Fig. B-4, TCDD-treated $B6^{+/+}$ mice responded as expected and were all consistently and profoundly suppressed in their CTL response. In contrast, TCDD-treated $B6^{\Delta 2/+}$ animals were quite variable in their responses, with 3/7 showing suppression similar to TCDD-treated $B6^{+/+}$ mice and 4/7 showing intermediate suppression. This variability was not the result of an assay inconsistency, as similar results were observed in three independent experiments. In fact, we have found that the CTL response of some of the heterozygous animals is not measurably suppressed by TCDD. Furthermore, when the alloantibody response was evaluated, we found that the $B6^{\Delta 2/+}$ mice were significantly ($p \leq 0.05$) less suppressed by TCDD than were the $B6^{+/+}$ mice. Specifically, the $titer_{30}$ was suppressed by 97% in $B6^{+/+}$ and by 75% in the $B6^{\Delta 2/+}$ animals. In this assay, variation between animals was observed in all groups. Taken together, these data suggest that a single copy of the AhR is not sufficient to induce full and consistent suppression of this immune response, and therefore the $B6^{\Delta 2/+}$ mice cannot be used as TCDD-sensitive controls.

DISCUSSION

Although previous reports have suggested that $AhR^{-/-}$ mice may have compromised immune systems, this conclusion is based largely on findings of altered splenic cellularity and architecture as well as the increased incidence of infection in aged animals (Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1995; Fernandez-Salguero *et al.*, 1997). We tested the functional immune response of $AhR^{-/-}$ mice following challenge with antigen and found that

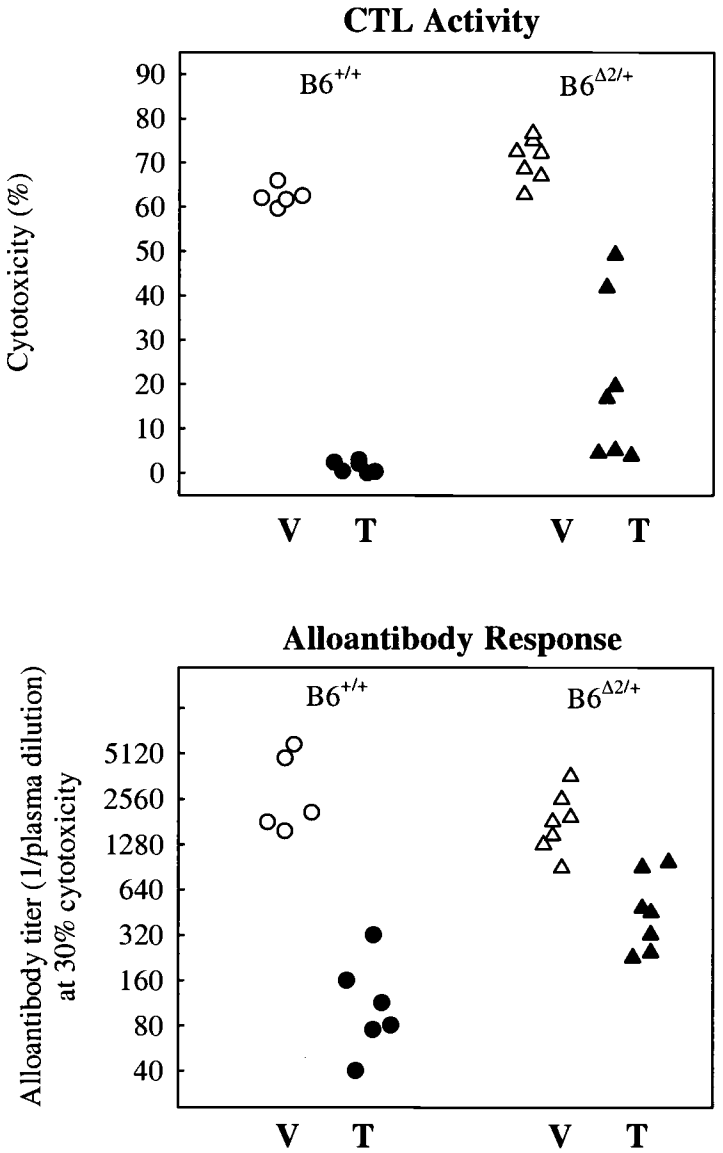

FIG. B-4. CTL and alloantibody response of heterozygous AhR$^{+/-}$ mice challenged with P815 tumor cells. B6$^{\Delta 2/+}$ and B6$^{+/+}$ male mice were treated with vehicle (V) or 15 ug/kg TCDD (T) one day prior to injection of P815 cells. CTL activity and alloantibody responses are shown for each individual animal. Data shown are the CTL response at the 100:1 E:T ratio, and the alloantibody response at the plasma dilution at which 30% lysis of ^{51}Cr-labeled P815 cells occurred. Data is shown for 5-7 mice per treatment.

the absence of the receptor did not affect the ability of the animals to generate normal immune responses. In our studies, antigen-challenged $AhR^{-/-}$ mice developed productive cell-mediated and humoral immune responses to allogeneic tumor cells and SRBC which were of equivalent magnitude to those generated in wildtype animals. Furthermore, we demonstrated that these results were not limited to a particular strain of $AhR^{-/-}$ mouse as both $B6/129^{\Delta 1\Delta 1}$ and $B6^{\Delta 2\Delta 2}$ animals developed normal CTL and alloantibody responses when challenged with allogeneic tumor cells.

Despite our conclusion that the AhR is not a critical factor in development of a functional immune system, it should be noted that $B6/129^{\Delta 1\Delta 1}$ animals develop significant splenic pathologies as they age (6-13 months) (Fernandez-Salguero *et al.*, 1997). Because all of our experiments were performed on animals at 7-12 weeks of age, we cannot rule out the possibility that the AhR does play a role in long term functional integrity of the immune system. In addition, although the responses tested in these experiments encompass the functions of numerous immune cells, they do not represent an exhaustive characterization of all types of immune responses, and therefore we cannot conclude that the immune system of the animals is entirely normal. For example, the function of NK cells is not reflected in either the response to SRBC nor to P815, nor does either model indicate how the mice will respond to pathogens. This latter point is particularly important due to the increased incidence of Helicobacter infection which has been reported in the $B6/129^{\Delta 1\Delta 1}$ mice (Fernandez-Salguero *et al.*, 1997).

Although the immune responses were similar in $AhR^{+/+}$ and $AhR^{-/-}$ animals, some differences in spleen cell recoveries were noted. In particular, the female $B6/129^{\Delta 1\Delta 1}$ mice responding to challenge with SRBC appeared to have abnormally high (30 million) numbers of spleen cells. Although we did not have genetically-matched animals as controls in this experiment, these cell numbers were substantially higher than were observed in the $B6^{+/+}$ mice in the same experiment (17 million) and were also higher than spleen cell numbers of male $B6/129^{+/+}$ animals responding P815 tumor (9 million, Table B-1). Male $B6/129^{\Delta 1\Delta 1}$ responding to challenge with P815 cells also had more spleen cells than $B6/129^{+/+}$ controls, although this difference did not appear to be as dramatic as in the female mice. Taken together, these observations in antigen-challenged mice differ with from those in other

studies using non-antigen-challenged B6/129$^{\Delta 1\Delta 1}$ mice which have reported that these animals have normal or reduced numbers of spleen cells depending on their age (Fernandez-Salguero *et al.*, 1995; Fernandez-Salguero *et al.*, 1997). In contrast to B6/129$^{\Delta 1\Delta 1}$ mice, none of the B6$^{\Delta 2\Delta 2}$ mice had increased numbers of spleen cells relative to B6$^{+/+}$ controls, a finding that was somewhat surprising due to a previous report which found that approximately 50% of these animals have enlarged spleens and increased numbers of cells (Schmidt *et al.*, 1996).

While immune responses appear to develop independently of the AhR, the receptor was found to play an obligatory role in suppression of immune responses by TCDD. Thus, following treatment with a highly immunosuppressive dose of TCDD, both strains of AhR$^{-/-}$ mice were able to develop normal cytotoxic T cell activity and produce cytotoxic alloantibodies in response to P815 tumor cells. In addition, TCDD did not cause an increase in splenic CD11b+ cells in the P815-challenged AhR$^{-/-}$ animals, an effect we have seen consistently in TCDD-treated B6$^{+/+}$ mice (Prell and Kerkvliet, 1997). TCDD likewise did not suppress the antibody response to SRBC in the B6/129$^{\Delta 1\Delta 1}$ animals, even when given at a dose which was more than 15-fold higher than the ID_{50} for B6$^{+/+}$ mice. These results were not unexpected given the large body of evidence from structure:activity relationships and studies with congenic mice that has shown HAH-induced immune suppression is AhR-dependent (Davis and Safe, 1988; Kerkvliet *et al.*, 1990a; Vecchi *et al.*, 1983). The current studies have proven this dependence by addressing the role of the receptor directly using the knockout mice.

One finding that was unexpected was that the CTL and alloantibody responses of B6$^{\Delta 2/+}$ heterozygous mice were less suppressed by a dose of TCDD that profoundly suppressed B6$^{+/+}$ mice. These results suggest that in terms of immune suppression, a single copy of the receptor may not confer complete sensitivity to TCDD. This conclusion differs from studies using other endpoints of toxicity such as EROD activity, CYP1A1 induction, and thymic atrophy (Schmidt *et al.*, 1996; Fernandez-Salguero *et al.*, 1996), each of which occurs to a similar degree in AhR$^{+/+}$ and AhR$^{+/-}$ animals. Notably, AhR$^{+/-}$ mice likewise exhibit intermediate sensitivity to TCDD-induced cleft palate (Mimura *et al.*, 1997). It was also interesting that the degree to which TCDD suppressed the CTL response to P815 cells

varied substantially among the individual heterozygous animals. It is unclear why the variability would be so pronounced in the heterozygous mice, however we can speculate that in a situation where the amount of receptor is limited, random variation in which DRE-containing genes are bound by the activated receptor could result in a wide spectrum of effects in targeted cells.

In conclusion, results from these experiments demonstrate that $AhR^{-/-}$ mice generate normal, productive immune responses to exogenous antigens, and thus that the receptor does not play an obligatory role in formation of a functional immune system. In contrast, we found that the receptor does play an obligatory role in TCDD-induced immune suppression as $AhR^{-/-}$ animals were resistant to suppression of both cell-mediated and humoral immune responses.

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