**AN ABSTRACT OF THE DISSERTATION OF**

David M. Shepherd for the degree of Doctor of Philosophy in Toxicology presented on July 28, 1999. Title: The Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on T Cell Activation.

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Nancy I. Kerkvliet

The immune system has been identified as a very sensitive target for the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD). Exposure to TCDD has been shown to disrupt the generation of both cell-mediated and humoral T cell-dependent immunity in laboratory animals; however, the mechanism remains unknown. In this dissertation, the hypothesis is tested that TCDD exposure alters T cell activation and differentiation either directly or by inhibiting the activation of antigen presenting cells (APC). Previous studies from our laboratory using the P815 tumor allograft model suggest that TCDD inhibited T cell activation by suppressing the induction of the costimulatory molecule CD86 on B220+ and Mac-1+ cells. To address the effects of TCDD on APC, we further characterized the activation of splenic APC in the P815 model and found that TCDD suppressed the induction of the accessory molecules CD86, CD54 and MHC II on APC as well as their production of IL-12. Although it was determined that the induction of these costimulatory molecules following P815 immunization was CD40-independent, their *in vivo* expression could be enhanced by administering an agonistic antibody to CD40 to mice. APC from anti-CD40 treated mice expressed significantly higher levels of these accessory molecules and IL-12, and this enhanced APC activation was largely unaffected by TCDD. However, TCDD-treated mice receiving both P815 and

anti-CD40 were unable to generate T cell-dependent allograft immunity suggesting that suppression of APC activation may not be underlying TCDD immunosuppression. To address the direct effects of TCDD on T cell activation, we adoptively-transferred DO11.10 TCR transgenic T cells into syngeneic recipients and monitored their activation *in vivo* following exposure to antigen. Although treatment of adoptively-transferred mice had no effect on the expansion or activation of the OVA-specific CD4+ T cells, the production of the T cell-derived cytokines IL-2, IFN-γ, IL-4 and IL-10 was suppressed. These data suggest that TCDD may suppress the differentiation of OVA-specific T cells into effector T helper cells which are capable of driving T cell-dependent immune responses.

# The Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on T Cell Activation

by

David M. Shepherd

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Dean of Graduate School

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

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David M. Shepherd, Author

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## CONTRIBUTION OF AUTHORS

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

| | |
|---|---|
| Choi, Jin-Young | Technical support. |
| Dearstyne, Erica A. | Technical support; critical review of the data and manuscript. |
| Hedstrom, Olaf R. | Technical support; critical review of the experimental design, data and manuscript. |
| Kerkvliet, Nancy I. | Critical review of the experimental design, data and manuscripts; financial support. |
| Steppan, Linda B. | Technical support. |

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## A LITTLE LEARNING IS A DANGEROUS THING

(From *Essay on Criticism,* II)

A little learning is a dangerous thing;
Drink deep, or taste not the Pierian spring:
There shallow draughts intoxicate the brain,
And drinking largely sobers us again.
Fired at first sight with what the Muse imparts,
In fearless youth we tempt the height of Arts,
While from the bounded level of our mind,
Short views we take, nor see the lengths behind;
But more advanced, behold with strange surprise
New distant scenes of endless science rise!
So pleased at first the towering Alps we try,
Mount o'er the vales, and seem to tread the sky,
Th' eternal snows appear already past,
And the first clouds and mountains seem to last;
But, those attained, we tremble to survey
The growing labors of the lengthened way,
Th' increasing prospect tires our wandering eyes,
Hills peep o'er hills, and Alps on Alps arise!

ALEXANDER POPE

# The Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on T Cell Activation

## CHAPTER I

## INTRODUCTION

The studies described in this dissertation focus on the mechanisms of TCDD-induced suppression of T cell activation. In this introductory chapter, the goal is to (1) outline the organization and function of the immune system, (2) describe the general properties of 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD), and (3) outline the toxicity associated with TCDD exposure with an emphasis on TCDD-induced immunotoxicity.

## THE IMMUNE SYSTEM

### Overview

The function of the immune system is to protect the body from damage caused by invading microorganisms including bacteria, viruses, fungi and parasites. The generation of immunity relies on the interaction of many different cells and molecules functioning in a coordinated manner to effectively eliminate pathogens. Antigens are any molecules which are recognized by the immune system and induce an immune reaction. Immune responses have traditionally been divided into two classifications depending on whether they are antigen-specific or not. Innate (or natural) immunity is dependent on a variety of effector mechanisms which are neither specific for particular antigens nor improved by repeated encounters with the same antigen. Conversely, acquired (or adaptive) immunity is antigen-specific and is characterized by five basic features which are essential for a functional host defense: (1) specificity; (2) diversity; (3) memory; (4) self-limitation; and (5) self-discrimination (Male, 1986; Abbas et al., 1994; Kuby, 1994). All antigen-specific immune responses are initiated by the recognition of antigen which leads to the activation of

lymphocytes and culminates in the development of effector mechanisms to eliminate the antigen. The specificity of immune responses is mediated by lymphocytes which are the only cells in the body capable of specifically recognizing and distinguishing different antigens (Abbas et al., 1994). Acquired immunity is characterized by two types of effector responses, (1) humoral and (2) cell-mediated (Kuby, 1994). Humoral immunity is mediated by B lymphocytes via the production of antibodies (or immunoglobulins) whereas cell-mediated immunity is mediated through cellular interactions orchestrated by T lymphocytes.

## Cells of the immune system

### B lymphocytes

B cells are the only cells capable of producing antibodies, the serum proteins which mediate humoral immunity (Abbas et al., 1994). In mammals, B cell maturation occurs in the bone marrow. The antigen receptors on B cells are membrane-bound forms of antibodies which possess very high affinity for a specific antigen. After recognition of antigen, B cell activation is initiated and culminates in the development of effector B cells which actively produce and secrete antibodies. In addition, antigen-induced cross-linking of surface immunoglobulins (Ig) on B cells may also activate these lymphocytes to operate as antigen presenting cells, a function which will be discussed later.

Humoral immune responses can be divided into two distinct types depending on their dependency on T cells and thus are named (1) thymus (T)-independent, or (2) T-dependent (Kuby, 1994). T-independent antibody responses develop after recognition of polymeric antigens such as polysaccharides, nucleic acids, and lipids which effectively cross-link antigen receptors on the B cells (Mond et al., 1995). These responses can develop in animals depleted of T cells and usually result in the production of lower affinity

IgM antibodies. The practical significance of T-independent humoral immunity is the generation of antibodies which effectively contribute to the resolution of bacterial and viral infections.

On the other hand, T-dependent humoral immune responses are primarily generated against proteinaceous antigens and require the interaction of B cells with T cells (Parker, 1993). Following recognition of antigen, B cells become activated and begin to proliferate. Then, subsequent interactions with activated T cells drive B cells to undergo differentiation which is characterized by antibody class switching, affinity maturation, and their development into antibody-secreting cells and/or memory cells. T cell-dependent activation of B cells occurs by both cognate and soluble interactions (MacLennan et al., 1997). The primary molecules involved in the cognate activation of B cells by T cells are CD40 and its ligand CD154, respectively (Laman et al., 1996). CD40 is a ~50 kD glycoprotein which is expressed on mature B cells and is upregulated following activation (Noelle et al., 1992a). It is a member of the TNF receptor family which includes TNF-RI (p55), TNF-RII (p75), Fas, CD27, OX-40 and 4-1BB (Stamenkovic et al., 1989; Baker and Reddy, 1996). Its ligand CD154 (CD40L, gp39) is expressed primarily on activated CD4+ T cells (Noelle et al., 1992b). The interaction of CD154 with CD40 occurs in a antigen non-specific and MHC-unrestricted fashion. In addition to B cell activation by physical contact, T cells also provide stimulation via their production of cytokines (Parker, 1993). These soluble mediators augment antibody production by amplifying B cell proliferation and differentiation, and also selectively promoting class switching of immunoglobulins to different isotypes. For example, the cytokine IFN-$\gamma$ been shown to promote antibody class switching to the IgG2a isotype, whereas IL-4 and IL-5 mediate switching to the IgE and IgG1 isotypes, respectively (Stavnezer, 1996). T cell deficiencies result in compromised humoral immune responses which are characterized by reduced antibody production by B cells or the production of only IgM. This is the case for mice which lack T cells by genetic mutation (nu/nu) or antibody-mediated depletion, and also those which possess defective T

cells such as cytokine- and CD154-knockout mice (Renshaw et al., 1994; Schijns et al., 1994; Malanchere et al., 1995; Pasparakis et al., 1996). In humans, defective T cell functions by pathological or genetic alterations also profoundly suppress antibody production as seen in individuals suffering from AIDS or hyper-IgM syndrome (HIGM1) (Notarangelo et al., 1992; Moja et al., 1997).

Ultimately, antibodies secreted by B lymphocytes neutralize antigens, activate the complement system, and opsonize antigens to enhance their clearance from the host by phagocytic cells (Medzhitov and Janeway, 1997). These effector functions effectively link the acquired and innate arms of the immune system to optimize the host defense against pathogenic insult.

**T lymphocytes**

Similar to B cells, T lymphocytes are also derived from the bone marrow. However, immature T cells migrate from the bone marrow to the thymus to undergo maturation. There they develop into two functionally distinct populations which are defined by the expression of the membrane proteins CD4 and CD8 (Abbas et al., 1994). The primary function of CD8+ T cells is to mediate cell-mediated immune responses whereas CD4+ T cells promote both humoral and cell-mediated immunity by contributing to the activation of B cells, and CD8+ T cells and macrophages, respectively. Both subsets of T cells recognize specific antigens by the expression of antigen receptors on their membranes. In contrast to Ig on B cells, T cell receptors (TCR) can not recognize soluble antigens in their native forms but instead respond only to processed antigenic peptide fragments. Antigen-specific T cell activation normally requires the interaction of T cells with antigen-presenting cells (APC) (Janeway and Bottomly, 1994; Jenkins, 1994; Mondino et al., 1996). Antigens may only be presented to the T cells by the APC in the context of major histocompatibility complex (MHC) proteins. Activation of T cells results

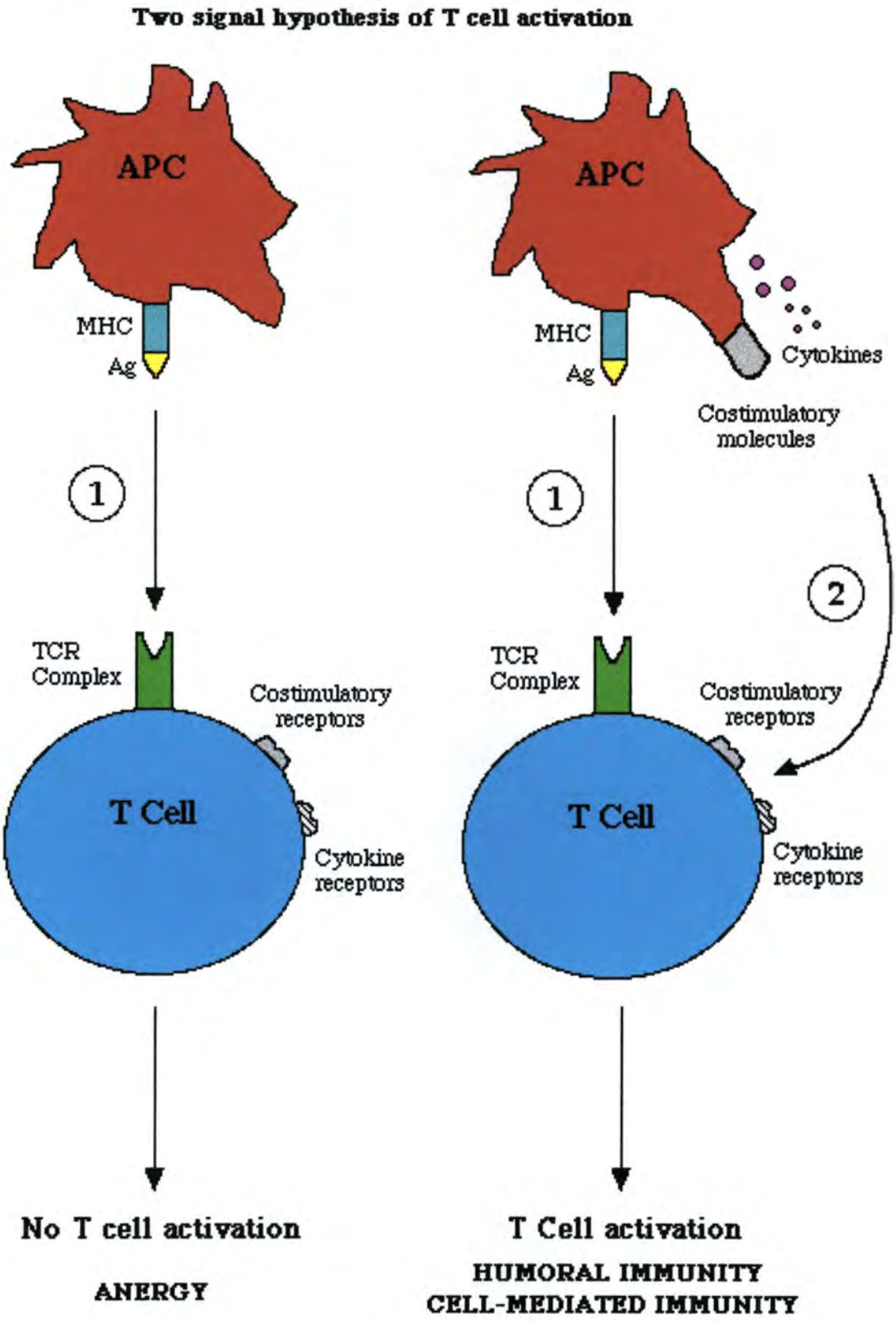
in their clonal expansion and differentiation into effector cells capable of promoting T-dependent immune responses.

The activation requirements for T cells vary depending on their state of differentiation (Croft, 1994). The current paradigm for the activation of naive T lymphocytes is the two-signal hypothesis (Figure I-1). In this model, T cells receiving antigenic stimulation through their TCR complex require some form of costimulation from cognate or soluble interactions with APC. T cells which receive both signals will become activated which is characterized by the production of IL-2 and clonal expansion. These activated T cells are capable of differentiating into effector T cells which can drive adaptive immune responses. On the other hand, T cells which only receive one signal (MHC: antigen) do not become activated but rather anergic and fail to generate T-dependent immunity. The activation requirements for memory T cells are not quite as strict as those of naive cells, but costimulation is required to a lesser degree for optimum responses and for responses to suboptimal antigen concentrations (Dutton et al., 1998). Antigen presenting cells have been shown to express costimulatory molecules on their cell membranes following activation with antigen and inflammatory mediators (Dubey and Croft, 1996; Croft and Dubey, 1997). These accessory proteins on APC then physically interact with membrane-bound receptors on the T cells and act synergistically with TCR-induced signals to amplify T cell activation. The best defined pathway for T cell costimulation involves the ligation of CD28 on the T cell by B7 costimulatory molecules expressed on APC (Lenschow et al., 1996). Ligation of CD28 promotes T cell proliferation and enhances the production of the autocrine growth factor IL-2. Disruption of the B7:CD28 pathway has been shown to significantly inhibit the generation of most T-dependent immune responses. The interaction of other accessory molecules such as CD54:CD11a and CD154:CD40 have also been shown to be important for the activation of naive T cells (Croft and Dubey, 1997). It has been suggested that the interaction of adhesion molecules like CD54 (ICAM-1) on APC and CD11a (LFA-1) on T cells may provide costimulation as well as

**Figure I-1. The two signal hypothesis of T cell activation.**

The activation of T cells requires two signals, ligation of the T cell receptor (TCR) with (1) antigen in the context of major histocompatibility complex (MHC) proteins, and (2) costimulation by costimulatory (or accessory) molecules and/or cytokines. T cells that receive both signals become activated and promote both humoral and cell-mediated immunity. In contrast, T cells that receive only one signal (MHC: antigen) do not become activated and are rendered anergic or unresponsive to antigenic stimulation.

Figure I-1.


Two signal hypothesis of T cell activation
APC
MHC
Ag
1
TCR Complex
Costimulatory receptors
T Cell
Cytokine receptors
No T cell activation
ANERGY
APC
MHC
Ag
Cytokines
Costimulatory molecules
1
2
TCR Complex
Costimulatory receptors
T Cell
Cytokine receptors
T Cell activation
HUMORAL IMMUNITY
CELL-MEDIATED IMMUNITY

increasing the duration and intensity of antigen-specific signal delivered through the TCR. Thus, in addition to the role that the CD154:CD40 pathway plays in the activation of B cells, the interaction of these molecules has been shown to augment T cell activation indirectly by increasing the costimulatory function of APC (Grewal and Flavell, 1998). Similarly, proinflammatory cytokines such as IL-1, TNF and IL-6 have been suggested to be capable of providing effective costimulation of T cells (Doherty, 1995). However, the effectiveness of these cytokines to perform this function has not been firmly established in vivo. Also, it is uncertain if these cytokines directly provide costimulatory signals to the T cells or indirectly via activation of APC.

The differentiation of activated CD4+ and CD8+ cells into T helper and cytotoxic effector cells is primarily mediated by cytokines (Swain, 1999). Furthermore, differentiated T cells are divided into two classifications based on their polarized production of certain cytokines. T cells which secrete IL-2, IFN-γ and TNF are type-1 effector cells, whereas type-2 effector T cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann et al., 1986). Furthermore, CD4+ and CD8+ type-1 effector T cells are referred to as Th1 and Tc1 cells, respectively. Conversely, CD4+ and CD8+ type-2 effectors are called Th2 and Tc2, respectively. Type-1 effector cells are primarily involved in cell-mediated inflammatory reactions, while type-2 effector cells aid in the generation of humoral and allergic responses. The differentiation of both types of effector cells may proceed through an intermediate phenotype expressing both cytokine patterns, or IL-2 exclusively. The presence of IFN-γ, IL-12 and TGF-ß during the initial stimulation of naive T cells results in their differentiation towards the type-1 phenotype, whereas IL-4 induces type-2 development (Sad and Mosmann, 1994). Recently, it has been proposed that differentiation of T cells into either phenotype depends on the expression of specific cytokine receptors (O'Garra, 1998). In this model, T helper cells which express IL-12 receptors (IL-12Rß2) differentiate into type-1 effector cells after encountering bioactive

IL-12. Conversely, T helper cells which express IL-1 receptors would differentiate into type-2 effectors following exposure to IL-1.

While both CD4+ and CD8+ T cells undergo many similar events during their activation and differentiation, the effector functions of each subset differs dramatically. The primary functions attributed to effector CD4+ T helper cells involve the secretion of cytokines, the activation of APC, the activation of B cells to produce antibody, and the activation of macrophages to mediate delayed-type hypersensitivity reactions (Kuby, 1994). Conversely, the effector functions of CD8+ T cells are somewhat more focused. Although activated CD8+ T cells produce significant amounts of cytokines, their primary effector function is as antigen-specific cytotoxic T lymphocytes (CTL) which mediate cell-mediated immunity (Abbas et al., 1994). The primary function of CTL is to kill virally infected cells, tumor cells and allogeneic cells which express foreign MHC class I molecules. They perform this killing function following the acquisition of two effector mechanisms; (1) the generation of specific lytic granules, and (2) the expression of membrane-bound death molecules.

Although T cells have been shown to be important for the generation of humoral immunity, they are essential for the generation of most cell-mediated immune responses. As one might expect, the absence of functional CD8+ T cells results in an inability to mount antigen-specific CTL responses. Interestingly, defective CD4+ T cell function or their depletion also profoundly affects the generation of cell-mediated immunity. This effect is thought to be due to defective APC activation, however, other explanations may exist such as insufficient cytokine production or the lack of cognate activation of phagocytic cells like neutrophils and macrophages. Regardless of the specific mechanism, defects in T cell functions result in compromised immunity to intracellular pathogens and tumor cells.

**Antigen presenting cells**

As previously discussed, T cells recognize antigen only when it has been processed into peptide fragments and presented in the context of MHC proteins. Also, for complete activation of T cells to occur they must receive some form of costimulation. Both of these functions are provided by APC. By definition, APC have been considered to be any cells which can process antigens and express MHC class II gene products (Male, 1986). However, functionally, this definition is amended to describe APC as cells which can provide costimulatory signals for T cells as well as presenting antigens. The activation of APC by inflammatory stimuli has been shown to enhance their costimulatory functions by increasing their production of cytokines and inducing costimulatory molecule expression (Croft and Dubey, 1997). Likewise, ligation of receptors such as CD40 and CD14 have also been demonstrated to transduce activating signals to APC and enhance their costimulatory capacities (Mahnke et al., 1997; Mackey et al., 1998).

The most effective and best characterized APC include dendritic cells, macrophages and B cells (Abbas et al., 1994). Dendritic cells (DC) are commonly referred to as "professional" APC because of their ability to phagocytose antigen and constitutively express costimulatory molecules. DC are competent at presenting antigens to T cells, including naive T cells which have not previously been exposed to antigen (Banchereau and Steinman, 1998). DC are approximately tenfold more stimulatory for naive T cells than activated B cells. Although DC are considered to possess constitutive costimulatory abilities, these functions increase following activation by CD40 ligation or LPS exposure (Kato et al., 1997). These activation stimuli can increase DC expression of accessory molecules, in addition to increasing their production of proinflammatory cytokines including TNF, and interleukins-1, -6, and -8.

Likewise, macrophages are phagocytic cells which participate in many inflammatory reactions (Kaye, 1995). These cells actively phagocytose a variety of infectious organisms such as bacteria and parasites, internalize and process them into

antigenic fragments and present them in association with MHC molecules to T cells. As participants in inflammatory responses, macrophages have been shown to generate and receive inflammatory stimuli which enhances their APC function (Kato et al., 1996). However, unactivated or resting macrophages are poor APC and do not induce activation of naive T cells.

In contrast to DC and macrophages, B cells are extremely efficient at capturing antigen because they express immunoglobulins on their cell surface which act as high-affinity antigen receptors. However, resting B cells have been shown to be very inefficient APC because they possess very little intrinsic costimulatory function (Eynon and Parker, 1992). Therefore, resting B cells which have been exposed to antigen have been shown to be capable of tolerizing antigen-specific T cells *in vivo* (Buhlmann et al., 1995; Parker et al., 1995). On the other hand, stimulation of B cells by inflammatory mediators, CD40 ligation or extensive crosslinking of surface Ig can sufficiently activate these cells to become competent APC with effective costimulatory function (Ranheim and Kipps, 1993; Kennedy et al., 1994; Lenschow et al., 1994). Therefore, unless the antigen recognized by the B cells is inherently stimulatory, these cells will not be induced to express multiple accessory molecules at a high enough level to allow them to produce a T cell response which will not lead to tolerance.

In summary, the role of the immune system is to protect the body by recognizing and destroying disease-causing agents. It is comprised of a complex network of immune cells which work together to perform this function. Disruption of immune processes may increase an individual's susceptibility to disease and even death. It is generally agreed that human health is influenced by the environment and that many diseases are caused by environmental factors. Therefore, it is of great importance to study the potential adverse effects to immune function that result from occupational or inadvertent exposure to environmental chemicals or pollutants. By increasing our understanding of the impact that

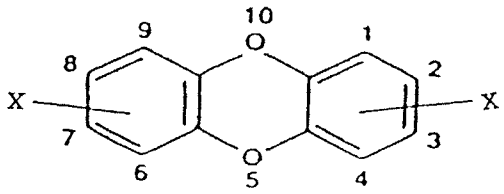
xenobiotics have on the immune system, we can better assess the potential risks to human health following exposure to these chemicals.

## 2,3,7,8-TETRACHLORODIBENZO-*p* -DIOXIN (TCDD)

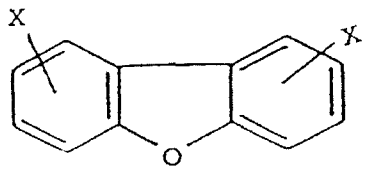
The halogenated aromatic hydrocarbons (HAHs) are a family of structurally related chemicals which includes polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and naphthalenes (Figure I-2). Collectively, these industrial compounds have been widely identified in the environment and are reported to be extremely potent at producing a variety of toxic effects in experimental animals (Schecter, 1994). 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD) is considered the prototypical HAH because it is the most potent of all these chemicals at producing toxicity. As predicted by its physiochemical properties, TCDD is a very stable compound with a half-life estimated to be approximately 10 years in soil (di Domenico et al., 1980). It is also very resistant to breakdown by acids, bases, hydrolysis and heat (Pohjanvirta and Tuomisto, 1994; Webster and Commoner, 1994). It has been suggested that the only significant natural process for TCDD degradation is photolysis. TCDD is highly lipophilic and can readily traverse biological membranes. These chemical properties contribute to the persistence of TCDD in the environment and its bioaccumulation in the food chain with residues being detected in fish, wildlife and vegetation (Startin, 1994). Human exposure to TCDD can occur by dermal exposure or inhalation, however, the most common route of exposure is believed to be through diet. The whole body half life of TCDD in humans is reported to be between 7-14 years (Olson, 1994).

Polychlorinated dibenzo-p-dioxins are not produced intentionally but are instead formed as unwanted byproducts of industrial processes. The five major sources which account for most of the TCDD released into the environment are: (1) incineration of hospital and municipal waste; (2) metals processing; (3) chlorine bleaching of pulp and paper;

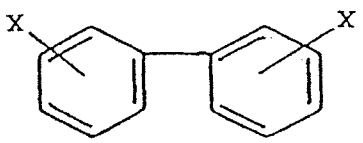
**Figure I-2. Structures of various halogenated aromatic hydrocarbons.**



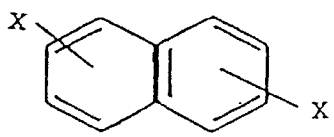

Dibenzodioxins




Dibenzofurans




Biphenyls




Naphthalenes

(4) combustion of home heating products; and (5) the manufacture of chlorine-based chemicals (Safe, 1990). However, dioxins have also entered the environment following other unintentional occurrences. For example, in 1976, approximately 300-2000 grams of TCDD was accidentally released into the surrounding countryside from a chemical plant in Seveso, Italy (Dickson and Buzik, 1993). Likewise, residents of Times Beach, Missouri were negligently exposed to TCDD-contaminated dust and soil when TCDD-contaminated oil was sprayed on roadways as a means of dust control (Tiernan et al., 1985). It has also been proposed in the "Trace Chemistries of Fire Hypothesis" that PCDDs may enter the environment through natural sources (Webster and Commoner, 1994). In this hypothesis, it was suggested that diverse combustion processes such as forest fires and volcanic eruptions generate PCDDs which subsequently contaminate the environment. However, results from the analysis of dated aquatic sediment samples have shown that increased PCDD concentrations correlated with the increased production and use of chlorinated industrial compounds. Therefore, these results indicate that the production of industrial halogenated organic chemicals and their subsequent incineration contribute more to the accumulation of PCDDs in the environment than do natural combustive processes.

## TOXIC EFFECTS OF TCDD

Numerous studies have reported that exposure to TCDD and related HAHs elicit a number of common toxic responses in animals (Mukerjee, 1998). In general, toxicity following exposure to TCDD is dependent on the animal species, strain, age and gender (Neubert, 1997). Interestingly, TCDD has been touted as "the most toxic synthetic chemical known to man" which explains the considerable amount of attention that it has received from the scientific community (Webster and Commoner, 1994). Surprisingly, despite extensive investigation, the underlying mechanisms of TCDD-induced toxicity remain to be determined.

**Laboratory Animals**

TCDD has been observed to cause numerous toxic effects in laboratory animals, including thymic atrophy, wasting syndrome, hepatotoxicity, teratogenic and fetotoxic effects, carcinogenesis, endocrine disruption and immunotoxicity (Safe, 1990; Dickson and Buzik, 1993; Lucier et al., 1993).

Although differences exist in the toxic responses of animals to TCDD, several effects have been reported for all animals following exposure. TCDD is lethal when administered at a sufficient dose (DeVito and Birnbaum, 1994). Unlike most toxicants which induce lethality within hours to days, TCDD-induced death takes weeks to manifest. Associated with this type of death is a wasting syndrome which involves severe weight loss and is accompanied by a dramatic reduction of both muscle and adipose tissue (Seefeld et al., 1984; Max and Silbergeld, 1987). Similarly, nonlethal doses of TCDD cause thymic atrophy in all experimental animals studied. Thymic atrophy is characterized by lymphocyte depletion in the thymic cortex (Staples et al., 1998). Although there has been a fair amount of research done on TCDD-induced thymic atrophy, the exact cellular targets and mechanistic actions have not been resolved. Another hallmark of TCDD exposure is chloracne. Chloracne results from overactive sebaceous glands producing vast excesses of keratin. Although best associated with human exposure, chloracne-like lesions also occur on rhesus monkeys, hairless mice, and on the inner surface of rabbit ears (McConnell and Moore, 1979; Kimbrough, 1984).

Other toxic effects of TCDD exposure are seen in many but not all species examined. For example, hepatoxicity is seen in rats and rabbits following exposure to TCDD. Hyperplasia and hypertrophy of parenchymal cells producing hepatomegaly are common observations in TCDD-exposed animals (DeVito and Birnbaum, 1994). Also, in mice, rats and monkeys, TCDD reduces fertility, litter size, and uterine weights (Kociba et al., 1976; Barsotti et al., 1979; Umbreit et al., 1987). It also has been shown to have antiestrogenic effects by decreasing the concentration of circulating estrogens, the levels of

estrogen receptors, and by disrupting estrogen receptor signal pathways (DeVito and Birnbaum, 1994; Safe et al., 1998). Alterations in testicular morphology have been reported in rhesus monkeys, rats, mice, guinea pigs and chickens following high-dose TCDD exposure (McConnell and Moore, 1979). In addition, TCDD has been shown to be a teratogen in several species as well as a developmental toxicant in rodents. Low doses of TCDD which do not produce any maternal or fetal toxicity may produce cleft palate and hydronephrosis in mice (Couture et al., 1990). Finally, TCDD has been shown to be a potent animal carcinogen at higher doses. Although TCDD is not directly mutagenic, it has been shown to be a potent tumor promoter in several different species of experimental laboratory animals (Wassom et al., 1977; DeVito and Birnbaum, 1994).

## Mechanisms of Action

The unusual potency of TCDD in eliciting its toxic effects suggested the possible existence of a specific receptor which recognized TCDD. Early studies by Poland et al. (1982) which utilized radiolabeled TCDD as a ligand demonstrated that a cytosolic fraction of mouse hepatocytes contained a protein that bound TCDD with high affinity (i.e. in the nanomolar range which is consistent with its biological potency in vivo). In addition, subsequent reports from Nebert and colleagues found that hepatic aryl hydrocarbon hydroxylase [AHH, now known as cytochrome P450 1A1 (CYP1A1)] activity could be highly induced in some strains of inbred mice (Nebert, 1989). Further studies showed that CYP1A1 activity was linked to the expression of the TCDD receptor which was mapped to a gene now referred to as the aromatic hydrocarbon (Ah) locus. Therefore, this protein was designated the Ah receptor (AhR) because it binds aromatic hydrocarbons and mediates their toxicity. TCDD is considered to be the prototypical AhR ligand because it displays the highest affinity for the AhR of all the HAHs. The AhR is considered to be an orphan receptor because its endogenous ligand is unknown. However, several possible endogenous ligands for the AhR have been postulated such as dietary lipophilic compounds

from plants, endogenous lipophilic compounds such as tryptophan and arachidonic acid metabolites (i.e tryptamine, indole acetic acid, and lipoxin A4), and bilirubin and biliverdin (Heath-Pagliuso et al., 1998; Phelan et al., 1998; Schaldach et al., 1999).

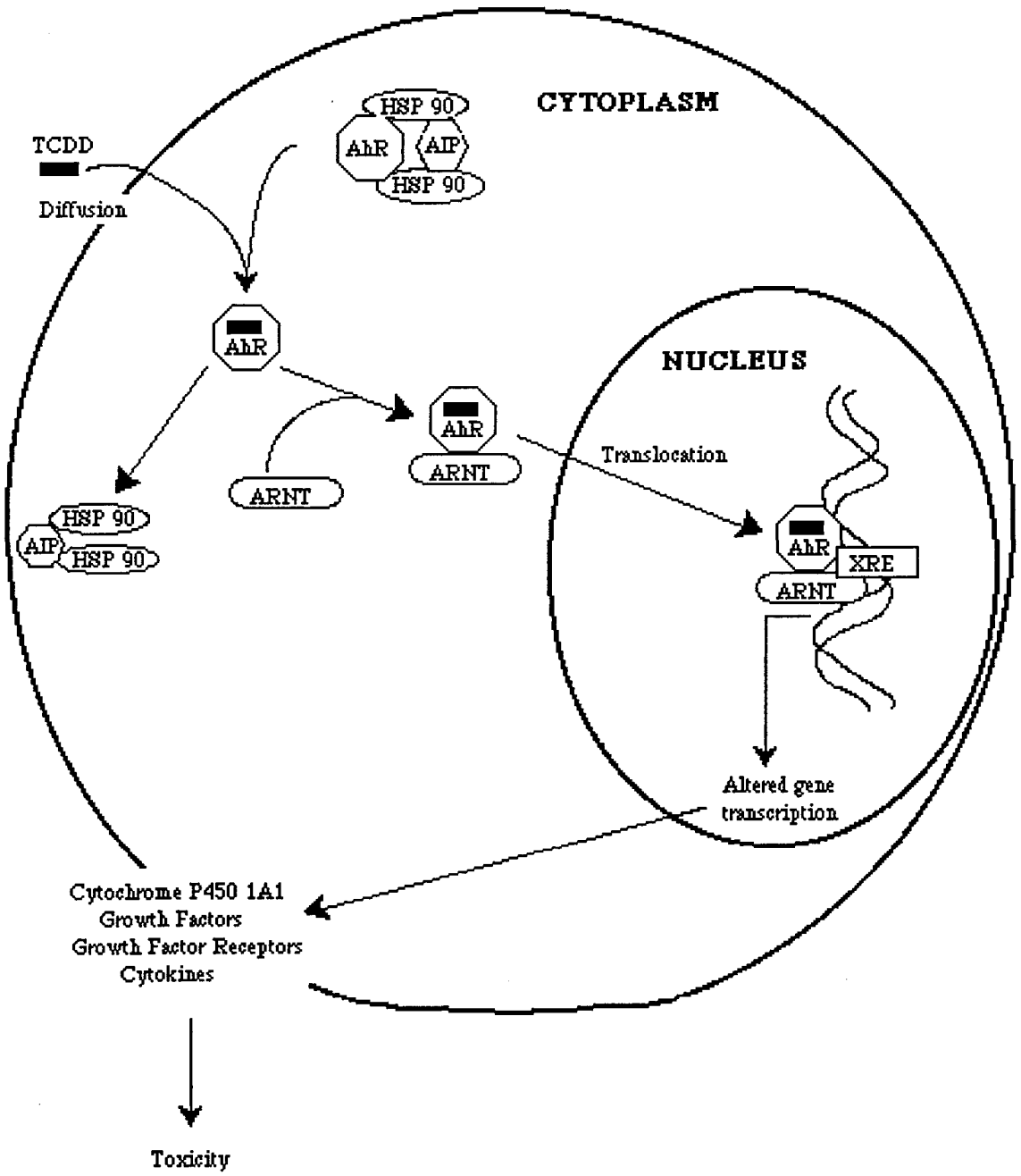
Inbred strains of mice differ quantitatively in their responsiveness to TCDD and other aromatic hydrocarbons and these differences have been attributed to a polymorphism in the Ah locus. For example, TCDD elicits its effects at about 10-fold lower concentrations in the more responsive mouse strains (such as C57Bl/6 and Balb/c which are $Ah^{b-1}$ and $Ah^{b-2}$, respectively) when compared to the less responsive strains (typified by DBA/2 which is $Ah^{d}$) (Poland and Glover, 1990). Numerous biological responses to TCDD exhibit a segregation pattern identical to that for binding of TCDD to the AhR. Thus, the genetic locus that governs the receptor polymorphism also governs the biological responses to TCDD. In fact, it has been demonstrated that most, if not all, toxicity induced by TCDD is mediated through the AhR (Safe, 1990; Schecter, 1994).

Cloning of the AhR has revealed important information regarding its structure and function (Whitlock, 1993). The AhR was found to contain a basic helix-loop-helix (bHLH) motif which has been previously characterized in other transcription factors that bind specific DNA sequences. According to recent reports, the AhR exists in an unliganded form as a multimeric protein complex of about 280 kD which normally resides in the cytoplasm (Hankinson, 1995; Denison and Heath-Pagliuso, 1998). As shown in Figure I-3, the AhR complex consists of the AhR, two 90 kD heat shock proteins (HSP), and a 50 kD AhR interacting protein (AIP) (Ma and Whitlock, 1997). Ligand binding triggers the dissociation of the AhR complex and translocation of the AhR from the cytoplasm to the nucleus. Upon entering the nucleus, the ligand-AhR pair associates with the AhR nuclear translocator protein (ARNT) to form a "transformed" heterodimeric complex. This complex binds the xenobiotic response element (XRE, also known as the dioxin response element or DRE) in responsive genes and alters transcription. The core consensus DNA sequence within the XRE has been defined as 5'-GCGTG-3'. Five

**Figure I-3. The Ah receptor model.**

A general schematic of the Ah receptor (AhR) model is shown. The AhR ligand (TCDD) diffuses into the cell where it binds to the AhR. Ligand binding causes release of heat shock proteins (HSP) and AhR interacting protein (AIP) from the AhR. The ligand-AhR complex then associates with the AhR nuclear translocator protein (ARNT). This "transformed" heterodimeric complex then enters the nucleus where it binds specific DNA sequences known as xenobiotic response elements (XREs). AhR complex binding to DNA then alters transcription of genes such as cytochrome P450 1A1 (CYP 1A1). Alteration of XRE-containing genes that encode for growth factors, growth factor receptors and cytokines may mediate the toxicity of TCDD and other xenobiotic AhR ligands.

**Figure I-3.**


CYTOPLASM
HSP 90
AhR
AIP
HSP 90
TCDD
Diffusion
AhR
NUCLEUS
AhR
ARNT
ARNT
Translocation
HSP 90
AIP
HSP 90
AhR
XRE
ARNT
Altered gene transcription
Cytochrome P450 1A1
Growth Factors
Growth Factor Receptors
Cytokines
Toxicity

functional XRE have been identified in the 5' promoter region of the CYP1A1 gene which have been shown to bind the TCDD-AhR-ARNT complex and increase the rate of transcription of that gene (Hankinson, 1995). In addition, this ligand-receptor complex has been shown to modulate the transcription of other genes such as CYP1A2 and glutathione-S-transferase which contribute to the metabolism of xenobiotics. Transcriptional activation or repression of other genes which regulate homeostasis is hypothesized to underlie the tissue- and cell-specific toxicity of TCDD.

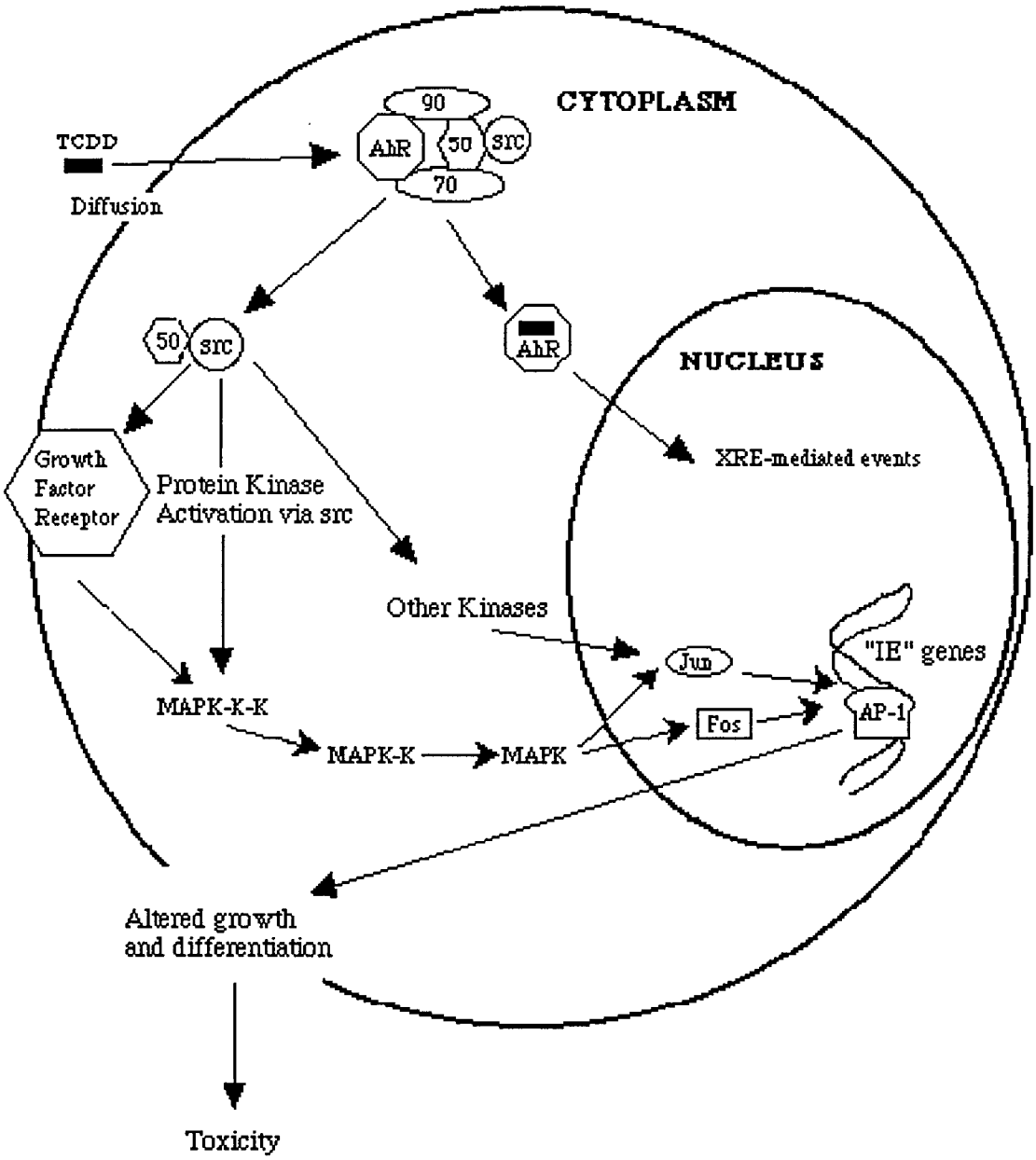
In addition, it has also been postulated that non-XRE events occur which mediate TCDD toxicity by activating a protein phosphorylation pathway, as shown in Figure I-4 (Matsumura, 1994). In this hypothetical scenario, AhR ligand (TCDD) passively diffuses into the cell and binds to the AhR causing dissociation of the AhR complex which consists of the AhR, HSP 90, 70, 50, and the protein kinase c-Src (Enan and Matsumura, 1996). Following dissociation, the ligand-AhR translocates from the cytoplasm to the nucleus, dimerizes with ARNT and subsequently interacts with XRE as previously described. At the same time, the departure of the ligand-AhR from the HSP complex activates the c-Src kinase. The c-Src kinase then activates the c-ras protein and MAP-kinase (MAPK) pathway leading to the activation of nuclear transcription factors such as AP-1 and inducing functional changes via primary response or "immediate early" (IE) genes. Although this pathway remains unproven, it does reconcile some unexplained aspects of TCDD-induced toxicity which are inconsistent with effects mediated exclusively through the XRE. For example, TCDD has been shown to induce certain biochemical changes in cells such as elevation of c-ras GTP-binding activity and an increase in jun-D and c-fos mRNA within 15 minutes which is unlikely to occur exclusively through AhR complex-mediated interactions with XRE (Puga et al., 1992).

Recently, AhR knockout mice were generated and utilized in studies to distinguish AhR-mediated toxicities from those resulting from alternative pathways (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). These studies found that $AhR^{-/-}$ mice were

**Figure I-4. XRE-independent mechanism of TCDD-induced toxicity.**

A diagram representing the hypothetical XRE-independent protein phosphorylation pathway is shown. The AhR ligand (TCDD) diffuses into the cells and binds to the AhR. After ligand binding, the AhR dissociates from its complex consisting of heat shock proteins 90, 70 and 50 and the protein kinase c-src. Following dissociation, the c-src kinase becomes activated and, in turn, activates other intracellular kinases such as c-ras and the mitogen activated protein kinase (MAPK) cascade. These activated kinases eventually phosphorylate nuclear transcription factors such as AP-1 and activate "immediate early" (IE) genes that control cellular growth and differentiation.

**Figure I-4.**


CYTOPLASM
TCDD
Diffusion
90
AhR
50
src
70
50
src
AhR
NUCLEUS
XRE-mediated events
Growth
Factor
Receptor
Protein Kinase
Activation via src
Other Kinases
Jun
"IE" genes
MAPK-K-K
Fos
AP-1
MAPK-K
MAPK
Altered growth
and differentiation
Toxicity

relatively unaffected by doses of TCDD (2000 μg/kg) which were 10-fold higher than that found to induce severe toxic and pathologic effects in AhR$^{+/+}$ littermates (Fernandez-Salguero et al., 1996). Analyses of liver, thymus, heart, kidney, pancreas, spleen, lymph nodes and uterus revealed no significant TCDD-induced lesions in AhR-deficient mice. These results suggested that pathological changes induced by TCDD were mediated entirely by the AhR.

## Humans

Although there exists limited information relating to the function of the AhR in humans, a number of studies have examined the toxic effects of TCDD following human exposure (Schecter, 1994; Neubert, 1997; Mukerjee, 1998). However, health-effect data obtained from humans in occupational settings are typically based on exposure to chemical mixtures contaminated with TCDD. Human exposure to TCDD has been associated with increased risk of severe skin lesions such as chloracne and hyperpigmentation, altered liver function and lipid metabolism, general weakness associated with drastic weight loss, changes in activities of various liver enzymes, depression of the immune system, and endocrine- and nervous-system abnormalities. Populations occupationally or accidentally exposed to chemicals contaminated with TCDD have also been reported to incur increased incidences of soft-tissue sarcoma and non-Hodgkin's lymphoma.

Epidemiological data from industrial workers exposed to TCDD indicates an overall increase in cancer mortality (Fingerhut et al., 1991). Similarly, studies of the general population exposed to TCDD following the Seveso disaster link exposure to multi-site tumor formation (Mocarelli et al., 1991). Recently, follow-up studies were conducted on human cohorts involved in this accidental TCDD exposure to investigate the occurrence of diseases other than cancer. It was found that the population most heavily exposed to TCDD exhibited increased incidences of chronic ischemic heart disease, chronic obstructive pulmonary disease and diabetes (Pesatori et al., 1998). However, it was suggested that

both TCDD exposure and stress associated with the accident might be responsible for the increased risk of these adverse health effects. In a study conducted by the CDC, Vietnam veterans which had been exposed to TCDD via contact with the pesticide Agent Orange were examined for toxic effects. The results of this study indicated that exposure to Agent Orange did not increase the risk of developing a variety of cancers (DeStefano, 1995; Kramarova et al., 1998). In contrast, reduced testosterone levels correlated with increased serum TCDD levels in men who handled Agent Orange in Vietnam suggesting that TCDD was potentially disrupting endocrine function in these individuals (Henriksen et al., 1996). Also, following the consumption of rice oil contaminated with mixtures of HAHs in the Yusho and Yu-cheng incidents, increased incidents of dermatological and neurological abnormalities were found in adults and infants (Schecter, 1994). Subsequent evaluation of these exposed populations suggested that exposure to high concentrations of HAHs may have long-term consequences on the health of children. Thus, although chloracne remains the most likely result of human exposure to TCDD, clearly the potential exists for serious health effects to occur if exposed to high enough levels or if chronic exposure leads to elevated body burdens of TCDD.

## Immunotoxicity

### Laboratory animals

The immune system has been identified as a sensitive target for the toxic effects of TCDD. In mice, acquired immunity has been shown to be especially sensitive to TCDD exposure, with both humoral and cell-mediated immune responses susceptible to suppression (Vos and Luster, 1989; Kerkvliet, 1995). Moreover, susceptibility to TCDD-induced immune suppression segregates with the AhR. The importance of the AhR in mediating the immunotoxic effects of TCDD has recently been elucidated in studies which utilized mice which were either congenic or deficient for the AhR. Studies by Kerkvliet

and colleagues demonstrated a reduced sensitivity for TCDD-induced suppression of both humoral and cell-mediated immune responses in C57Bl/6 mice congenic at the Ah locus to express the low-affinity AhR phenotype (Kerkvliet et al., 1990a). Similarly, AhR-deficient mice were totally refractory to the immunosuppressive effects of TCDD proving the importance of this receptor in mediating toxicity (Kerkvliet, 1999).

Since the ultimate function of the immune system is to protect an organism from infectious and neoplastic diseases, it is of great importance that TCDD has been shown to increase the susceptibility of animals to bacterial, viral, parasitic, and neoplastic diseases (Kerkvliet, 1998). It has been reported that exposure to TCDD decreases the host resistance of laboratory animals to bacterial infections. Several studies have shown an increased susceptibility of rodents to bacterial infections by *Salmonella*, a gram-negative bacterium (Hinsdill et al., 1980). Likewise, increased mortality was reported in TCDD-treated mice exposed to the gram-positive bacterium, *Streptococcus pneumoniae* (White et al., 1986). Also, under certain experimental conditions, TCDD has been shown to increase susceptibility to infection by *Listeria monocytogenes* (Hinsdill et al., 1980).

Additionally, exposure of laboratory animals to TCDD has been shown to decrease resistance to viral and parasitic infections, as well as neoplastic disease. Studies by House et al. (1990) and Burleson et al. (1996) have reported increased mortality of TCDD-treated mice which had been challenged with influenza virus. Likewise, TCDD exposure resulted in an increased mortality of mice to *herpes simplex type II* virus but not *herpesvirus suis* infection (Thigpen et al., 1975; Clark et al., 1981). Tucker et al. (1986) observed a TCDD-induced increase in the magnitude and duration of peak parasitic infection in mice by *Plasmodium yoeli*, a nonlethal strain of malaria. Finally, mice which had been pre- and post-natally exposed to low levels of TCDD exhibited an increased growth of transplanted tumors when compared to control-treated mice (Luster et al., 1980). Although TCDD exposure has been shown to reduce host resistance in several different experimental

models, the specific immunologic functions targeted by TCDD in these models remain to be fully defined.

In contrast to the suppressive effects on host resistance, TCDD exposure has been shown to enhance inflammatory responses in several animal models. TCDD has been shown to produce neutrophilia in the SRBC model which may have resulted from an enhanced production of inflammatory mediators such as TNF and IL-1 (Moos et al., 1994). Furthermore, increased inflammatory mediator production may underlie the enhanced rat paw edema response to dextran and carrageenan in rats treated with TCDD (Katz et al., 1984). In other studies, an augmented inflammatory response was observed in the liver and skin following TCDD exposure which was characterized by the accumulation of activated macrophages and neutrophils (Weissberg and Zinkl, 1973; Puhvel and Sakamoto, 1988). Although recent studies have defined a beneficial role for inflammation in the initiation of acquired immune responses by providing "danger signals", paradoxically, TCDD-induced hyperinflammation is associated with suppressed adaptive immunity. Moreover, although often postulated, no direct evidence has been reported to establish a direct cause-and-effect relationship between enhanced inflammation following TCDD exposure and suppression of antigen-specific immunity (Kerkvliet, 1998).

One of the hallmarks of TCDD exposure in all species tested is thymic involution. Although there does not exist a direct relationship of TCDD-induced thymic atrophy causing peripheral immune suppression in the adult animal, there is supporting evidence that it causes damage to the developing immune system of neonates (Kerkvliet and Burleson, 1994). For example, rodents which were exposed to TCDD during the pre/neonatal period are more sensitive to immune suppression when compared to exposed adults (Luster et al., 1980). Likewise, TCDD has also been shown to alter *in vivo* fetal thymocyte differentiation in developing rodents (Blaylock et al., 1992). It has been postulated that thymic involution is due to increased apoptosis of thymocytes following exposure to TCDD, and recently, TCDD was demonstrated to mediate these effects via

modulation of the thymic cells which were derived from the hemopoietic compartment (Staples et al., 1998b). Therefore, the age at which an animal is exposed to TCDD can be a critical factor affecting the impact that thymic -related changes will have on the generation of immunity.

TCDD exposure has been shown to disrupt the generation of T cell-dependent immunity in laboratory animals. Chronic as well as acute exposure to TCDD has been reported to suppress the CTL response in mice responding to allogeneic tumor cells (Clark et al., 1983; Kerkvliet et al., 1990b). In one study by Kerkvliet et al. (1996), suppression of the alloantigen-specific CTL response correlated with decreased CD8+ T cell activation which was characterized by reduced cytokine production and lack of expansion of effector CTL. Suppression of delayed-type hypersensitivity (DTH) responses has been demonstrated in guinea pigs, rabbits and mice following chronic exposure to low doses of TCDD (40 ng/kg of body weight/week) (Kerkvliet, 1998). Likewise, humoral immunity generated against the T-dependent antigen sheep red blood cells (SRBC) has been shown to be consistently suppressed in mice following exposure to <1μg/kg TCDD (Hinsdill et al., 1980; Vecchi et al., 1980; Clark et al., 1981; Holsapple et al., 1986b; Kerkvliet et al., 1990b). Another study compared the sensitivity of antigens which generate antibody production but differ on their dependency for T cell help. Following the immunization of 1,2,3,4,6,8-HpCDD-treated mice with either SRBC, or the T-independent antigens DNP-Ficoll and TNP-LPS, $ID_{50}$ (the dose of toxicant which inhibits 50% of the maximal response) values were found to be 53, 127, 516 μg/kg, respectively (Kerkvliet and Burleson, 1994). Although the doses needed to suppress the SRBC response with HpCDD are greater than TCDD, these results indicate that the exquisite sensitivity of the antibody response to PCDD exposure would hinge on the dependency on T cells. However, relatively few reports exist which have examined the effects of TCDD exposure on effector functions associated with CD4+ T helper cells. In one report by Lundberg et al. (1992), the proliferation of OVA-specific T cells from TCDD-treated mice was shown to be

suppressed following *in vitro* restimulation with antigen. Another report by Prell et al. (1995) showed no TCDD effects on the proliferation of CD4+ T cells following *in vivo* activation with the polyclonal activator anti-CD3. However, Tomar and Kervliet (1991) reported that exposure of SRBC-primed mice to a dose of 5 μg/kg suppressed the *in vivo* response of T helper cells to TNP-conjugated SRBC. Therefore, at this time, it is unclear what effects TCDD is having on CD4+ T helper cell activation.

In contrast to the immunotoxic effects of TCDD observed in animal models, TCDD does not consistently produce toxic effects on *in vitro* generated immune functions. While some TCDD effects have been reported from *in vitro* experiments, often these results are incongruous with *in vivo* generated data. For example, several studies have reported suppression of the antibody response to SRBC generated *in vitro* when TCDD was directly added to the cultures, but these effects were not dependent on the AhR (Holsapple et al., 1986a; Tucker et al., 1986). In addition, the concentrations of TCDD needed to suppress immune function have been reported to be much higher for *in vitro* responses than for those generated in the animal. However, some laboratories have demonstrated direct effects of TCDD on B cell function. Karras et al. (1996) reported that *in vitro* exposure of resting B lymphocytes to TCDD induced an increase in intracellular $Ca^{2+}$ concentrations. The authors suggested that inappropriate elevation of $Ca^{2+}$ was responsible for suppressing surface Ig- but not CD40-mediated antibody production. Also, Luster et al. (1979) observed that while B cell viability and antigen-induced viability were unaltered, B cell differentiation and antibody secretion were suppressed following *in vitro* exposure to TCDD. In contrast, T cell functions appear to be unaffected by *in vitro* TCDD exposure. Interestingly, a recent study by Lawrence et al. (1996) found that although T cells expressed both AhR and ARNT, this complex was unable to bind the consensus XRE following exposure to TCDD. These results suggest that T cells may not be directly affected by TCDD, however, this conclusion remains to be proven.

**Humans**

In contrast to the wide variety of immunosuppressive of TCDD effects documented in experimental animals, the impact of TCDD on the human immune system remains controversial. Several studies have reported adverse health effects in humans following occupational or accidental exposure to TCDD which may be attributable to decreased immune function. For example, Tonn et al. (1996) examined the long term effects of TCDD exposure on German industrial workers who were exposed to high doses of TCDD for several years. In this study, they found that TCDD body burdens in exposed individuals were at least 10 times higher (between 43 and 874 pg/g blood fat) than in the control population even 20 years after the last reported exposure. TCDD-exposed individuals showed no significant differences in the expression of immune surface markers of B or T lymphocytes or their mitogen-induced proliferation when compared to controls. In contrast, lymphocytes from TCDD-exposed subjects generated reduced allogeneic and IL-2-boosted proliferative responses following *in vitro* stimulation. The authors concluded that TCDD had a long-term immunosuppressive effect on T helper cell function which was mediated by reduced functionality of cells rather than by a reduction in absolute numbers in the peripheral blood. Another study by Webb et al. (1989) reported on the immunologic effects of persons from Missouri who were exposed to TCDD in occupational, recreational, and residential environments. The adipose levels of TCDD in these people were documented to be between <20-750 parts per trillion (ppt ). Increased exposure to TCDD correlated with an increased percentage of CD8+ and T11+ but not CD4+ T cells. No significant effects of TCDD exposure were measured in the proliferative responses to Con A, PHA, PWM, or tetanus toxoid, or in the generation of CTL activity. Although serum IgA levels were found to be increased in TCDD-exposed individuals, no effect was observed on IgG levels. Moreover, no adverse clinical disease was associated with TCDD exposure in these subjects.

Following the explosion at the herbicide plant in Seveso, Italy, studies were conducted to evaluate immunotoxic parameters from children who were exposed to TCDD. In the initial evaluation, no significant TCDD effects were observed on serum levels of Ig or complement, or lymphoproliferative responses to mitogens (Mocarelli et al., 1986). In another study which evaluated a different cohort of TCDD-exposed children, researchers found significantly increased levels of serum complement (Tognoni and Bonaccorsi, 1982). In addition, these subjects also had elevated numbers of peripheral blood lymphocytes and increased lymphoproliferative responses, although again, no adverse clinical effects were correlated with TCDD exposure.

In contrast, in a study conducted by the Centers for Disease Control (CDC), Vietnam veterans who were exposed to TCDD through the use of the herbicide Agent Orange did not exhibit significant differences in levels of lymphocyte subsets or serum Ig when compared to non-exposed soldiers (CDC, 1988). In a separate study by Wolfe et al. (1990), U.S. Air Force veterans who were exposed to TCDD displayed increased levels of serum IgA while other immunological parameters were unaffected.

Recently, several laboratories have investigated the toxic effects of TCDD on *in vitro* generated immune responses of human leukocytes. Neubert et al. (1991) have reported a direct effect of TCDD on decreasing the percentages and numbers of both CD4+ T cells and B cells at concentrations of $10^{-12}$ and $10^{-14}$ M TCDD, respectively. These decreases occurred in lymphocyte cultures following activation with PWM from which the authors concluded that lymphoproliferation in these particular subsets was directly affected by TCDD. In contrast, a subsequent study by Lang et al. (1994) found no significant effects of TCDD on the *in vitro* activation of human lymphocytes. In this study, lymphocytes from healthy individuals were treated with $10^{-7}$-$10^{-14}$ M TCDD in the absence or presence of stimulation with PWM or anti-CD3 mAb (OKT3). Although significant CYP1A1 activity could be detected in these samples, neither alteration in surface marker expression nor suppression of lymphocyte proliferation could be demonstrated in mitogen-

activated cells following TCDD exposure. The authors concluded from these results that the induction of CYP1A1 enzyme activity in human peripheral blood lymphocytes (PBL) does not directly correlate with immunotoxic effects. In two separate studies by Wood and colleagues, TCDD was demonstrated to have differential effects on the activation of human lymphocytes by mitogens. In the first report, $3x10^{-8}$-$3x10^{-10}$ M TCDD had no effect on the superantigen-induced proliferation of both T and B cells, but did significantly suppress the production of IgM antibodies (Wood et al., 1992). In the other study, no significant effect was observed on either PWM-induced proliferation or antibody production in human tonsillar lymphocytes treated with $3x10^{-8}$ M TCDD (Wood and Holsapple, 1993).

In summary, the data relating to the potential effects of TCDD on the human immune system are inconsistent and often conflicting. The basis for the lack of consistent effects may depend on several factors such as insensitive immunological assays (i.e. mitogen responsiveness), lack of accurate exposure documentation, and genetic variations in outbred human populations. Therefore, future research should strive to establish sensitive biomarkers in humans which could be used to assess potential immunotoxic effects following the exposure of individuals to TCDD. Continued research using experimental animals will help identify and validate potential biomarkers of TCDD exposure on the immune system while also helping to elucidate possible mechanisms of action.

## HYPOTHESIS

The hypothesis tested in this dissertation is that TCDD-induced immunosuppression is mediated by alterations in T cell activation involving either the T cell or the antigen presenting cell. To address this hypothesis, two different approaches were undertaken. In Chapter 2, we assessed the effects of TCDD on the activation of antigen-presenting cells. We extended previous observations from our laboratory which demonstrated that exposure to TCDD suppressed the induction of costimulatory molecules on splenic APC following

immunization with alloantigen. To perform these studies, we utilized the P815 tumor allograft model which measures the activation of CD8+ T cells from precursor cells into CTL effector cells possessing alloantigen-specific lytic activity. Since TCDD was shown to suppress APC activation during this immune response, we evaluated if this suppression could be overcome by providing a potent activation stimulus for the APC. By administering agonistic antibodies to murine CD40, we could induce detectable activation profiles on the APC and determine what effects TCDD might be having on this process. Furthermore, in Chapter 3, we investigated the role of the CD154:CD40 pathway in the generation of allograft immunity. Studies were performed to determine what role CD40 ligation plays in the generation of the allogeneic response to P815 tumor cells. The activation of APC and CD8+ T cells was examined in mice which were deficient for CD154.

In Chapter 4, studies were performed to evaluate the effects of TCDD exposure on the *in vivo* activation of naive antigen-specific CD4+ T cells. To permit these investigations, we utilized the DO11.10 adoptive transfer model to allow us to monitor the fate of T helper cells from DO11.10 mice which express a transgenic TCR that specifically recognizes the 323-339 peptide of chicken ovalbumin (OVA) in the context of I-$A^d$ class II MHC. After adoptively-transferring a small number of DO11.10 T cells into syngeneic recipient mice, we measured the activation of these OVA-specific T cells following immunization with antigen. More importantly, for the first time, we were able to directly measure the effects of TCDD exposure on the *in vivo* activation of antigen-specific CD4+ T cells.

Finally, the results presented in this thesis are summarized in Chapter 5. The implications of these investigations are discussed and future areas of study will be proposed.

# CHAPTER II

## ANTI-CD40 INDUCED ACTIVATION OF ANTIGEN PRESENTING CELLS FAILS TO OVERCOME TCDD-INDUCED SUPPRESSION OF ALLOGRAFT IMMUNITY

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## ABSTRACT

We have previously demonstrated that 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD) suppressed the induction of the costimulatory molecule CD86 (B7-2) on B220+ and Mac-1+ spleen cells in the P815 allograft model. In this report, we extend that observation by describing TCDD modulation of CD86, CD54, and major histocompatibility complex (MHC) class II expression on B220+, Mac-1+, and CD11c+ splenic antigen presenting cells (APC). We show secretion of interleukin-12 (IL-12) from spleen cells was significantly suppressed from P815-immunized mice exposed to TCDD, presumably via suppressed transcription of the p40 subunit mRNA. From these data, we hypothesized that TCDD suppresses the activation of APC by inhibiting the induction of costimulatory functions. To test this hypothesis, we injected vehicle- and TCDD-treated mice with an agonistic antibody to murine CD40 to determine if exogenous stimulation could influence the *in vivo* activation of APC. The administration of anti-CD40 increased the expression of CD86, CD54, and MHC II on splenic APC, and highly elevated the production of interleukin-12. Treating mice with TCDD had minimal effects on the anti-CD40 induced expression of accessory molecules on splenic APC. TCDD exposure had no effect on anti-CD40 induced IL-12 in the plasma but suppressed its production from cultured spleen cells. Surprisingly, although stimulation via CD40 increased the activation of APC, allograft effector functions were not restored in TCDD-treated mice, perhaps due to persistent defects in antigen processing and presentation, cytokine production, or other uncharacterized functions of APC.


## INTRODUCTION

The activation of naive T lymphocytes is dependent on two distinct signals, ligation of the T cell receptor (TCR) by specific antigen complexed with MHC proteins and costimulation delivered via interactions with antigen presenting cells (APC) such as dendritic cells (DC), macrophages, and activated B cells(Janeway and Bottomly, 1994;

Jenkins, 1994). The activation of APC by "danger signals" such as inflammatory cytokines and adjuvants enhances their costimulatory functions by increasing their expression of accessory molecules and production of cytokines(Matzinger, 1994). Accessory molecules expressed on APC including CD80 (B7-1), CD86 (B7-2), CD54 (ICAM-1), CD40, MHC, etc. have been shown to provide costimulatory signals for T cells(Croft, 1994; Cai et al., 1998). Likewise, many cytokines produced by activated APC such as TNF and IL-1 function to augment T cell costimulation(O'Garra, 1998). Cytokines have also been shown to contribute to the ultimate differentiation of T lymphocytes into effector cells(Swain, 1999). For example, the differentiation of Th1 effector cells is thought to be driven primarily by IL-12 which is produced by macrophages and dendritic cells. On the other hand, defective costimulation has been shown to profoundly affect the activation of T cells in several experimental systems, leaving them tolerized and unable to respond appropriately to antigen(McAdam et al., 1998).

Recently, the interaction of CD154 with CD40 has been demonstrated to play an important role in the generation of cell-mediated immunity (Grewal and Flavell, 1998; Mackey et al., 1998). Ligation of CD40, which is expressed on APC, by its ligand CD154 (CD40 ligand, gp39), which is expressed transiently on activated T cells, results in enhanced activation of APC. This activation has been characterized by the increased expression of accessory molecules on APC as well as enhanced production and secretion of IL-12 from these cells (Ranheim and Kipps, 1993; Roy et al., 1993; Cella et al., 1996; Kato et al., 1996; Koch et al., 1996). Thus, the CD154:CD40 pathway contributes to the APC costimulation of T cells by inducing both cognate (accessory molecules) and soluble (cytokine) interactions. Incomplete activation of naive T cells following insufficient TCR engagement, costimulation, or differentiation leads to antigen-specific T cell unresponsiveness or anergy (Schwartz, 1990; Guerder et al., 1994). Therefore, proper T cell activation by activated APC is essential for the generation of effective T cell-mediated

immunity and disruption of this process may compromise the capacity of an organism to respond to pathogenic insult.

The immune system has been identified as a sensitive target of TCDD (Vos and Luster, 1989; Kerkvliet and Burleson, 1994). Moreover, T cell functions have been shown to be disrupted in laboratory animals following exposure to TCDD (Kerkvliet, 1998). Although immune suppression by TCDD has been demonstrated to be dependent on the Ah receptor, the specific mechanism of immune suppression remains unknown. Our laboratory has recently reported that TCDD suppression of *in vivo* T cell-mediated functions may not be mediated through direct effects on T lymphocytes, but rather through suppression of APC activation (Lawrence et al., 1996; Prell and Kerkvliet, 1997). Specifically, TCDD suppressed the expression of CD86 on B220+ and Mac-1+ spleen cells in the P815 allograft model, and ultimately inhibited the generation of allograft effector responses. Injection of TCDD-treated mice with B7-transfected P815 cells generated a CTL but not alloantibody response demonstrating that CD8+ T cells were capable of responding to alloantigen if provided sufficient costimulation. Collectively, this information prompted us to further analyze the effects of TCDD on APC activation.

In this report, we show that TCDD treatment of mice reduced the expression of the accessory molecules CD86, CD54, and MHC II on splenic B220+, Mac-1+ and CD11c+ cells following P815 immunization. Suppressed IL-12 production from splenic APC was also measured over the course of the alloimmune response. To test the hypothesis that TCDD suppresses the *in vivo* activation of APC, we have characterized the activation of APC in vehicle- and TCDD-treated mice following the administration of an agonistic antibody to murine CD40. Furthermore, we have examined if anti-CD40 stimulated APC could overcome the immunosuppressive effects of TCDD and generate allograft effector responsiveness in TCDD-treated mice.

# MATERIALS AND METHODS

## Animals

Five- to six-week old male C57Bl/6 and female DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in accordance with National Research Council guidelines.

## P815 mastocytoma cells

Wild-type P815 tumor cells, derived from a methylcholantherene-induced mastocytoma were maintained by weekly passage in DBA/2 mice.

## TCDD exposure

2,3,7,8-Tetrachlorodibenzo-*p* -dioxin (Cambridge Isotope Laboratories, Inc., Woburn MA) was dissolved in anisole and diluted in peanut oil. A vehicle solution of anisole in peanut oil was prepared similarly. Mice were given a single dose of TCDD (7.5-15 μg/kg) or vehicle by gavage 1 day prior to P815 immunization.

## Reagents

HPLC-purified, agonistic anti-CD40 mAb (FGK45) used for the *in vivo* activation of APC was kindly provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). Rat IgG was purchased from Cappel (Organon Teknika, West Chester, PA) and used as an Ig control for the *in vivo* CD40 ligation experiments. Spleen cell phenotypes were determined by flow cytometric analysis using the following Abs: FITC-conj. anti-CD54 (3E2), FITC-conj. anti-CD11c (HL3), FITC-conj. anti-CD62L (MEL-14), PE-conj. anti CD86 (GL1), PE-conj. anti-CD44 (IM7), PE-conj. anti-CD54, Biotin-conj. anti-$IA^b$ (AF6-120.1), CyChrome-conj. anti-B220 (RA3-6B2), and CyChrome-conj. anti-CD8α (53-6.7) from PharMingen (San Diego, CA); Tri-color conj.

anti-Mac-1 (M1/70.15) from Caltag Labs (Burlingame, CA), and the second step reagent streptavidin-Red613 from Gibco BRL (Gaithersburg, MD).

## Animal treatments and experimental design

Mice were injected ip with $1x10^7$ P815 tumor ascites cells on day 0. Initial studies characterizing the *in vivo* effects of anti-CD40 treatment were conducted in both vehicle- and TCDD-treated mice by ip administration of 0, 5, 25, or 100 μg of FGK45 or rat IgG. Mice were then sacrificed by $CO_2$ overdose on day 2 relative to injection. Alternatively, vehicle- or TCDD-treated mice were treated with 5 μg of FGK45 or rat IgG on days 0, 2, and 4 and mice were sacrificed on day 5. Spleens, livers and thymi were removed aseptically and blood was collected by heart puncture into heparinized syringes. Plasma was separated by centrifugation and stored at -70°C. Several experiments included fixation of tissue samples in 10% formalin for subsequent histological analyses. To measure allograft effector responses, vehicle- and TCDD-treated mice immunized with P815 tumor cells on day 0 and administered 5 μg of FGK45 or rat IgG on days 0, 2, and 4 were sacrificed on day 10 and effector functions evaluated as described further in the *Materials and Methods*.

## Preparation of spleen cells

Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Erythrocytes were removed by hypotonic lysis. Cells were washed once and resuspended in cold HBSS/5% FBS with 20 mM HEPES, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate.

## Flow cytometric analysis of spleen cells

Aliquots of spleen cells were treated with 10 μg of rat IgG to block nonspecific staining, and then stained with optimal concentrations of fluorochrome-conjugated mAb.

Appropriately labeled, isotype-matched Igs were used as controls for nonspecific fluorescence. Twenty thousand to 100,000 events were collected by listmode acquisition from freshly-stained cells using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WinList (Verity Software House, Topsham, ME). Expression of the cell-surface proteins CD86, CD54, and MHC II ($I\text{-}A^b$) was measured on gated $B220^+$, $Mac\text{-}1^+$, and $CD11c^+$ spleen cells as well as on the total viable spleen cell population. CTL effectors ($CTL_E$) were identified by first gating on the $CD8^+$ T cells and then identifying the $CD44^{hi}/CD62L^{lo}$ population, as previously described (Mobley and Dailey, 1992).

## Histological analysis of hepatic and splenic tissue

In some experiments, tissue samples were collected, weighed and prepared for histopathological examination. Samples were fixed in 10% buffered formalin, sectioned, and stained with hematoxylin and eosin.

## CTL assay

The cytolytic activity of spleen cells against P815 tumor cells was measured in a standard 4 h $^{51}Cr$-release assay as previously described (Kerkvliet et al., 1996). The percentage of cytotoxicity at each effector:target (E:T) ratio was calculated using the following equation:

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

where Er = the experimental release using spleen cells from P815-immunized mice, Nr = the nonspecific release using splenocytes from naive mice, Mr = maximal release of $^{51}Cr$ from cells incubated with 1% SDS, and Sr = spontaneous release of $^{51}Cr$ incubated in medium alone. Duplicate wells were tested from E:T ratios of 200:1 to 6.25:1.

**Cytotoxic antibody assay**

Cytotoxic alloantibody titers in plasma were determined using a complement-dependent $^{51}Cr$ release assay as previously described (Kerkvliet et al., 1996). Briefly, serial two-fold dilutions (1/10 to 1/2560) of heat-inactivated plasma were incubated with $^{51}Cr$-labeled P815 cells for 20 min at 37°C in 5% $CO_2$. Low-Tox-M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added for 45 min at 37°C. The amount of $^{51}Cr$ released into the supernatant was measured by gamma counting and specific cytotoxic activity was calculated. All samples were tested in duplicate on separate plates. The Ab titer was defined as the highest dilution of plasma at which a minimum of 20% specific cytotoxicity was measured.

**Cytokine analysis**

Spleen cells ($1x10^7$) were incubated in RPMI/10% FBS supplemented with 1.5 mM sodium pyruvate, 20 mM HEPES, and 50 μg/ml gentamicin for 6 h with $1x10^6$ P815 tumor cells at 37°C in 5% $CO_2$ in 1.5 ml silicone-treated polypropylene eppendorf tubes (to prevent nonspecific production of IL-12 by adherent spleen cells). Supernatants from cultured P815 tumor cells alone and naive spleen cells with P815 were also collected as controls for each experiment. Levels of IL-12 were determined from each supernatant by sandwich ELISA using a noncompeting pair of anti-IL-12 (C15.6 and C17.8) mAbs from Genzyme (Cambridge, MA). ELISA was performed according to manufacturer's directions, with known amounts of recombinant murine IL-12 (Genzyme) used to generate standard curves for comparison. Biotinylated detection anti-IL-12 was complexed with avidin-peroxidase and visualized with ABTS substrate. Absorbance was measured at 405 nm using a plate reader (Bio-Tek Instruments, Wincoski, VT) and cytokine values were then determined using Immunosoft software (Dynatech Labs, Alexandria, VA).

**RNA preparation and reverse transcription (RT)-PCR**

Spleen cells were restimulated *in vitro* with P815 cells for 6 h before RNA extraction. RNA extractions, RT-PCR, and analysis of DNA products was performed as described previously by Kerkvliet et al. (1996). Briefly, RNA extraction was performed by lysis of spleen cells in 4M guanidine-thiocyanate. cDNA was synthesized using RNA extracted from $1x10^6$ spleen cells using oligo(dt)primers and MMLV reverse transcriptase. PCR was performed for 30 cycles with a 1 min denaturing step at 94°, 1 min annealing step at 60°, and a 2 min extension phase at 72° using the DNA Engine (PTC-200) from MJ Research (Watertown, MA). DNA products were separated by agarose gel electrophoresis and visualized using ethidium bromide. All cDNA samples were initially analyzed for expression of the housekeeping gene ß2-microglobulin to determine relative amounts and integrity of mRNA/cDNA. Samples displaying low levels of ß2-microglobulin gene expression or degraded DNA amplification products were discarded.

Primers for IL-12 p40, IL-12 p35 and ß2-microglobulin were designed using RightPrimer (BioDisk Software, San Francisco, CA) to span introns of the respective genes to allow for discrimination between amplified genomic DNA and cDNA. The primer sequences used were: ß2-microglobulin, 5'-ATGGCTCGCTCGGTGACCCT and 3'-TCATGATGCTTGATCACATG; IL-12 p40, 5'-CGTGCTCATGGCTGGTGCAAAG and 3'-CTTCATCTGCAAGTTCTTGGGC; IL-12 p35, 5'-GGTGTCTTAGCCAGTCCCGAAA and 3'-GCGCAGAGTCTCGCCATTATGA.

**Statistical Analysis**

Results are presented as the mean ± SE of six mice per group unless indicated otherwise. Most experiments were repeated at least once. Analysis of variance modeling was performed using Statview statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using the least significant difference multiple

comparison *t* test or Dunnet's *t* test for pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### The effects of TCDD exposure on APC costimulatory molecule expression in the P815 model

We have previously demonstrated in the P815 tumor allograft model that B220+ and Mac-1+ spleen cells from TCDD-treated mice expressed significantly less CD86 when compared to vehicle-treated controls (Prell and Kerkvliet, 1997). We extend those observations in this report to further characterize the effect of TCDD on the expression of the accessory molecules CD54 (ICAM-1) and MHC II, in addition to CD86. Furthermore, we measured the expression of these activation markers on not only splenic B220+, Mac-1+, but also CD11c+ cells. As shown in Table II-1, on day 2 post-P815 immunization, TCDD did not suppress the expression of CD86, CD54 or MHC II on splenic APC. Conversely, the expression of CD86 on Mac-1+ cells, and CD54 and MHC II on CD11c+ cells was significantly increased following TCDD treatment.

In contrast, on day 5 post-immunization, the expression of P815-induced accessory molecules was suppressed on spleen cells from TCDD-treated mice. Specifically, TCDD suppressed the induction of CD86, CD54 and MHC II on B220+ cells when compared to the vehicle-treated controls. Also, TCDD significantly reduced the expression of MHC II on Mac-1+ cells and lowered the expression of CD86 and CD54. The percentage of CD11c+ cells which expressed either CD86 or MHC II was significantly reduced while the expression of CD54 on CD11c+ cells was not affected. Taken together, these data extend our previous results and further demonstrate the suppressive effects of TCDD on costimulatory molecule induction in the P815 allograft model.

**Table II-1.**

**The effects of TCDD on the expression of accessory molecules on splenic antigen presenting cells in the P815 model[a]**

| | Day 2 | | Day 5 | |
|---|---|---|---|---|
| | Vehicle | TCDD | Vehicle | TCDD |
| **B220+ cells** | | | | |
| % CD86+ | 24.4 (1.4) | 27.6 (2.0) | 33.8 (2.9) | 20.9 (2.7)* |
| MCF CD86 | 5.6 (0.1) | 6.0 (0.3) | 10.7 (0.9) | 7.2 (0.6)* |
| % CD54+ | 57.8 (0.5) | 59.2 (1.7) | 39.1 (2.1) | 30.2 (2.2)* |
| MCF CD54 | 7.6 (0.1) | 7.8 (0.3) | 8.6 (0.5) | 6.8 (0.4)* |
| % MHC II+ | 99.1 (0.1) | 99.1 (0.1) | 96.5 (0.8 | 87.3 (5.6) |
| MCF MHC II | 354 (25) | 325 (13) | 289 (25) | 199 (21)* |
| **Mac-1+ cells** | | | | |
| % CD86+ | 37.9 (0.9) | 42.7 (1.2)* | 41.2 (4.8) | 26.8 (4.4) |
| MCF CD86 | 10.8 (0.2) | 11.9 (0.3) | 30.1 (2.8) | 23.7 (1.3) |
| % CD54+ | 43.3 (1.8) | 46.4 (1.7) | 36.5 (3.7) | 29.7 (3.5) |
| MCF CD54 | 14.3 (0.5) | 15.2 (0.5) | 13.4 (2.0) | 10.5 (1.1) |
| % MHC II+ | 42.0 (1.9) | 42.0 (2.7) | 28.8 (4.3) | 16.7 (2.3)* |
| MCF MHC II | 625 (50) | 503 (43) | 61.2 (7.3) | 37.7 (3.6)* |
| **CD11c+ cells** | | | | |
| % CD86+ | 70.9 (2.9) | 71.4 (1.8) | 90.7 (1.6) | 81.1 (2.1)* |
| MCF CD86 | 45.5 (2.5) | 60.8 (7.4) | 54.1 (9.4) | 71.0 (9.6) |
| % CD54+ | 99.2 (0.1) | 99.3 (0.1) | 99.9 (0.1) | 99.9 (0.1) |
| MCF CD54 | 138 (4.4) | 156 (4.6)* | 324 (18) | 309 (15) |
| % MHC II+ | 89.8 (1.1) | 91.9 (1.0) | 98.4 (0.4) | 96.4 (0.3)* |
| MCF MHC II | 107 (2.8) | 127 (6.1)* | 167 (7.0) | 124 (6.8)* |

[a] Vehicle- and TCDD treated mice were immunized with $1x10^7$ P815 cells. Two and five days later, splenic B220+, Mac-1+ and CD11c+ were stained and analyzed by flow cytometry for their expression of the accessory molecules CD86, CD54 and MHC II. Values represent the mean percent positive staining and median channel fluorescence (MCF) of samples with SEM in parentheses. Data represent values from 4-6 mice/treatment with each experiment being repeated at least twice. * indicates $p \leq 0.05$.

**The effects of TCDD exposure on IL-12 production**

IL-12 is a cytokine produced by antigen presenting cells which drives T cell differentiation and promotes the generation of Th1-mediated immunity (Gately et al., 1998). The bioactive 70 kD heterodimeric protein, comprised of the constitutively expressed p35 subunit and the inducible p40 subunit, binds to specific receptors on T cells and NK cells and induces IFN-γ production. Since we have previously reported that TCDD significantly suppressed the production of IFN-γ in the P815 model, we examined if TCDD was producing similar effects on the production of IL-12. Following immunization of mice with P815, the production of IL-12 by spleen cells was measured on days 1-9 following *in vitro* restimulation (Figures II-1A and II-1B) with P815 tumor cells. Levels of IL-12 gradually increased from day 4 through day 9 with peak production of approximately 750 pg/ml on day 9. TCDD-treated mice produced similar levels of IL-12 on days 1-3 but produced significantly less IL-12 on days 4, 5 and 7-9 when compared to vehicle-treated mice. Furthermore, reduced splenic levels of mRNA for the inducible IL-12 p40 gene were observed from TCDD-treated mice when compared to vehicle-treated mice on days 5-9 post-P815 immunization (Figure II-1C). No TCDD effects were observed on the mRNA levels for the constitutively-expressed IL-12 p35 subunit, or $ß_2$-microglobulin (data not shown). Collectively, these data characterizing the effects of TCDD on costimulatory molecule expression and IL-12 production suggests that TCDD inhibits the activation of splenic APC in mice immunized with allogeneic P815 tumor cells.

**Characterization of APC activation following administration of an agonistic antibody to CD40**

CD40 ligation has previously been shown *in vitro* to induce the expression of costimulatory molecules on antigen presenting cells while also promoting their production of IL-12 (Ranheim and Kipps, 1993; Roy et al., 1993; Cella et al., 1996; Kato et al., 1996;

**Figure II-1. The effects of TCDD on IL-12 production in the P815 allograft model.**

Levels of IL-12 p70 protein were measured from ex vivo generated culture supernatants on days 1-5 (A), and days 6-9 (B). Spleen cells ($1x10^7$) from vehicle- (open squares) or TCDD-treated (filled squares) mice were restimulated with P815 tumor cells ($1x10^6$) for 6 hours. Additionally, mRNA was extracted from some of the spleen cell cultures and the levels of IL-12 p40 mRNA were evaluated by RT-PCR (C). mRNA was extracted from spleen cell samples on days 1-5 and 5-9 in two separate experiments, however, IL-12 p40 mRNA expression was not induced above the non-immune control until days 5-9. No IL-12 p40 mRNA was detected from P815 cells alone (not shown). Data points represent the mean ± SE for duplicate samples from 4-6 mice/treatment group. Comparison to vehicle-treated controls, * indicates $p \leq 0.05$.

A
B
C
IL-12 (Pg/ml)
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
Day Post P815
5
6
7
8
9
Vehicle
Non-Immune
TCDD

Figure II-1.

Koch et al., 1996). However, the effects of exogenous stimulation of CD40 following the administration of an agonistic antibody have not been characterized in mice. Therefore, we evaluated the efficacy of treating mice with anti-CD40 specifically for the purpose of generating activated APC.

The administration of 100 μg anti-CD40 mAb to mice significantly enhanced the expression of CD86 on their spleen cells and increased their plasma levels of IL-12 by 50-fold when compared to rat IgG-treated controls (Figures II-2A and II-2B). The increased CD86 expression on spleen cells was found to occur in a time- and dose-dependent manner (Figures II-2C and II-2D). Anti-CD40 induced CD86 expression was detectable within 8 hours after administration and continued to increase through 48 hours. Anti-CD40 treatment of mice significantly increased their splenic weights and cell numbers by 48 hours (Table II-2). In mice receiving anti-CD40 mAb, the percentage of B220+ and CD11c+ cells increased by 16% and 160%, respectively, while no change was observed in the percentage of Mac-1+ spleen cells.

As a result of the anti-CD40 treatment, toxicity was observed in mice administered high doses of anti-CD40 mAb. A significant reduction in body weight was measured within 48 hours after the administration of either the 25 or 100 μg dose of anti-CD40. In contrast, no significant changes in body weight were observed in mice injected with either 5 μg of anti-CD40, or 100 μg of a relevant control Ig (data not shown). Similarly, anti-CD40 treatment produced significant pathological damage in the hepatic and splenic tissue of mice which had received the 25 and 100 μg doses of the antibody. However, only minor histological changes were observed in mice administered the control Ig or the 5 μg dose of anti-CD40 (Figures II-3A and II-3C). Mice receiving higher amounts of anti-CD40 (≥25 μg) displayed marked lymphoproliferative responses in their spleens which were characterized by enlarged lymphoid follicles containing minimal areas of red pulp (Figure II-3B). Mitotic figures were frequently observed within these follicles (Figure II-3D). Although the livers of mice injected with 5 μg of anti-CD40 showed some areas of

**Figure II-2. The administration of anti-CD40 to mice modulates splenic expression of CD86 and plasma IL-12 levels.**

Mice were treated with 100 μg anti-CD40 mAb (FGK45) (black histogram) or rat IgG (white histogram) and analyzed two days later for splenic CD86 expression (A). Also, plasma IL-12 levels (B) from these mice were evaluated by ELISA. Subsequently, experiments were performed to evaluate the kinetics and dose-response of CD86 induction on spleen cells following anti-CD40 treatment. CD86 induction on spleen cells was measured at 0 (white histogram), 8 (light gray), 24 (dark gray) and 48 h (black) after the administration of 100 μg FGK45 to mice (C). In a separate experiment, mice were treated with 0 (white histogram), 5 (light gray), 25 (dark gray) and 100 μg (black) FGK45 and splenic CD86 expression was measured two days later (D). Each experiment was repeated at least twice with 3-5 mice/treatment group with representative samples shown.

**Figure II-2.**

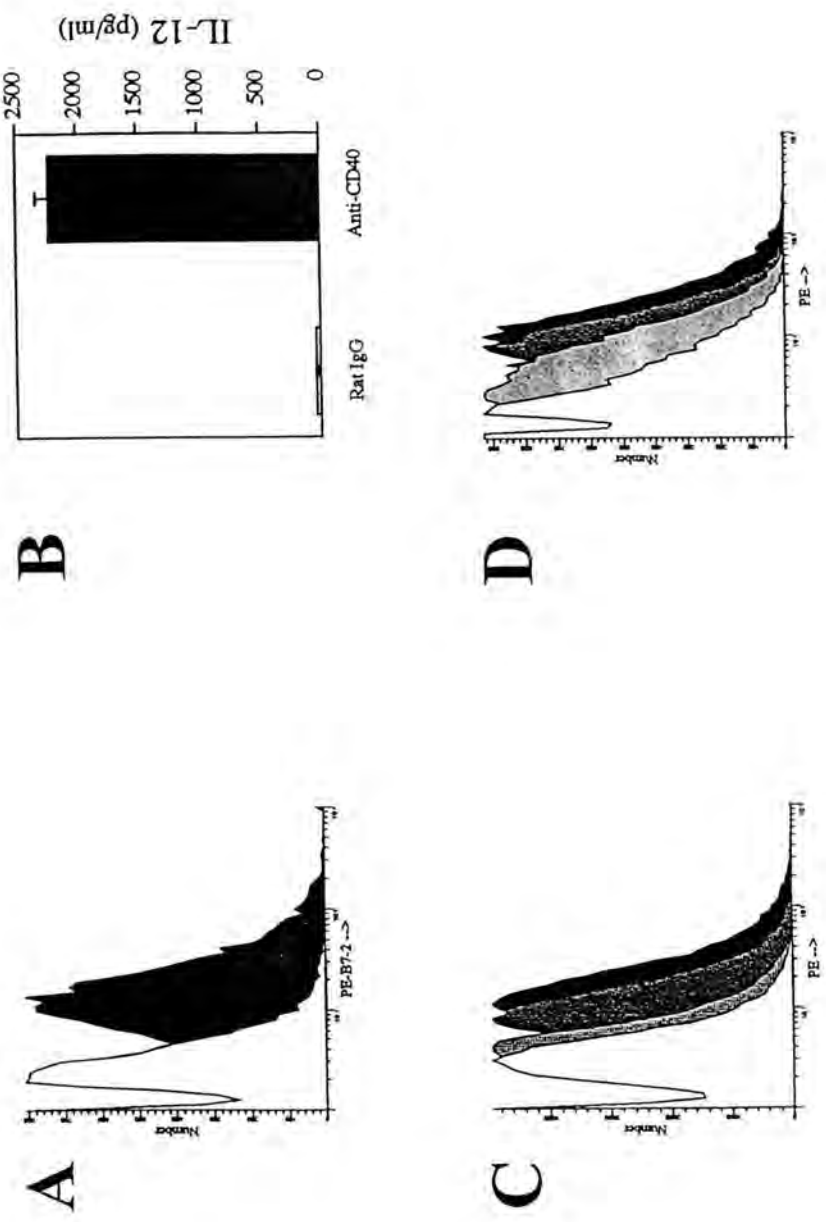

A
B
C
D
IL-12 (pg/ml)
2500
2000
1500
1000
500
0
Rat IgG
Anti-CD40
PE-B7-2 -->
PE -->
Number

Table II-2.

The effects of anti-CD40 treatment on
spleen cell weights, numbers and phenotypes[a]

| | Spleen weight (mg) | Spleen cell number ($1x10^6$) | % B220+ | % Mac-1+ | % CD11c+ |
|---|---|---|---|---|---|
| Rat IgG | 82.2 (4.9) | 114 (5.6) | 69.2 (1.3) | 2.9 (0.2) | 1.5 (0.1) |
| Anti-CD40 | 132 (6.8)* | 154 (6.6)* | 80.6 (1.2)* | 2.9 (0.3) | 3.9 (0.1)* |

[a] Mice were treated with 5 μg anti-CD40 mAb. Two days later, spleens were removed, weighed, and spleen cells were prepared and counted. Spleen cells were stained and analyzed for the expression of B220, Mac-1 and CD11c as described in the *Materials and Methods*. Values represent the mean weights, numbers, and percent positive staining with SEM in parentheses. Data represent values from 4-6 mice/treatment, and * indicates $p \leq 0.05$.

**Figure II-3. Histological appearance of spleen (A-D) and liver (E and F) after two days of anti-CD40 treatment.**

Splenic tissue sections from mice treated with 5 μg (A and C) or 100 μg (B and D) FGK45. Mice receiving 25 and 100 μg FGK45 displayed enlarged lymphoid follicles with minimal areas of red pulp; magnification x100 (A and B) and x250 (C and D). Note the detection of mitotic figures in the sections from mice receiving the 100 μg (D) but not the 5 μg (C) dose of FGK45. Hepatic tissue sections from mice treated with 5 μg (E) or 100 μg (F) FGK45. The treatment of mice with 100 μg (F) FGK45 produced areas of hepatic necrosis whereas the 5 μg (G) FGK45 dose did not generate comparable toxicity; magnification x100. Symbols used in micrographs: c = central vein; n = necrosis; and T = thrombi.

**Figure II-4. The treatment of mice with anti-CD40 affects the generation of allograft effector responses.**

Mice were immunized with $1x10^7$ P815 cells on day 0 and administered 5 or 100 μg FGK45, or 100 μg rat IgG on days 0, 2, 4, 6 and 8. On day 10 post-immunization, CTL activity (A) and alloantibody activity (B) were measured as described in the *Materials and Methods*. Data points represent the mean ± SE from 4-6 mice/treatment group. Comparison to rat IgG-treated controls, * indicates $p \leq 0.05$.

**Figure II-3.**

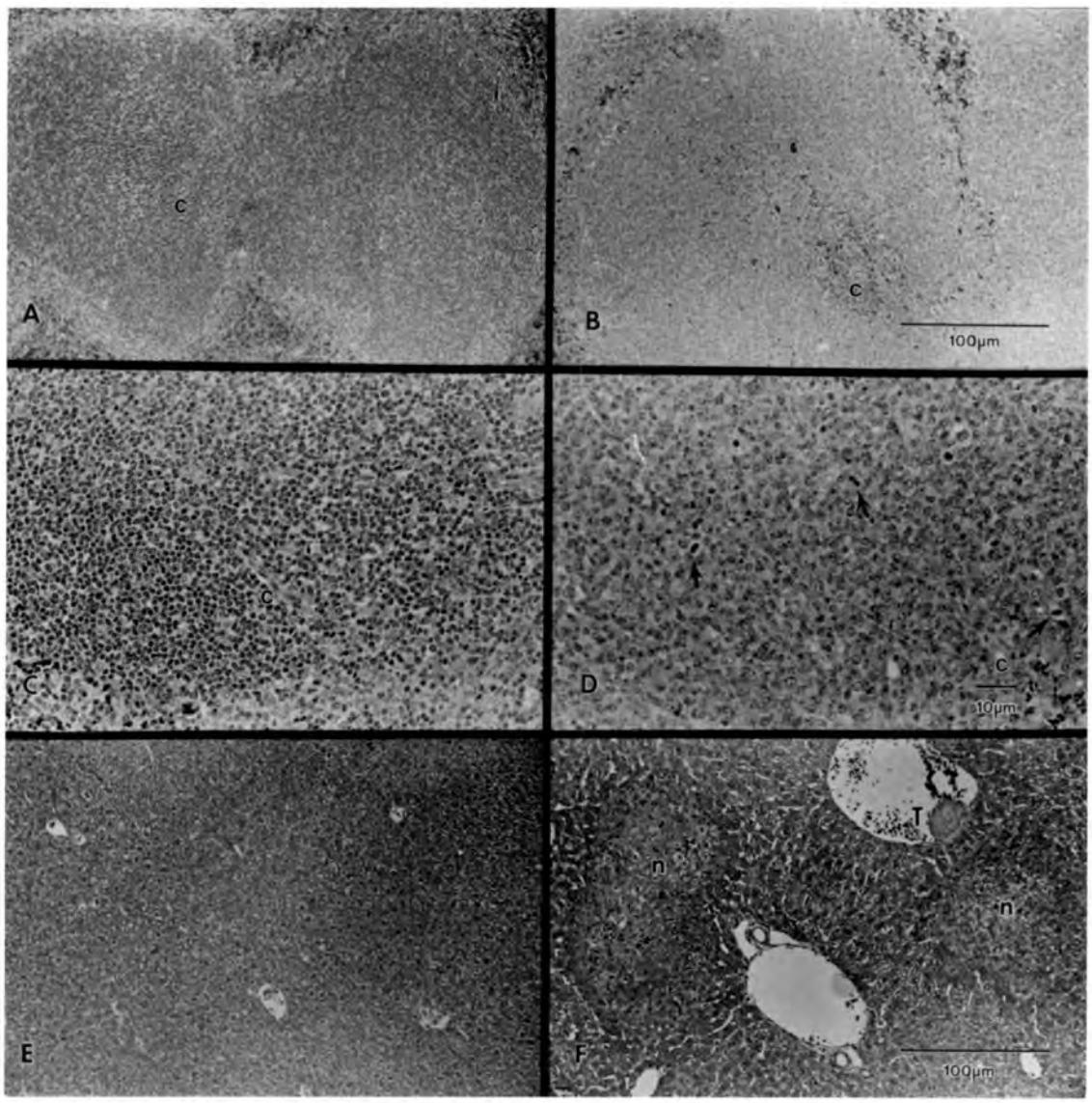
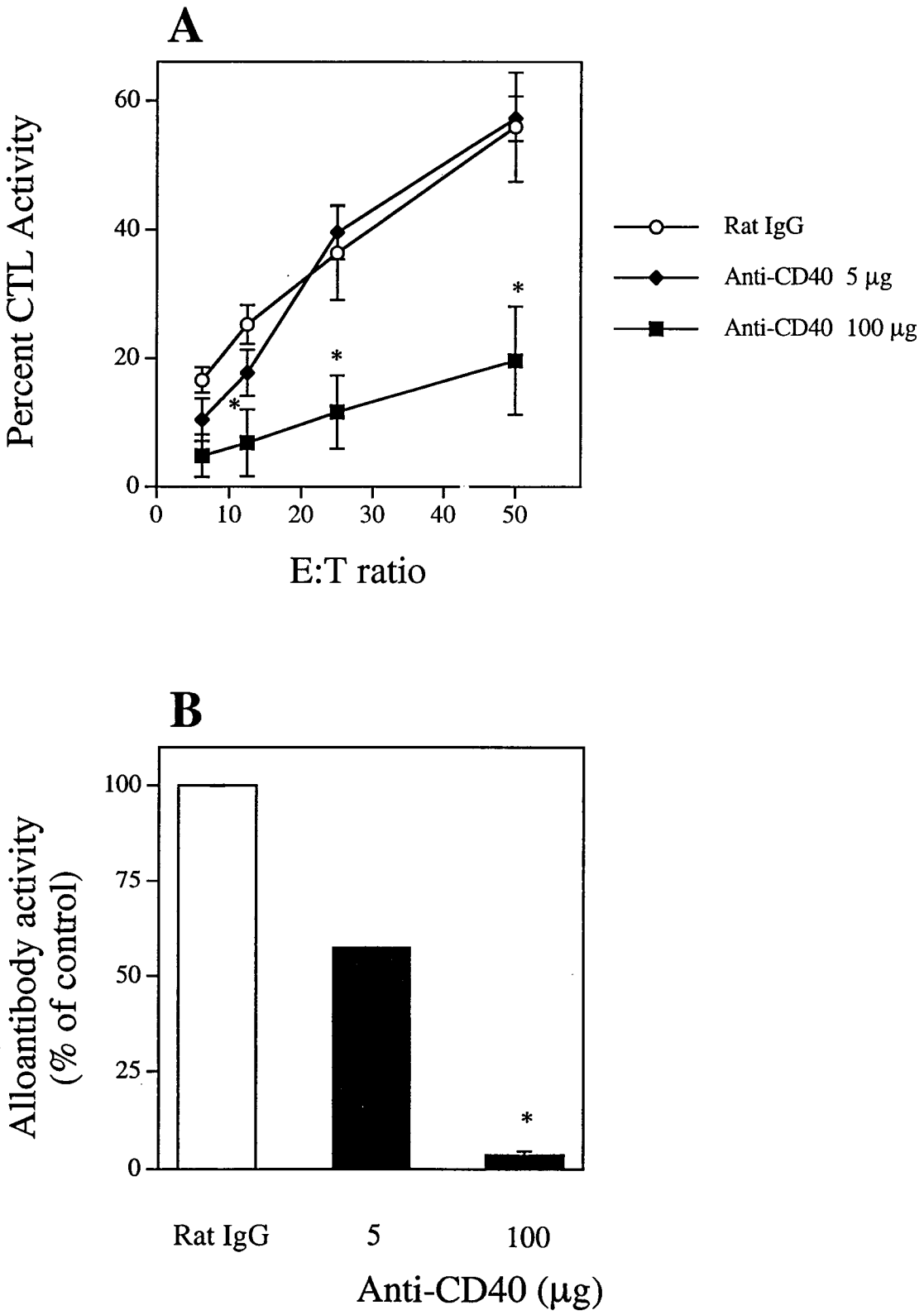


**Figure II-4.**


A
Percent CTL Activity
60
40
20
0
0
10
20
30
40
50
E:T ratio
*
*
*
Rat IgG
Anti-CD40 5 μg
Anti-CD40 100 μg
B
Alloantibody activity
(% of control)
100
75
50
25
0
*
Rat IgG
5
100
Anti-CD40 (μg)

multifocal coagulative necrosis, it was negligible when compared to the tissue damage observed in the livers of mice given either the 25 or 100 μg dose (Figures II-3E and II-3F). Additionally, dose-dependent thymic atrophy was also observed in mice injected with anti-CD40 (data not shown).

To determine what effects anti-CD40 treatment would have on the generation of allograft immunity, we treated P815-immunized mice with anti-CD40 and measured allograft effector functions on day 10. As shown in Figures II-4A and II-4B, treating mice with the 100 μg dose of anti-CD40 suppressed the generation of both the CTL and alloantibody responses in P815-immunized mice. In contrast, the 5 μg dose had no effect on CTL generation and only slightly diminished alloantibody activity.

## The effects of TCDD on CD40-induced expression of APC costimulatory molecules

Following the characterization of the *in vivo* effects of anti-CD40 treatment on APC activation, we tested our hypothesis that TCDD suppresses the activation of APC by treating vehicle- and TCDD-treated mice with anti-CD40 and measuring the subsequent expression of accessory molecules on their splenic APC populations.

As shown in Table II-3, anti-CD40 treatment significantly augmented the induction of accessory molecules on splenic APC, especially on the B220+ cells. On day 2, splenic B220+ cells from anti-CD40 treated, P815-immunized mice expressed significantly higher levels of CD86 and MHC II compared to splenic B220+ cells from mice immunized with P815 cells alone (Table II-3 vs. Table II-1). Although TCDD slightly inhibited the anti-CD40 induced expression of CD54 on the B220+ cells, it had no effect on the CD40-mediated expression of CD86 or MHC II. In a similar fashion, enhanced accessory molecule expression was detected on CD11c+ spleen cells from anti-CD40 treated mice when compared to mice injected with P815 cells alone. Although TCDD slightly reduced the expression of MHC II on CD11c+ cells, it increased the expression of CD54 and had

**Table II-3.**

**The effects of TCDD on the anti-CD40 induced expression of accessory molecules on splenic antigen presenting cells in the P815 model[a]**

| | Day 2 | | Day 5 | |
|---|---|---|---|---|
| | Vehicle | TCDD | Vehicle | TCDD |
| **B220+ cells** | | | | |
| % CD86+ | 70.8 (1.0) | 70.9 (1.8) | 50.6 (3.5) | 43.3 (4.1) |
| MCF CD86 | 17.8 (0.3) | 18.6 (0.6) | 17.1 (2.0) | 14.3 (1.8) |
| % CD54+ | 95.2 (0.1) | 93.8 (0.4) | 76.2 (1.1) | 73.7 (3.3) |
| MCF CD54 | 24.2 (0.3) | 22.7 (0.5)* | 18.2 (1.3) | 18.2 (2.1) |
| % MHC II+ | 98.6 (0.3) | 98.5 (0.3) | 98.1 (0.2) | 95.7 (0.2) |
| MCF MHC II | 459 (29) | 429 (10) | 702 (17) | 640 (38) |
| **Mac-1+ cells** | | | | |
| % CD86+ | 47.8 (3.5) | 36.4 (2.8)* | 39.7 (3.2) | 38.0 (5.3) |
| MCF CD86 | 13.8 (1.4) | 10.0 (1.5) | 28.6 (1.3) | 27.6 (2.2) |
| % CD54+ | 55.2 (4.0) | 48.0 (2.9) | 34.7 (2.4) | 37.9 (4.5) |
| MCF CD54 | 28.6 (4.4) | 19.5 (2.8) | 14.2 (1.5) | 16.5 (2.4) |
| % MHC II+ | 52.4 (3.3) | 42.4 (3.3) | 31.1 (3.1) | 28.2 (3.9) |
| MCF MHC II | 359 (8) | 469 (21)* | 72.0 (6.4) | 70.5 (5.2) |
| **CD11c+ cells** | | | | |
| % CD86+ | 91.3 (0.9) | 93.1 (0.6) | 90.0 (1.5) | 84.8 (1.3)* |
| MCF CD86 | 99.0 (2.6) | 102 (3.4) | 40.7 (4.5) | 43.0 (4.8) |
| % CD54+ | 99.5 (0.1) | 99.9 (0.1)* | 99.9 (0.1) | 99.9 (0.1) |
| MCF CD54 | 277 (34.2) | 442 (7.1)* | 357 (24) | 386 (25) |
| % MHC II+ | 97.0 (1.1) | 99.0 (0.5) | 97.8 (0.2) | 96.9 (0.3)* |
| MCF MHC II | 212 (1.8) | 181 (4.0)* | 167 (12) | 147 (8.8) |

[a] Vehicle- and TCDD treated mice were immunized with $1x10^7$ P815 cells and administered anti-CD40 mAb. Two and five days later, splenic B220+, Mac-1+ and CD11c+ were stained and analyzed by flow cytometry for their expression of the accessory molecules CD86, CD54 and MHC II. Values represent the mean percent positive staining and median channel fluorescence (MCF) of samples with SEM in parentheses. Data represent values from 4-6 mice/treatment with each experiment being repeated at least twice. * indicates $p \leq 0.05$.

no effect on CD86 expression. In contrast to the effects measured on both B220+ and CD11c+ cells, only moderate increases in accessory molecule expression were detected on the Mac-1+ cells of mice administered anti-CD40. In addition, the induction of CD86 was suppressed on Mac-1+ spleen cells from TCDD-treated mice, and the expression of CD54 and MHC II was reduced relative to that observed in the vehicle-treated controls.

On day 5 post-immunization, only the B220+ spleen cells from P815-immunized mice administered anti-CD40 continued to display elevated expression of accessory molecules when compared to mice immunized with P815 cells alone (Table II-3 vs. Table II-1). TCDD had no significant effect on the expression of CD86, CD54 or MHC II detected on the B220+ and Mac-1+ spleen cells from anti-CD40 treated mice. Conversely, TCDD did produce small but significant suppressive effects on the expression of CD86 and MHC II, but not CD54, on the splenic CD11c+ cells from mice receiving anti-CD40 treatment. Taken together, these data suggest that the administration of anti-CD40 to mice induces the expression of accessory molecules on splenic APC populations and this activation process is largely unaffected by TCDD.

**The effects of TCDD on CD40-induced IL-12 production**

As previously reported, spleen cells from P815-immunized mice produced increasing amounts of IL-12 throughout the course of the P815 allogeneic immune response and TCDD significantly suppressed this production. On the other hand, plasma IL-12 levels were significantly increased in mice receiving anti-CD40 treatment. Therefore, we analyzed IL-12 production in P815-immunized mice which had been exposed to TCDD to determine if anti-CD40 treatment could bypass this defect.

Spleen cells from vehicle- or TCDD-treated mice which had been injected with P815 cells and administered either anti-CD40 or rat IgG were restimulated *ex vivo* with P815 cells to determine their capacity to produce IL-12. On day 2, spleen cells from vehicle treated-mice which had been given anti-CD40 antibodies *in vivo* showed a trend

towards producing more IL-12 when compared to the rat IgG controls (Figure II-5A). In contrast, splenic IL-12 production from TCDD-treated mice was reduced independent of CD40 stimulation. By day 5, significant suppression of IL-12 production was measured from spleen cells of TCDD-treated mice when compared to the vehicle-treated controls (Figure II-5B). In addition, spleen cells from TCDD-treated mice that had been given anti-CD40 produced lower levels of IL-12 following *ex vivo* restimulation when compared to the vehicle-treated mice which had been injected with anti-CD40. These results suggest that exogenous CD40 stimulation is unable to overcome the TCDD-induced defect in splenic IL-12 production.

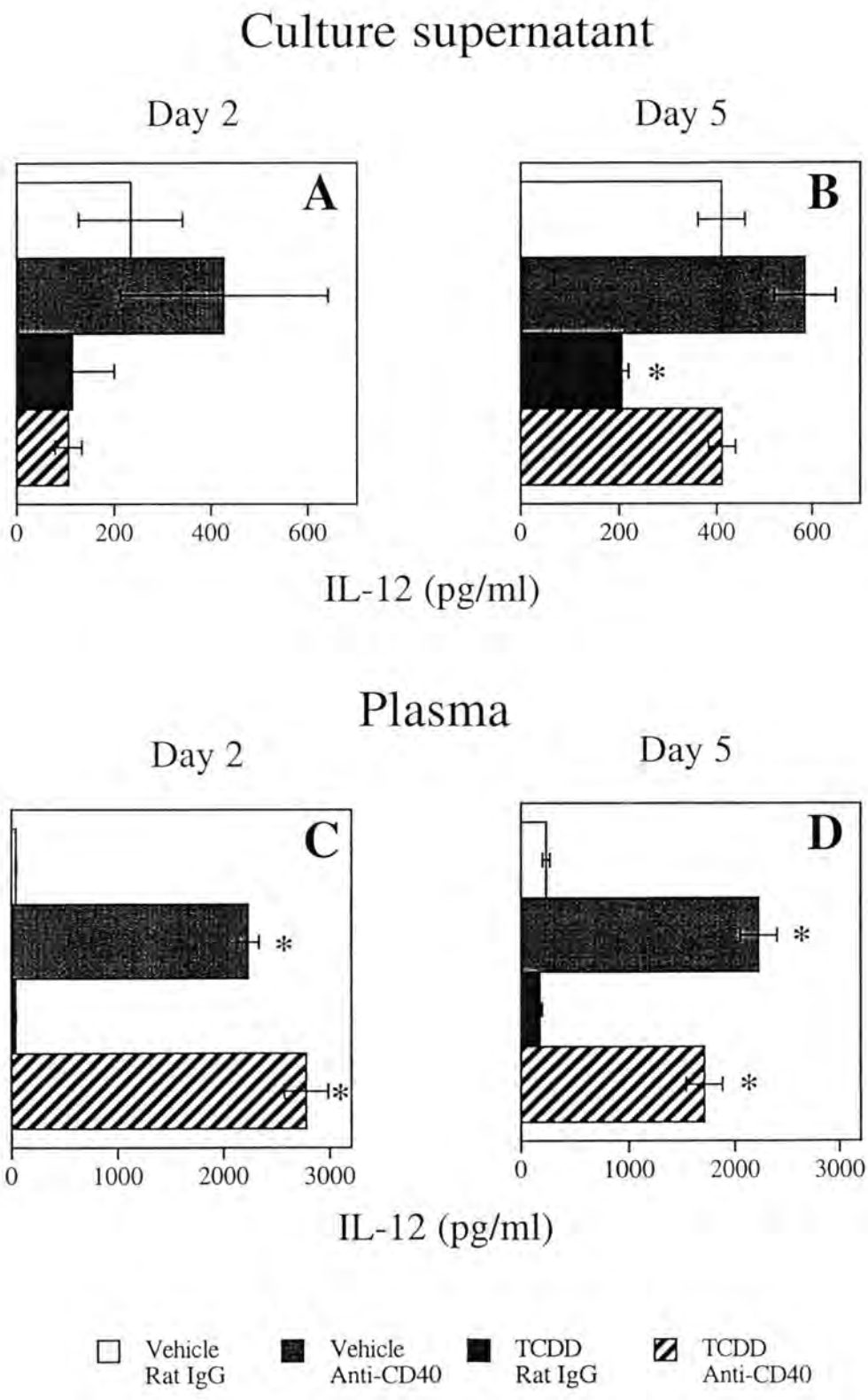
To further assess the impact of TCDD exposure on CD40-induced IL-12 production, we measured IL-12 levels in the plasma of mice on days 2 and 5 following P815 injection (Figures II-5C and II-5D). Anti-CD40 treatment significantly increased plasma IL-12 levels in mice on both days independent of TCDD exposure. In contrast to the anti-CD40 treated mice, plasma levels of IL-12 in mice injected with P815 but administered rat IgG were barely detectable on days 2 and 5. Furthermore, plasma IL-12 levels remained elevated through day 10 in vehicle- and TCDD-treated mice receiving anti-CD40 treatment but never increased in mice receiving the rat IgG control (data not shown).

## The effects of TCDD on the generation of allograft immunity in mice administered anti-CD40

TCDD has been shown to suppress the generation of the allogeneic immune response to P815 tumor cells (Clark et al., 1981; Kerkvliet et al., 1990; De Krey and Kerkvliet, 1995; Kerkvliet et al., 1996). We postulated that the generation of activated APC following anti-CD40 treatment would be sufficient to ultimately restore T cell allograft effector functions in mice exposed to TCDD. To test this possibility, we analyzed the effects of anti-CD40 administration on the day 10 allograft effector responses in vehicle- or

**Figure II-5. The effects of TCDD on anti-CD40 induced IL-12 production.** Vehicle- and TCDD-treated mice were immunized with P815 cells ($1x10^7$) on day 0, received either 5 μg of FGK45 or rat IgG on days 0 and 2 (A), or days 0, 2 and 4 (B), and were sacrificed on day 2 or 5, respectively. days Spleen cells ($1x10^7$) were cultured with P815 tumor cells ($1x10^6$) for 6 h and IL-12 p70 protein levels were measured from supernatants by ELISA. Additionally, plasma collected from these treated mice were assayed for levels of IL-12 on day 2 (C) and day 5 (D). Data represent the mean ± SE from 4-6 mice/treatment group. Comparison to vehicle-treated rat IgG controls, * indicates $p \leq 0.05$.

**Figure II-5.**


Culture supernatant
Day 2
Day 5
A
B
*
0
200
400
600
IL-12 (pg/ml)
Plasma
Day 2
Day 5
C
D
*
*
*
0
1000
2000
3000
IL-12 (pg/ml)
Vehicle Rat IgG
Vehicle Anti-CD40
TCDD Rat IgG
TCDD Anti-CD40

TCDD-treated mice which had been injected with P815 cells on day 0. Comparable CTL responses were measured from vehicle-treated mice injected with either anti-CD40 or rat IgG, demonstrating that the administration of anti-CD40 had not adversely affected the generation of CTL activity (Figure II-6A). Moreover, the administration of anti-CD40 effectively doubled the number of CTL effector cells generated in vehicle-treated mice on day 10 post-immunization (Figure II-6B). Surprisingly, anti-CD40 treatment of mice exposed to TCDD did not significantly elevate their CTL activity above the levels measured from TCDD-treated mice administered rat IgG. Similarly, no significant increase was observed in the number of CD8+ CTL effector cells generated in TCDD-treated mice given anti-CD40. Likewise, the generation of alloantibody activity was also not increased in TCDD-treated mice administered anti-CD40 when compared to the rat IgG controls (Figure II-6C).

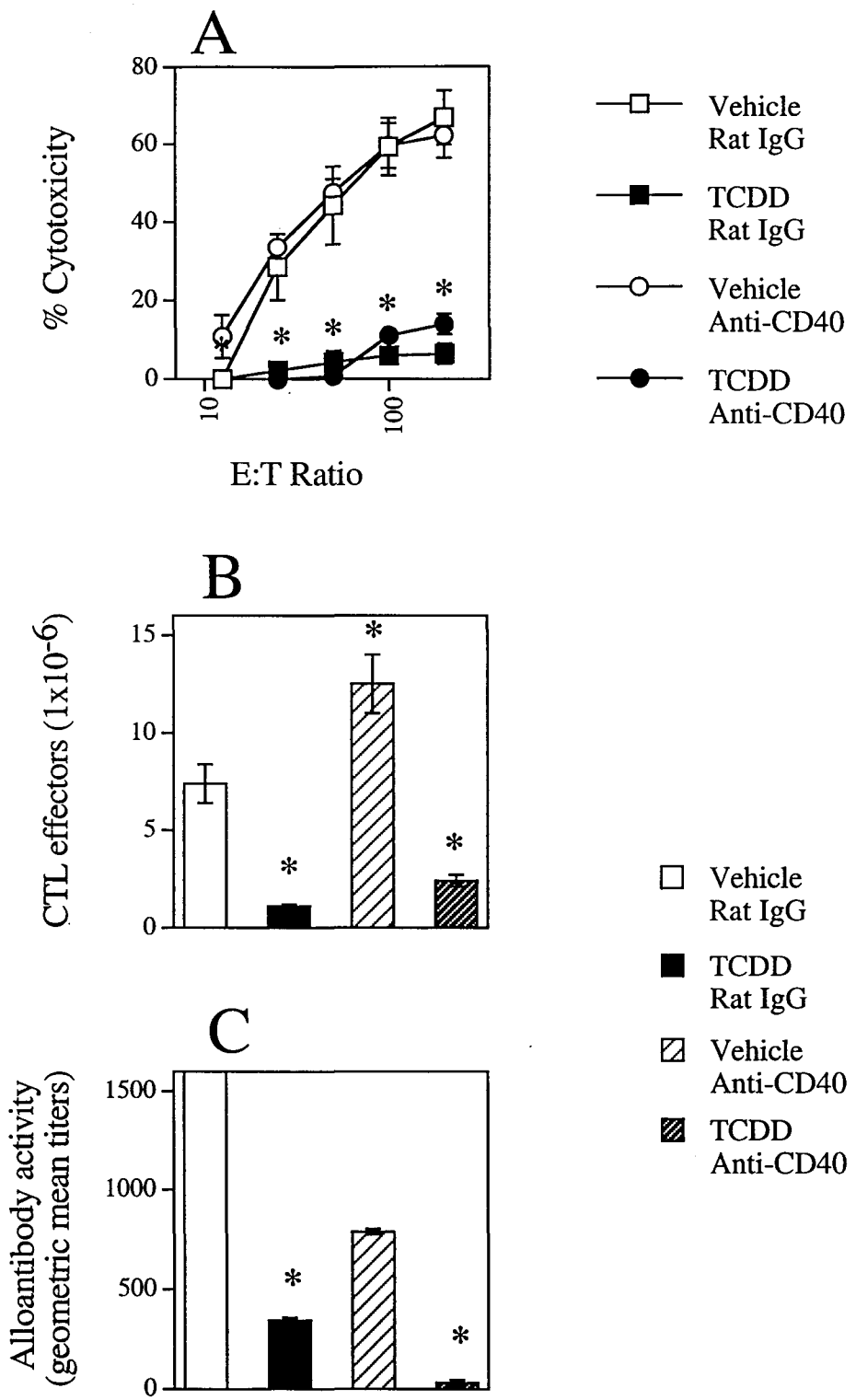
## DISCUSSION

In this report, we have characterized the effects of TCDD exposure on APC activation in the P815 allograft model. We found a significantly reduced induction of the accessory molecules CD86, CD54, and MHC II on splenic APC following P815 immunization in TCDD-treated mice when compared to vehicle controls. The production of IL-12 from spleen cells of TCDD-treated mice was also impaired during the development of the allogeneic immune response to P815 cells. The objective of the studies presented herein was to test the hypothesis that TCDD suppresses the activation of APC. We demonstrated that *in vivo* stimulation of APC via exogenous ligation of CD40 was largely unaffected in TCDD-treated mice. The anti-CD40 induced expression of CD86, CD54 and MHC II on splenic B220+, Mac-1+ and CD11c+ was minimally affected by TCDD exposure and plasma IL-12 production was completely unaffected. Much to our surprise, despite the significant activation of APC that developed in TCDD-exposed mice which had

**Figure 6. The effects of TCDD on the anti-CD40 mediated generation of allograft effector responses.**

Vehicle- and TCDD-treated mice were immunized with P815 cells (1x107) on day 0 and administered 5 μg of FGK45 or rat IgG on days 0, 2 and 4. On day 10 post-immunization, splenic CTL activity (A) was measured from these mice as described in the *Materials and Methods*. In addition, the number of splenic CTL effector ($CD8^+/CD62L^{low}/CD44^{high}$) cells was determined for all treatment groups by flow cytometry (B). Alloantibody activity was also measured from plasma samples collected from treated mice (C). Data represent the mean ± SE from 4-6 mice/treatment group. Comparison to vehicle-treated rat IgG controls, * indicates $p \leq 0.05$.

**Figure II-6.**


A
% Cytotoxicity
80
60
40
20
0
10
100
E:T Ratio
Vehicle
Rat IgG
TCDD
Rat IgG
Vehicle
Anti-CD40
TCDD
Anti-CD40
B
CTL effectors (1x10-6)
15
10
5
0
C
Alloantibody activity
(geometric mean titers)
1500
1000
500
0
Vehicle
Rat IgG
TCDD
Rat IgG
Vehicle
Anti-CD40
TCDD
Anti-CD40

received anti-CD40 treatment, allograft effector functions were not restored in these mice by day 10 post-immunization.

The generation of the allogeneic immune response to P815 tumor cells has been shown to require the costimulatory molecule CD86 (Prell and Kerkvliet, 1997). Previous studies from our laboratory have demonstrated TCDD-induced suppression of CD86 expression on splenic B220+ and Mac-1+ cells from mice immunized with P815 allogeneic tumor cells (Prell and Kerkvliet, 1997). The data presented in this report have confirmed these previous results and further demonstrated TCDD suppression of two other accessory molecules, CD54 and MHC II, on APC during the development of this allogeneic immune response. The percentage of CD11c+ dendritic cells which expressed CD86 and MHC II was also shown to be significantly reduced in TCDD-treated mice. To our knowledge, this is the first report demonstrating the effects of TCDD on dendritic cell activation. Therefore, TCDD suppression of APC activation may represent a mechanism by which TCDD induces immune suppression.

It is generally accepted that the production of IL-12 by APC contributes to the development of Th1-driven cell-mediated immune responses (Gately et al., 1998). Therefore, we measured IL-12 production through the course of the P815 allograft immune response. IL-12 was detectable on all days tested in this allogeneic response with maximal production measured on day 9 post-P815 immunization. TCDD suppressed the production of IL-12, and the decreased levels of IL-12 protein corresponded with decreased levels of mRNA for the p40 but not the p35 subunit of the IL-12 gene. Interestingly, subsequent scanning of the DNA database identified a putative DRE in the upstream promoter region of both the IL-12 p40 and p35 gene suggesting that TCDD might be directly altering IL-12 transcription via an AhR-mediated mechanism. However, our results suggest that the inducible IL-12 p40 gene may be more sensitive to the effects of TCDD than the constitutively expressed IL-12 p35 gene as no TCDD effects were detected on the splenic levels of IL-12 p35 mRNA in P815-immunized mice. Further investigations are currently

underway to determine the functionality of the putative DRE in each of these two genes. Taken together, these data suggest that TCDD inhibits the *in vivo* activation of APC and that this suppression may play an important role in mediating the immunotoxic effects which are associated with TCDD exposure.

Ligation of CD40 by either CD154 or exogenously with agonistic antibodies induces measurable APC activation. Stimulation through CD40 has been shown primarily through *in vitro* studies to increase expression of accessory molecules, enhance antigen processing and presentation, provoke secretion of certain cytokines, and modulate survival of leukocytes (Ranheim and Kipps, 1993; Roy et al., 1993; Caux et al., 1994; Kennedy et al., 1994; Cella et al., 1996; Bennett et al., 1998; De Smedt et al., 1998; Maxwell et al., 1999). In this report, we have characterized the application of agonistic antibodies to CD40 in an *in vivo* model of APC activation. Anti-CD40 treatment of mice generated activated APC by increasing their expression of accessory molecules and inducing IL-12 production. However, stimulation through CD40 also produced adverse toxicity in mice as illustrated by histological analysis of livers and spleens of mice receiving 25 and 100 μg doses of anti-CD40. It is important to note that treatment of P815-challenged mice with the 100 μg dose but not the 5 μg dose of anti-CD40 resulted in significant suppression of both the CTL and alloantibody responses on day 10. Therefore, by treating mice with the 5 μg dose of anti-CD40, we effectively enhanced their APC activation as defined by increased accessory molecule expression and IL-12 production and minimized any associated toxicity.

Following the characterization of anti-CD40 induced activation of APC *in vivo*, we then determined what effects TCDD exposure would have on this process. In contrast to the suppressive effects of TCDD in the P815 model, TCDD exposure produced only minor effects on APC activation in mice receiving anti-CD40 stimulation. The administration of anti-CD40 generated highly-activated splenic B220+ and CD11c+ cells, even in TCDD-treated mice. These results are consistent with a previous report by Karras et al.

(1996) that showed B cell stimulation by fixed and activated T cell membranes expressing CD154 was refractory to suppression by TCDD . In contrast, the splenic Mac-1+ population showed the greatest sensitivity to the effects of TCDD as illustrated by the significant suppression of anti-CD40 induced CD86 expression on day 2. This effect of TCDD on the Mac-1+ cells could possibly be explained by their less vigorous response to CD40 stimulation when compared to the B220+ cells in the spleen.

In addition to increasing their expression of accessory molecules, activated APC produce proinflammatory cytokines which contribute to the activation and differentiation of T cells (Matzinger, 1994). For example, CD40 ligation has been shown *in vitro* to induce IL-12 production from macrophages and dendritic cells and to a lesser extent B cells (Grewal and Flavell, 1998). In the P815 model, anti-CD40 treatment increased the levels of IL-12 detected in spleen cell culture supernatants but TCDD significantly suppressed this production. In contrast, when we evaluated the plasma from these mice, TCDD exposure had no effect on the circulating levels of IL-12. These results suggest that TCDD may be selectively suppressing the production of IL-12 from cells in the spleen but not elsewhere. Since CD40 is expressed on many cell types in various tissues, it is conceivable that extra-splenic cells produced the IL-12 which was found circulating in TCDD-treated mice which had been administered anti-CD40. In spite of the effects of TCDD on splenic IL-12 production, our results indicated that TCDD-treated mice administered anti-CD40 produced high levels of systemic IL-12 which should have contributed to the development of Th1 immunity. It remains to be determined what role, if any, IL-12 plays in the generation of the P815 allograft response. Collectively, these results suggest that APC in TCDD-treated mice can respond *in vivo* to CD40 stimulation, however, no conclusions can be made regarding their functionality.

The allosensitization of mice with P815 tumor cells leads to the generation of allograft effector responses (Kerkvliet et al., 1996). TCDD has been shown to suppress allograft effector functions in C57Bl/6 mice in a dose-dependent manner (De Krey and

Kerkvliet, 1995). Therefore, we attempted to overcome TCDD suppression of the P815 allograft response by administering anti-CD40 mAb to TCDD-treated mice, in hopes of generating activated APC and ultimately restoring T cell effector functions. Surprisingly, no beneficial effects were detected on day 10 allograft effector responses in TCDD-treated mice which had received anti-CD40. These results were completely unexpected since the administration of CD40 antibodies had impressive effects on increasing the expression of costimulatory molecules and the production of systemic IL-12.

Several possibilities exist to explain the persistent TCDD-induced suppression of the allograft response in mice administered antibodies to CD40. First, although we measured anti-CD40 induced increases in APC expression of CD86, CD54 and MHC II, it is possible that TCDD is affecting the expression of other accessory molecules found on APC which are critical for T cell activation. The expression of several accessory molecules such as OX40L, CD70 and 4-1BBL has recently been reported to be induced on activated APC and to play a role in T cell activation and survival (Gramaglia et al., 1998; Saoulli et al., 1998; Akiba et al., 1999; Takahashi et al., 1999). If ligation of CD40 did not induce the expression of these additional molecules, or if TCDD suppressed their induction following anti-CD40 treatment, then the APC might not become sufficiently activated and remain unable to activate naive T cells. An analogous situation could exist for TCDD-suppression of cytokines produced by APC which are needed for generation of the allograft effector response. Although ligation of CD40 induced significantly increased production of systemic IL-12 in mice, it remains a possibility that additional cytokines are required in this response. The role of IL-12 in the P815 model was ambiguous following the observation that TCDD-exposed mice treated with anti-CD40 produced high-levels of IL-12 in their plasma without any beneficial effect on the generation of allograft effector responses. It is possible that other proinflammatory cytokines such as IL-18, IL-1 or TNF are essential for the generation of allograft effector responses. Also, it has recently been suggested that the expression of the IL-12 receptor (IL-12Rß2) is critical for the generation

of Th1 effector cells (Szabo et al., 1997; O'Garra, 1998). Therefore, it is possible that TCDD is affecting the expression of this cytokine receptor on T cells, thus rendering them unable to respond to increasing amounts of IL-12. Current studies are in progress to further refine the role of IL-12 and its receptor, as well as other APC-derived cytokines in the P815 allograft model.

Second, we did not address the functional capacity of the activated APC generated in P815-immunized mice which had received anti-CD40 treatment. Several reports have suggested that premature activation of APC prior to antigen encounter compromises their ability to internalize antigens (De Smedt et al., 1996; Winzler et al., 1997; De Smedt et al., 1998; Leenen et al., 1998). Recent studies from our laboratory have characterized an increase in costimulatory molecule expression on dendritic cells from TCDD-treated mice which have not been exposed to antigen (B.A. Vorderstrasse and N.I. Kerkvliet, manuscript in preparation). These results suggest that TCDD might be inappropriately activating APC and compromising their ability to process and present antigens. However, this possibility remains to be proven. Likewise, it is conceivable that anti-CD40 treatment of mice exposed to TCDD effectively upregulates essential accessory molecules on APC but does not restore their ability to process and present alloantigens to antigen-specific T cells. In this scenario, anti-CD40 would generate activated APC in TCDD-treated mice which would be void of antigen and thus unable to initiate a subsequent immune response. Therefore, future studies are needed to investigate what effects, if any, TCDD might be having on the processing and presentation of antigens by APC.

Third, the administration of anti-CD40 mAb is unlikely to completely mimic CD154 ligation by activated T cells. In addition to stimulating APC via ligation of CD40, activated T cells express other adhesion/costimulatory molecules and cytokines that could influence APC activation. Also, the timing of the anti-CD40 treatments in our studies may not have mimicked the normal expression of CD154 on the activated T cells or their interactions with the APC. Therefore, by ligating CD40 inappropriately on APC, we may have adversely

affected APC activation in TCDD-treated mice.

Fourth, the suppression of allograft immunity in TCDD-treated mice could possibly be occurring via direct effects on the T cells themselves. We have recently demonstrated that CD154:CD40 interactions are essential for the generation of allograft immunity in the P815 model (Shepherd and Kerkvliet, 1999). We suggested that the lack of CTL activity in CD154-/- mice injected with P815 cells was possibly due to the absence of signalling into the T cell via CD154. Also, it is interesting to note in the P815 model, that TCDD treatment suppressed the splenic expression of CD154 mRNA on days 7-9 post-immunization (D.M.S, unpublished observation). Therefore, it is plausible to consider that TCDD decreases CD154 expression on T cells to levels that are insufficient to transduce activation signals necessary for full activation of these antigen-specific cells. These results are consistent with an inability of anti-CD40 treatment to restore T cell-mediated allograft effector responses in TCDD-treated mice while providing stimulation for APC. Interestingly, allograft effector functions were also not restored in CD154-/- mice that had been injected with P815 cells and administered anti-CD40 suggesting that a costimulatory signal through CD154 is essential for T cell activation in allogeneic immune responses (Shepherd and Kerkvliet, 1999). Thus, further studies directed at defining possible direct effects of TCDD on both antigen presenting cells and T cells should provide further insight into possible mechanisms of TCDD-induced immune suppression.

# CHAPTER III

## DISRUPTION OF CD154:CD40 BLOCKS GENERATION OF ALLOGRAFT IMMUNITY WITHOUT AFFECTING APC ACTIVATION

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## ABSTRACT

CD154 (CD40L, gp39) interaction with its receptor CD40 has been shown to be critically important for the generation of cell-mediated as well as humoral immunity. It has been proposed that ligation of CD40 on APCs, presumably by activated Th cells, leads to increased APC function as defined by upregulation of costimulatory molecules and enhancement of IL-12 production. In this report, we directly examined the contribution of the CD154:CD40 pathway in a murine model of allograft rejection. Generation of both the CTL and alloantibody responses following injection with allogeneic P815 tumor cells was severely compromised in CD154 knockout mice and wild-type C57Bl/6 mice treated with the anti-CD154 mAb, MR1. Splenic production of IL-2, IFN-γ and TNF was significantly suppressed from CD154-deficient mice, indicating a lack of T cell priming. However, splenic cells from CD154 knockout mice induced comparable levels of CD86 expression and IL-12 production when compared to their wild-type littermates. The treatment of $CD154^{-/-}$ mice with the agonistic anti-CD40 mAb, FGK45, generated activated APCs yet failed to restore either the CTL or alloantibody responses to P815. Likewise, immunization with B7-transfected P815 tumor cells failed to generate expansion of the CTL effector population in $CD154^{-/-}$ mice. These results suggest that the generation of allograft immunity is dependent on the interaction of CD154 with CD40 but not primarily for the activation of APCs.


## INTRODUCTION

It is widely accepted that activation of naive T cells requires at least two signals: Ag/MHC stimulation of the TCR/CD3 complex and costimulation (Janeway and Bottomly, 1994; Jenkins, 1994). The interaction of B7 molecules on APCs with their counter-receptor, CD28, on T cells is believed to be the primary form of costimulation. However, other accessory molecules such as CD54/LFA-1 and CD2/LFA-3 also contribute significantly to T cell activation through adhesive interactions (Springer, 1990).

Additionally, the differentiation of activated T lymphocytes into competent effector cells is dependent on cytokines (Paul and Seder, 1994). Both IL-12 and IL-4 have been shown to direct the development of T cells into a Th1 or Th2 phenotype, respectively. Disruption of either costimulation or differentiation of Ag-activated T cells ultimately leads to a conditional unresponsiveness termed tolerance, and a compromised state of immunity (Schwartz, 1990; Guerder et al., 1994).

CD154 is a member of the tumor necrosis factor (TNF) family (Armitage et al., 1992; Noelle et al., 1992), which includes TNF-α, CD95L, LTα, LTß, and the ligands for CD27, CD30, OX-40, and 4-1BB (Baker and Reddy, 1996). Expression of CD154 is found primarily on activated CD4+ T cells (Roy et al., 1993), but has also been reported on CD8+ T cells, mast cells, and basophils (Gauchat et al., 1993; Roy et al., 1993; Hermann et al., 1995; Sad et al., 1997). CD40, the receptor for CD154, is expressed on APCs such as B cells (Stamenkovic et al., 1989), macrophages (Alderson et al., 1993), and dendritic cells (Inaba et al., 1994). It is a member of the TNF receptor family (Stamenkovic et al., 1989), which includes TNF-RI (p55), TNF-RII (p75), CD95, CD27, CD30, OX-40, and 4-1BB (Stamenkovic et al., 1989; Baker and Reddy, 1996). The interaction of CD154 with CD40 has been identified as a major pathway for the activation of APC and is essential for the generation of many cell-mediated immune responses (Larsen and Pearson, 1997; Grewal and Flavell, 1998; Mackey et al., 1998). Disruption of this pathway leads to increased susceptibility to opportunistic pathogens, illustrated by the prevalence of *Pneumocystis* and *Cryptosporidium* infections in hyper-IgM syndrome (HIGM1) patients who fail to express functional CD154 (Notarangelo et al., 1992; Hayward et al., 1997). Experimental animal models to investigate this immunodeficiency have been established by administering CD40-Ig fusion protein or antagonistic CD154 antibodies to block CD154:CD40 interactions (Grewal and Flavell, 1998). Recently, the creation of CD154-

and CD40-knockout mice have allowed further investigation of the important role of this pathway in the development of effective immunity (Kawabe et al., 1994; Xu et al., 1994).

It has been shown that CD40 ligation induces accessory molecules such as CD86, CD80, CD54 and MHC II on APC populations (Ranheim and Kipps, 1993; Roy et al., 1993; Grewal and Flavell, 1996). It has also been determined that stimulation via CD40 induces secretion of IL-12 from both dendritic cells and macrophages (Cella et al., 1996; Kato et al., 1996; Koch et al., 1996). Recently, several reports suggested that a primary role of activated T helper cells in the generation of CD4-dependent cell-mediated immunity was to provide CD40 ligation on APC, presumably to increase B7 expression and IL-12 secretion (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). This activation step then empowered the APC to successfully activate $CD8^+$ cells and drive their differentiation into CTL effectors. Therefore, we hypothesized that a lack of CD154:CD40 interaction would prevent generation of allograft immunity by failing to increase B7 expression and induce IL-12 secretion from APC. To test this hypothesis, we utilized the P815 tumor allograft model and followed the activation of B cells, macrophages, and dendritic cells from $CD154^{-/-}$ and wild-type mice over the course of the allogeneic response. However, despite a significantly reduced capacity of CD154-knockout mice to generate alloimmunity, we found no differences in the induction of costimulatory molecule expression or IL-12 secretion between $CD154^{+/+}$ and $CD154^{-/-}$ mice. These results suggest that the generation of allograft immunity is dependent on the interaction of CD154 with CD40 but not primarily for the activation of APCs.

# MATERIALS AND METHODS

## Animals

Five- to six-week old male C57Bl/6, CD154-/- (B6,129-Cd40l), CD154+/+ wild-type (B6,129 $F_2$), and female DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in front of a laminar flow unit.

## P815 mastocytoma cells

Wild-type P815 tumor cells, derived from a methylcholantherene-induced mastocytoma were maintained by weekly passage in DBA/2 mice. CD86-transfected P815 (clone HTR.C/B7-2) and its vector-transfected control (clone HTR.C/C) were generously provided by Dr. Thomas Gajewski (University of Chicago Medical Center, Chicago, IL). Transfected cells were maintained in vitro in RPMI 1640 medium supplemented with 10% FBS (Hyclone; Logan, UT) and 1 mM gentamicin at 37° in 5% $CO_2$.

## Reagents

HPLC-purified, antagonistic anti-CD154 mAb (MR1) used for in vivo blocking experiments, anti-CD4 (GK1.5) used for depletion studies, and agonistic anti-CD40 mAb (FGK45) used for exogenous CD40 ligation experiments were all kindly provided by Dr. Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH). Hamster IgG and rat IgG were purchased from Cappel (Organon Teknika, West Chester, PA) and used as Ig controls for the in vivo blocking and CD40 ligation experiments, respectively. Spleen cell phenotypes were determined by flow cytometric analysis using the following Abs: FITC-conjugated anti-CD54 (3E2), FITC-conj. anti-CD11c (HL3), FITC-conj. anti-CD62L (MEL-14), PE-conj. anti CD86 (GL1), PE-conj. anti-CD44 (IM7), PE-conj. anti-CD54, Biotin-conj. anti-$IA^b$ (AF6-120.1), CyChrome-conj. anti-B220 (RA3-6B2),

and CyChrome-conj. anti-CD8α (53-6.7) from PharMingen (San Diego, CA); Tri-color conj. anti-Mac-1 (M1/70.15) from Caltag Labs (Burlingame, CA), and the second step reagent streptavidin-Red613 from Gibco BRL (Gaithersburg, MD). For cytokine ELISA assays, capture and biotinylated detection antibody pairs and their respective standards were purchased from PharMingen, except for IL-12 which was purchased from R&D Systems (Minneapolis, MN) and IL-4 which was obtained from Genzyme (Cambridge, MA).

**Animal treatments**

Studies to evaluate the role of CD154 in allograft rejection were conducted in CD154-/- mice and their wild-type littermates. Alternatively, C57Bl/6 mice were treated i.p. with 250 μg of MR1 on day 0 relative to P815 immunization to block CD154:CD40 interactions. In vivo depletion of CD4+ T cells was performed by injecting mice with 0.25 mg GK1.5 on day -2 relative to CD86-P815 injection. Preliminary studies determined that >99% of naive ($CD44^{high}CD45RB^{low}$) CD4+ T cells were depleted for at least 10 days using this treatment protocol. Mice were injected i.p. with either $1x10^7$ tumor ascites P815 or CD86-transfected P815 cells on day 0. The protocol for providing exogenous ligation of CD40 involved i.p. injection of both CD154-/- and +/+ mice with 5 μg of FGK45 on days 0, 2, 4, 6, and 8 relative to P815 injection. All mice were sacrificed by $CO_2$ asphyxiation on days 5-8, or 10 relative to injection. Spleens were removed aseptically and blood was collected by heart puncture into heparinized syringes. Plasma was separated by centrifugation and stored at -70°C.

**Preparation of spleen cells**

Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Erythrocytes were removed by hypotonic lysis. Cells

were washed once and resuspended in cold HBSS/5% FBS with 20 mM HEPES, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate.

## Flow cytometric analysis of spleen cells

Expression of the cell-surface proteins CD86, CD54, and I-$A^b$ was determined from viable spleen cells and then further measured on gated populations of $B220^+$, Mac-$1^+$, and $CD11c^+$ cells. A distinct population of spleen cells expressing high levels of CD11c ($CD11c^{hi}$) was determined to represent dendritic cells by profiles of costimulatory molecule expression. These $CD11c^{hi}$ cells constitutively expressed high levels of CD86, CD54, and I-$A^b$ similar to levels found on dendritic cells enriched over BSA-"dense" gradients as previously described (Nussenzweig and Steinman, 1980). In a typical splenic preparation, 1.5-3.3% of the cells were $CD11c^{hi}$ of which >88.4% were $CD86^+$, >99.5% were $CD54^+$, and >95.5% were I-$A^+$. CTL effectors ($CTL_E$) were identified by first gating on the $CD8^+$ T cells and then identifying the $CD44^{hi}/CD62L^{lo}$ population, as previously described (Mobley and Dailey, 1992). Nonspecific binding was blocked with 10 μg of rat IgG, and then the cells were stained with optimal concentrations of fluorochrome-conjugated mAb. Appropriately labeled, isotype-matched Igs were used as controls for nonspecific fluorescence. 20,000 to 100,000 events were collected by listmode acquisition from freshly-stained cells using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WinList (Verity Software House, Topsham, ME).

## CTL assay

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

1996). The percentage of cytotoxicity at each effector:target (E:T) ratio was calculated using the following equation:

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

where Er = the experimental release using spleen cells from P815-immunized mice, Nr = the nonspecific release using splenocytes from naive mice, Mr = maximal release of $^{51}Cr$ from cells incubated with 1% SDS, and Sr = spontaneous release of $^{51}Cr$ incubated in medium alone. Duplicate wells were tested at E:T ratios of 200:1 to 6.25:1.

## 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 two-fold dilutions (1/10 to 1/2560) of heat-inactivated plasma were incubated with $^{51}Cr$-labeled P815 cells for 20 min at 37°C in 5% $CO_2$. Low-Tox-M rabbit complement (1/12, Cedarlane Laboratories, Hornby, Ontario, Canada) was added for 45 min at 37°C. The amount of $^{51}Cr$ released into the supernatant was measured by gamma counting and specific activity was calculated. All samples were tested in duplicate on separate plates. The Ab titer was defined as the highest dilution of plasma at which a minimum of 20% specific cytotoxicity was measured.

## Cytokine analysis

Spleen cells ($1x10^7$) were incubated in RPMI/10% FBS supplemented with 1.5 mM sodium pyruvate, 20 mM HEPES, and 50 μg/ml gentamicin for 6 h with $1x10^6$ P815 tumor cells at 37°C in 5% $CO_2$ in 1.5 ml polypropylene Eppendorf tubes that were silicone-treated to prevent nonspecific production of IL-12 by adherent spleen cells. Supernatant cultures from P815 tumor cells alone and naive spleen cells with P815 were

also collected as controls for each experiment. Levels of IL-2, IL-4, IL-12, and IFN-γ were determined for each supernatant by using specific Ab sandwich ELISAs. Secondary biotinylated detection reagents were complexed with avidin-peroxidase and visualized with ABTS substrate. Absorbance was measured at 405 nm using a plate reader (Bio-Tek Instruments, Wincoski, VT) and cytokine values were then determined using Immunosoft software (Dynatech Labs, Alexandria, VA). TNF levels were measured by standard bioassay utilizing L929 fibroblasts (Kerkvliet et al., 1996).

### Statistical Analysis

Results are presented as the mean ± SE of six mice per group unless indicated otherwise. Most experiments were repeated at least once. Analysis of variance modeling was performed using Statview statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using the least significant difference multiple comparison *t* test or Dunnet's *t* test for pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### Disruption of the CD154:CD40 pathway suppresses generation of allograft immunity in the P815 model

Previous studies have established the importance of CD154 in the generation of cell-mediated immunity, including models of allograft rejection (Hancock et al., 1996; Larsen et al., 1996). We investigated the role of CD154 in the generation of immune responsiveness to allogeneic P815 tumor cells. Following immunization of C57Bl/6 mice with P815 tumor cells, the concomitant development of CTL and alloantibody responses effectively rejects these allogeneic cells. The allograft response peaks within 10 days of

immunization and correlates with the generation of CTL activity specific for H2-$D^d$ bearing cells (Kerkvliet et al., 1996), and the emergence of alloantibodies in the plasma (Figures III-1A and III-1B). CD154 knockout mice failed to generate allo-CTL activity following immunization with P815 tumor cells (Figure III-1A). Similar results were also observed in P815-immunized $CD154^{+/+}$ mice which had been treated with the anti-CD154 blocking antibody, MR1. Furthermore, the production of cytotoxic alloantibody was suppressed by greater than 90% in both the $CD154^{-/-}$ and MR1-treated mice when compared to their appropriate controls (Figure III-1B). Although it has already been established that IgM is the isotype primarily responsible for cytotoxicity in this alloantibody assay, we further evaluated the effect of blocking CD154:CD40 interactions on the generation of other alloantibody isotypes. Consistent with previously published reports which illustrate the critical role of CD154 in the generation of humoral immunity (Kawabe et al., 1994; Renshaw et al., 1994; Xu et al., 1994), blocking CD154 ligation of CD40 resulted in significant suppression of not only IgM, but also IgG2a, and IgG1 anti-P815 antibodies, as measured by flow cytometry (data not shown).

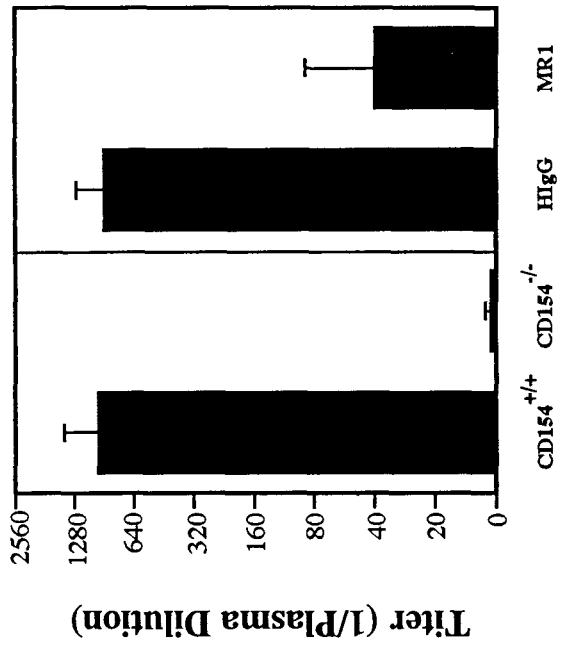
**Lack of T cell priming occurs in mice deficient of CD154**

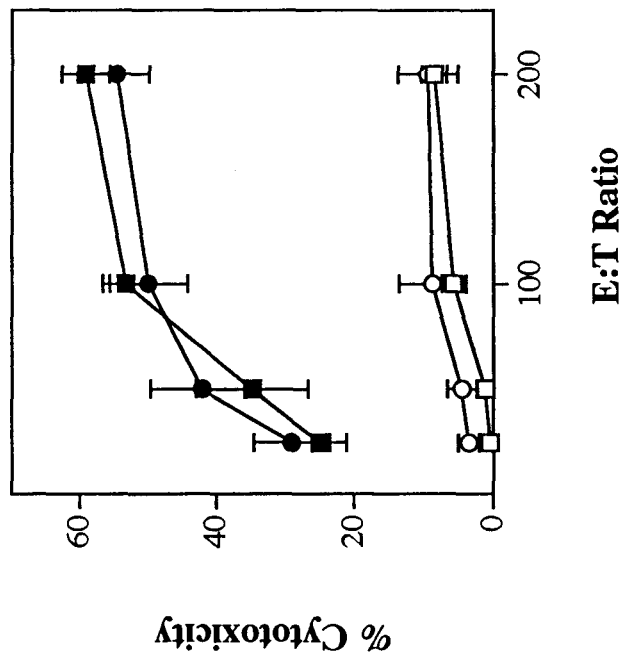
The inability to mount an effective CTL response to allogeneic P815 cells could be due to either lack of priming of T cells or defective effector function. Therefore, we analyzed the splenic $CD8^+$ cells from P815-immunized $CD154^{-/-}$ and $CD154^{+/+}$ mice for the generation of cells bearing the CTL effector ($CTL_E$) phenotype. Expression of the $CD8/CD44^{high}/CD62L^{low}$ phenotype correlates with cytolytic activity, as shown previously by Mobley and Dailey (1992). Ten days after P815 immunization, $CD154^{+/+}$ mice had generated approximately $3x10^7$ $CTL_E$ while the number of effector CTLs detected from the spleens of $CD154^{-/-}$ mice did not differ significantly from that found in non-immunized

**Figure III-1. Disruption of CD154:CD40 suppresses the generation of allograft immunity.**

(A) Splenic CTL activity was measured from $CD154^{-/-}$ mice (open squares), $CD154^{+/+}$ mice (filled squares), and C57Bl/6 mice treated with 1.25 mg MR1 (open circles) or Hamster IgG (filled circles), 10 days after injection with $1x10^7$ allogeneic P815 tumor cells. (B) Cytotoxic alloantibody titers were determined on day 10 post-immunization from mice with functional ($CD154^{+/+}$ or HIgG treatment) or disrupted ($CD154^{-/-}$ or MR1 treatment) CD154:CD40. Data represent mean ± SEM of 6 mice/treatment group; one representative experiment of three is shown.

**Figure III-1.**


Titer (1/Plasma Dilution)
2560
1280
640
320
160
80
40
20
0
CD154+/+
CD154-/-
HIgG
MR1


% Cytotoxicity
60
40
20
0
100
200
E:T Ratio

mice (Figure III-2A). These results indicate that the lack of CTL activity in CD154$^{-/-}$ mice was due to an absence of $CTL_E$ and not an inability of CTL cells to perform their effector function.

To evaluate whether T cells from immunized CD154-knockout and wild-type mice were primed, we measured cytokine production in this response. We have previously shown in the P815 model that 6-hour restimulation of immunized spleen cells permits detection of IL-2, IFN-γ, and TNF on days 5-10 following immunization (Kerkvliet et al., 1996). As shown in Figures III-2B-D, increased levels of IFN-γ and TNF were observed on days 6-8, and increased IL-2 production on days 5-8 from wild-type but not CD154-deficient mice. The reduced cytokine production from knockout mice continued through day 10 (data not shown) when both the CTL and alloantibody assays were performed. Additionally, the production of IL-4 was barely detectable on any day tested (data not shown) and did not differ between wild-type and knockout mice. These results indicate that a lack of CD154:CD40 interaction prevents proper T cell priming although not via immune deviation (the switch from a beneficial Th-1 type of response to an inappropriate Th-2 type) as previously reported by Hancock et al. (1996) in the cardiac allograft model.

## APC activation is unaffected in CD154-deficient mice following immunization with P815 tumor cells

To examine if disruption of the CD154:CD40 pathway would render mice incapable of priming alloantigen-specific T cells by failing to activate APC, we analyzed the production of IL-12 and induction of costimulatory molecules from the spleen cells of immunized mice. Ligation of CD40 has been shown to induce IL-12 production, primarily from APC, thereby regulating the differentiation of activated T cells into Th1 effector cells and promoting cell-mediated immunity (Stuber et al., 1996). Due to the considerable

**Figure III-2. T cell priming to allogeneic P815 tumor cells requires CD154.**

(A) Number of splenic $CTL_E$ ($CD8^+/CD62L^{low}/CD44^{high}$) cells was measured from $CD154^{-/-}$, $CD154^{+/+}$, and non-immunized mice on day 10 relative to P815 injection. *Ex vivo* cytokine production was measured from CD154-/- (open circles), CD154+/+ (filled circles), or non-immune mice (filled triangle) during the allogeneic response to P815 tumor cells. Spleen cells ($1x10^7$) were restimulated with $1x10^6$ P815 tumor cells for 6-hours and the culture supernatants were assayed for production of IL-2 (B) and IFN-γ (C) by cytokine-specific ELISAs, or TNF (D) by L929 bioassay. Data points represent the mean ± SEM for duplicate samples from six mice/treatment group. P815 cells cultured alone for 6 h produced < 2 pg/ml of TNF and undetectable amounts of IL-2 or IFN-γ.

A
# $CTL_E$ ($1x10^{-6}$)
30
20
10
0
Wild Type
CD154 -/-
Naive


B
IL-2 (pg/ml)
600
400
200
0
0 5 6 7 8
Day After P815 Injection


C
IFN-γ (pg/ml)
12000
8000
4000
0
0 5 6 7 8
Day After P815 Injection


D
TNF (pg/ml)
20
15
10
5
0
0 5 6 7 8
Day After P815 Injection

Figure III-2.

influence CD40-mediated IL-12 production has on priming Th1 effector T cells and the absence of Th1 priming previously described in CD154-deficient mice, we examined the ability of spleen cells from P815 immunized mice to produce IL-12. As shown in Figure III-3A, spleen cells from both CD154 knockout and wild-type mice produced comparable levels of IL-12 on days 5-8 post-immunization. Although levels were higher in cultures from wild-type mice on day 8, these levels were not significantly different from those produced by CD154 knockout mice, a relationship that continued through day 10 (data not shown).

Previously, we reported that spleen cells from C57Bl/6 mice increase their expression of CD86 on days 5-8 following immunization with P815 tumor cells (Prell and Kerkvliet, 1997). Furthermore, mAb blocking experiments demonstrated that CD86 was the primary B7 molecule involved in this allograft model as treatment with anti-CD86 rendered mice unresponsive to P815 challenge while administration of anti-CD80 did not. Therefore, we evaluated the role of CD154 in the induction of CD86 on spleen cells after P815 injection. As shown in Figure III-3B, comparable CD86 expression was exhibited on days 5 through 8 from $CD154^{+/+}$ and $CD154^{-/-}$ mice. Similarly, CD154-deficiency did not alter normal Ag-induced increases in CD54 or $I\text{-}A^{b}$ expression on spleen cells on days 5 through 8 (data not shown). As it is unknown exactly which splenic APC population is responsible for the activation of allo-specific T cells in the P815 model, expression of accessory molecules on $B220^{+}$, $Mac\text{-}1^{+}$ and $CD11c^{+}$ cells was determined. Shown in representative histograms in Figures III-3C-E, CD154 deficiency did not alter CD86 expression on any of these APC populations following P815 immunization. Histograms are shown for day 6 data, but are representative of all days (5-8) tested . Antigen-induced increases in splenic CD86 expression were observed to be slight on the B cells (mean channel fluorescence (MCF= 9), significant for the dendritic cells (MCF= 44), while decreased expression was exhibited on the macrophage population (MCF= 5.5). This apparent reduction in CD86 expression on $Mac\text{-}1^{+}$ cells following P815 injection may be

**Figure III-3. Disruption of CD154:CD40 does not alter APC activation, as defined by splenic IL-12 production and CD86 expression.**

(A) The *ex vivo* production of IL-12 was measured by ELISA from 6-hour restimulated spleen cell culture supernatants from $CD154^{-/-}$ (open circles), $CD154^{+/+}$ (filled circles), or non-immune mice (filled triangle) over the course of the P815 response. (B) Splenic CD86 expression from non-immunized, or P815-immunized $CD154^{-/-}$ and $CD154^{+/+}$ mice was determined for days 5-8 by flow cytometric analysis. (C) Representative histograms showing day 6 expression of CD86 from $CD154^{-/-}$ (dashed line), $CD154^{+/+}$ (solid line), or non-immune (dotted line) from gated populations of $B220^{+}$, Mac-$1^{+}$, and $CD11c^{+}$ spleen cells. Also, represented is the isotype-specific control antibody sample (filled area). Data is shown from one of 6 representative mice/treatment group.

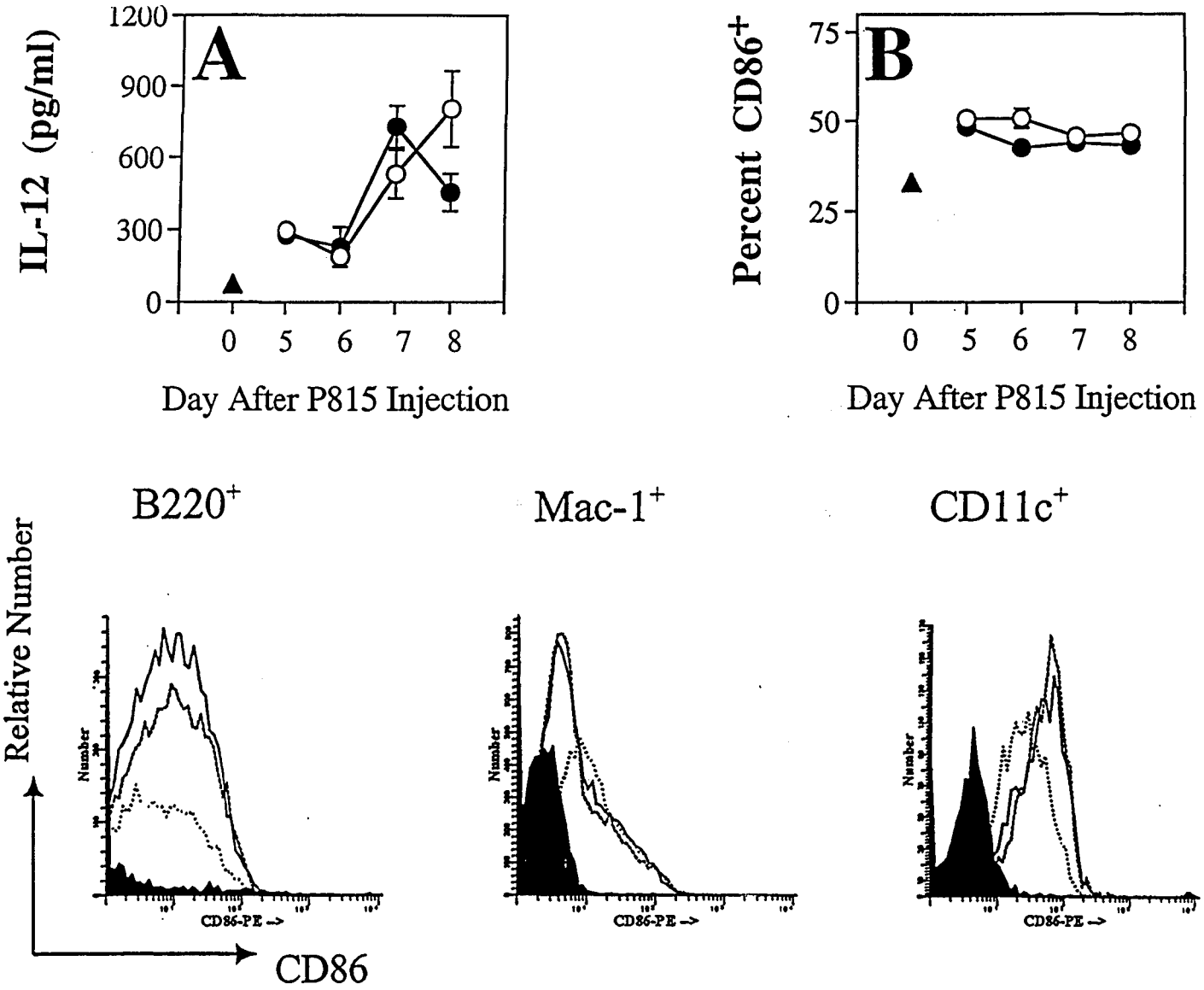

A
IL-12 (pg/ml)
1200
900
600
300
0
0 5 6 7 8
Day After P815 Injection
B
Percent CD86+
75
50
25
0
0 5 6 7 8
Day After P815 Injection
C
B220+
Mac-1+
CD11c+
Relative Number
Number
CD86-PE -->
CD86

Figure III-3.

attributable to an influx of Mac-1+ cells into the spleen (2% in naive mice versus 10% in mice injected with P815. However, this increased Mac-1+ population was seen in both the wild-type as well as the knockout mice, yielding no explanation for the absence of T cell priming in mice defective for CD154. Similar patterns of CD54 and I-$A^b$ expression were also detected on days 5-8 for each of the splenic APC populations examined without significant effects of CD154-deficiency (data not shown). Although these markers of APC activation are by no means exhaustive, the data taken together fail to establish any measurable differences in CD40-mediated APC activation between P815-immunized CD154-deficient or wild-type mice. The apparent lack of effect of the CD154 mutation on induction of the accessory molecules CD86, CD54 and I-$A^b$, combined with an unaltered capacity to produce IL-12 in the knockout mice suggests that CD40 interaction with its ligand is not an essential requirement for activation of APC in this model of allograft immunity.

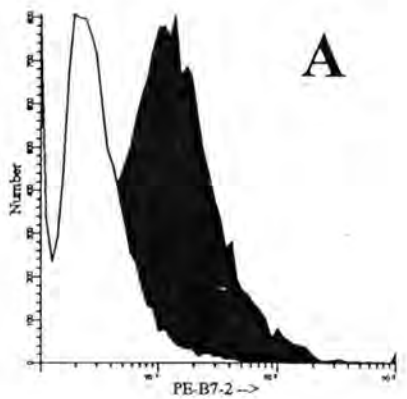
## Exogenous ligation of CD40 fails to restore allograft immunity in CD154-deficient mice

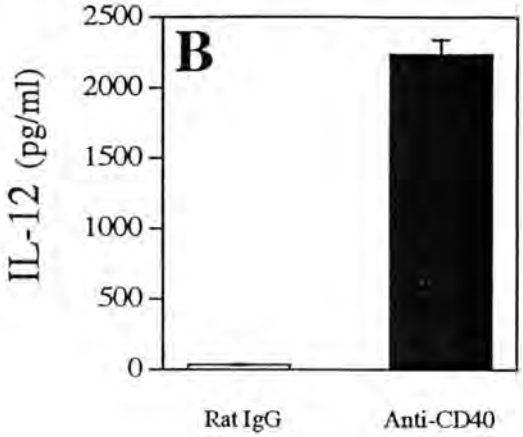
Since CD154$^{-/-}$ mice are genetically incapable of expressing functional CD154, we hypothesized that exogenous ligation of CD40 would circumvent the need for its ligand and restore allograft effector functions in these mice. To test this hypothesis, both wild-type and CD154-deficient mice were immunized with P815 tumor cells and then administered FGK45, an agonistic anti-murine CD40 mAb. Treatment of mice with the 5 μg dose of FGK45 produced highly elevated expression of CD86 on splenic APC and significantly increased plasma levels of IL-12 within 48 hours (Figures III-4A and III-4B). Surprisingly, exogenous ligation of CD40 with FGK45 did not restore CTL activity in CD154$^{-/-}$ mice to levels seen from wild-type mice (Figure III-4C). Although a small increase in CTL activity was observed in CD154$^{-/-}$ mice which had received anti-CD40

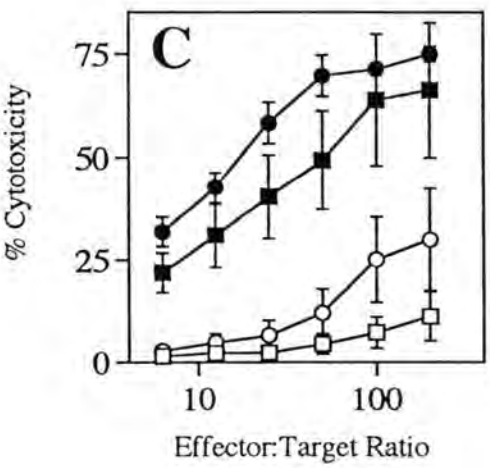
**Figure III-4. Addition of agonistic anti-CD40 mAb fails to restore allo-CTL or cytotoxic alloantibody activity in CD154-deficient mice.**

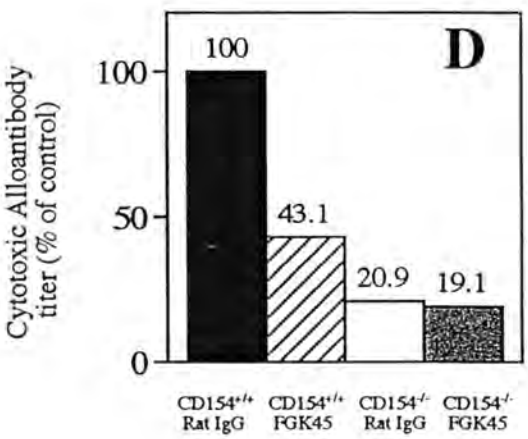
Splenic CD86 expression (A) or plasma IL-12 levels (B) were determined from C57Bl/6 mice treated with 5 μg FGK45 for 2 days. 10 days after P815 injection, (C) CTL, or (D) cytotoxic alloantibody activity was measured from $CD154^{-/-}$ (open symbols) and $CD154^{+/+}$ (filled symbols) mice treated on days 0, 2, 4, 6, and 8 with either 5 μg FGK45 (circles) or Rat IgG (squares). Data represent mean ± SEM of 6 mice/treatment group for CTL activity and comparison of cytotoxic antibodies from all mice relative to the $CD154^{+/+}$ control mice receiving Rat IgG treatment.

**Figure III-4.**


A
Number
PE-B7-2 -->


B
IL-12 (pg/ml)
2500
2000
1500
1000
500
0
Rat IgG
Anti-CD40


C
% Cytotoxicity
75
50
25
0
10
100
Effector:Target Ratio


D
Cytotoxic Alloantibody titer (% of control)
100
50
0
100
43.1
20.9
19.1
CD154+/+ Rat IgG
CD154+/+ FGK45
CD154-/- Rat IgG
CD154-/- FGK45

treatment, this increase was not statistically significant at any of the effector:target ratios examined. It is important to note that splenic T cells from CD154$^{-/-}$ mice are not intrinsically incapable of generating CTL effector cells as shown in a recent report by Buhlmann et al. (1999). In this report, spleen cells from CD154$^{-/-}$ and wild-type mice generated comparable CTL activity after being cultured in vitro for 6 days with allogeneic stimulator cells. In addition to a lack of restoration of the CTL response by anti-CD40, no enhancement of cytotoxic alloantibody plasma titers was observed in anti-CD40 treated CD154$^{-/-}$ mice when compared to the appropriate controls (Figure III-4D). Interestingly, treatment of CD154$^{+/+}$ mice with a 5 μg dose of FGK45 partially suppressed the generation of cytotoxic alloantibodies on day 10 post-P815 immunization, yet had no adverse effect on CTL activity. The dose of FGK45 administered to mice in these experiments proved to be of critical importance as higher amounts of anti-CD40 mAb (25 and 100 μg) produced moderate to severe organ-specific toxicity including thymic atrophy, hepatic coagulative necrosis and neutrophilia, and splenic lymphoproliferation within the lymphoid follicles (D.M.S. and O.R. Hedstrom, unpublished observations). Ultimately, any beneficial effects of increasing costimulatory molecule expression and systemic IL-12 production by APCs following anti-CD40 treatment failed to enhance the generation of allograft effector function.

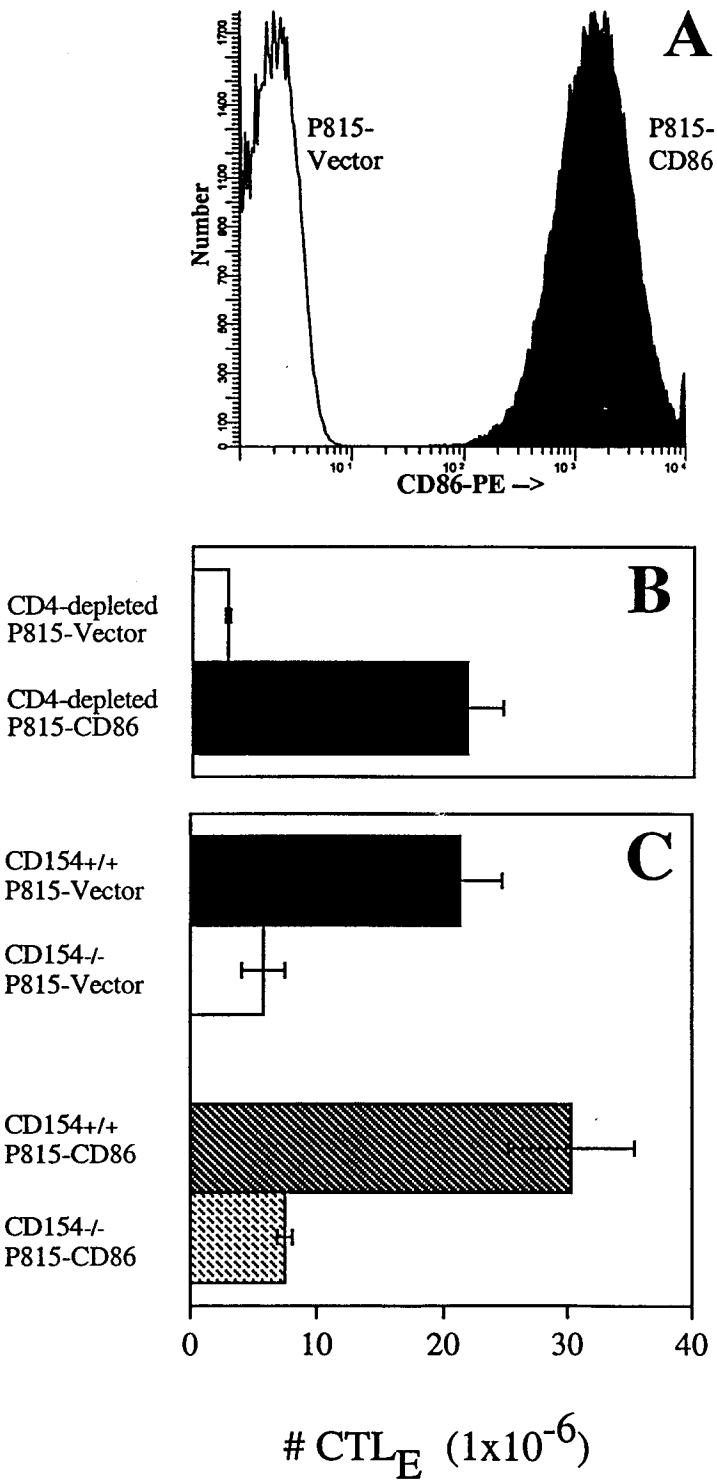
## Injection of B7-transfected P815 tumor cells fails to generate CTLE in CD154-knockout mice

We have previously reported that C57Bl/6 mice depleted of CD4+ T cells failed to generate CTL activity following P815 immunization (Kerkvliet et al., 1996). However, when P815 cells transfected to express high levels of murine CD86 (Figure III-5A) were used to immunize CD4-depleted mice, increased numbers of CTL effector cells were generated when compared to CD4-depleted mice injected with vector-transfected P815 cells

**Figure III-5. Immunization of CD154$^{-/-}$ mice with CD86-transfected P815 tumor cells fails to increase numbers of $CTL_E$.**

CD86 expression was evaluated on P815-vector and P815-CD86 tumor cells (A). Splenic $CTL_E$ numbers were determined by flow cytometric methods from CD4-depleted mice (B), or CD154$^{-/-}$ and CD154$^{+/+}$ mice (C) immunized with either $1x10^7$ P815-vector or P815-CD86 tumor cells, 9 or 10 days post-immunization, respectively. Data represent the mean ± SEM of 4-6 mice/treatment group.

**Figure III-5.**


A
P815-
Vector
P815-
CD86
Number
CD86-PE –>
B
CD4-depleted
P815-Vector
CD4-depleted
P815-CD86
C
CD154+/+
P815-Vector
CD154-/-
P815-Vector
CD154+/+
P815-CD86
CD154-/-
P815-CD86
0
10
20
30
40
# CTL$_E$ (1x10$^{-6}$)

(Figure III-5B). The dual expression of both H2-$D^d$ (signal one) and B7 (signal two) provides necessary stimulation of alloreactive CD8+ cells to drive their differentiation from precursor CTL (CTLp) into $CTL_E$ possessing full cytolytic activity. To examine a possible role for CD154 in CD8+ T cell activation, we immunized $CD154^{-/-}$ mice with B7-transfected P815 tumor cells. By providing the requisite two signals (Ag and costimulation) for T cell activation, CD154 knockout mice (similar to CD4-depleted mice) should be capable of generating $CTL_E$ unless there is a requirement for CD154 in this process. On day 10 post-immunization, the numbers of $CTL_E$ were determined for both $CD154^{-/-}$ and $CD154^{+/+}$ mice. In contrast to the results observed in CD4-depleted mice, $CD154^{-/-}$ mice injected with P815-CD86 failed to generate comparable numbers of $CTL_E$ when compared to wild-type mice which had received either P815-vector or P815-CD86 (Figure III-5C). Also, following immunization with either P815-vector or P815-CD86, $CD154^{-/-}$ mice failed to generate CTL activity or splenic IFN-γ production comparable to that measured from $CD154^{+/+}$ mice (data not shown).

## DISCUSSION

It is well-established that CD154:CD40 pathway is important in the generation of cell-mediated immunity (Grewal and Flavell, 1998; Mackey et al., 1998). For example, the increased susceptibility of patients with Hyper IgM syndrome to intracellular pathogens is highly suggestive of defective T cell immunity in the absence of functional CD154 expression (Hayward et al., 1997). Many experimental systems have also shown the critical role of this pathway in generating T-cell mediated immune responses. Increased susceptibility of CD154-deficient mice to both intra- and extra-cellular pathogens, as well as numerous viruses has been shown to be due to compromised Th1 immunity (Wiley and

Harmsen, 1995; Campbell et al., 1996; Kamanaka et al., 1996; Lu et al., 1996; Soong et al., 1996). Additionally, models of allogeneic immunity have also been shown to depend on successful interaction of CD40 with CD154. In a model of graft-vs-host disease (GVHD), it was shown that $F_1$ recipients which received T cells from CD154$^{-/-}$ mice failed to succumb to GVHD unlike recipients injected with wild-type T cells (Buhlmann and Noelle, 1996). Similarly, in a study by Parker et al. (1995) which evaluated the role of CD154:CD40 in a transplantation model, blocking CD154:CD40 led to enhanced tolerance in chemically-induced diabetic mice. Finally, in several studies examining the role of CD154 in allograft rejection, disruption of the CD154:CD40 pathway conferred increased acceptance of cardiac allografts (Hancock et al., 1996; Larsen et al., 1996; Niimi et al., 1998). Similar to the results presented in this report, generation of effector allograft immunity was critically dependent on the successful ligation of CD40 by CD154.

The mechanism by which disruption of the CD154:CD40 pathway suppresses cell-mediated immunity, however, varies depending on the experimental model examined. Our results show that immunization of CD154-deficient mice with allogeneic P815 cells failed to properly prime allo-specific T cells. Inadequate priming of T cells due to the absence of CD154 has been shown in several other models of T-dependent immunity. Studies by Grewal et al. (1995) demonstrated suppression of Ag-specific expansion and effector cytokine production by CD4$^+$ T cells from mice lacking CD154. Likewise, Gray and co-workers showed that T cells activated in the absence of CD40 were unable to help normal B cells undergo Ig class switching or germinal center formation (van Essen et al., 1995). While data exist implicating CD154 involvement in T helper cell priming, studies examining its role in priming of CD8$^+$ cells are less clear. A limited number of studies suggest that activation of CD8$^+$ CTLs following viral infection is unaffected in CD154$^{-/-}$ mice (Borrow et al., 1996; Oxenius et al., 1996; Whitmire et al., 1996). However, a role for CD154 was indicated for the maintenance of CTL memory cells, as the anti-viral memory CTL response was defective in mice deficient for CD154 (Borrow et al., 1996).

Currently, while the importance of CD154 in the generation of the allograft response is accepted, the precise role is unclear. In a study by Larsen et al. (1996), the predominant effect of CD154 blockade was found to be defective effector T cell function and not the priming of these cells. However, in a study by Hancock et al. (1996), T cells were found to have been inappropriately primed causing them to deviate from the normal Th1 phenotype into suppressive Th2 cells which produced increased levels of IL-4 and IL-10. In our model, failure to generate alloimmunity was due to defective T cell priming, and not due to immune deviation (as IL-4 levels were undetectable), or compromised T cell effector function (no $CTL_E$ were generated).

Currently, a potential explanation for the suppressed cell-mediated immunity observed in CD154-deficient mice revolves around inadequate APC activation attributable to deficient CD40 ligation. CD40 ligation has been shown to induce increased APC activity by upregulating costimulatory molecule expression and increasing the production of IL-12 as well as other proinflammatory cytokines (Ranheim and Kipps, 1993; Kiener et al., 1995; Cella et al., 1996; Koch et al., 1996). In CD154-deficient mice infected with an adenovirus, a lack of CD40 signalling resulted in a failure to generate both CTL and antibody responses (Yang and Wilson, 1996). Reduced expression of CD86 was noted on spleen cells from these adenovirus-infected CD154-knockout mice. Moreover, treatment of these animals with an agonistic anti-CD40 mAb restored their immune responsiveness, an outcome the authors attributed to increased CD86 expression. Likewise, in a murine model of EAE, Grewal et al. (1996b) showed that CD154-deficient mice which carried a transgenic TCR for myelin basic protein failed to generate the disease after antigenic immunization. Similar to the adenoviral model, injection of these mice with APC expressing transgenic-B7 allowed subsequent development of acute EAE indicating that CD154 was required for induction of costimulatory activity on the APC. These reports contrast with our results which suggest that APC activation is a CD40-independent event following immunization with allogeneic P815 tumor cells. Although some evidence exists

identifying the dendritic cell as the key APC in our model (Moser et al., 1995), we evaluated costimulatory profiles for all putative splenic APC populations to best identify any effect of CD154-deficiency. However, no differences in CD86, CD54, or I-$A^b$ induction were observed on the B220+, Mac-1+ or CD11c+ cells from CD154$^{-/-}$ and CD154$^{+/+}$ mice following P815 immunization. Additionally, no effect was measured in the ability of spleen cells from both types of mice to produce IL-12 following activation. Although in contrast to the adenoviral and EAE models, these results are consistent with three reports examining the role of CD154 in a cardiac allograft model, in which CD154-deficiency did not alter CD86 induction/expression (Hancock et al., 1996; Larsen et al., 1996; Niimi et al., 1998). The recent study by Niimi et al. (1998) clearly demonstrated the importance of the CD40 interaction in the immune response to alloantigen and suggested that the effect of this pathway may be independent of its effect on the B7/CD28 pathway. These data taken together suggest that dependency of CD40 ligation for APC activation may be related to characteristics inherent to specific antigens. Uptake of antigens such as viruses and soluble proteins by APCs may require CD40 ligation to induce competent costimulatory activity, whereas ingestion of particulate antigens such as allogeneic cells may sufficiently activate APC and therefore have less dependence on CD40. This would provide one potential explanation for the normal expression of costimulatory molecules on APCs from CD154$^{-/-}$ mice in models of allograft rejection but not in other experimental systems.

The failure to restore allograft immunity in CD154$^{-/-}$ mice immunized with P815 tumor cells and treated with an agonistic anti-CD40 mAb is an interesting result. If the only contribution of CD154 toward the generation of this alloimmune response was to provide ligation of CD40, then it should follow that providing that stimulus would restore allograft effector functions. This result did not occur in our studies, possibly because ligation of CD40 with agonistic antibodies did not reproduce the same physiological effects as CD154. Also, it is possible that signalling could be occurring directly into the T cells via

CD154 following ligation of CD40. Interestingly, many potentially therapeutic effects were observed in our mice receiving anti-CD40 treatment. These included induction of high levels of costimulatory molecule expression on splenic APC combined with an increased production of IL-12. Recently, it has been shown by Bennett et al. (1998) that similar anti-CD40 treatment of CD4-depleted mice provided necessary signals to induce competent cross-priming of Ag-exposed APC. It is conceivable then that exogenous ligation of CD40 in our system also enhanced cross-priming in our APC populations. However, no restoration of CTL activity was seen in the P815-immunized CD154-deficient mice which received anti-CD40 treatment suggesting that the defect may not reside in the APC but in the T cell. Our results differ from those reported by Yang and Wilson (1996), and several recent reports in which anti-CD40 mAb treatment of $CD154^{-/-}$ or CD4-depleted mice, restored CTL activity (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). All of these studies, however, evaluated CTL activity from anti-CD40-treated mice only after driving potential CTLp in vitro for approximately 5-7 days with antigen, APC, and even added growth factors, making it difficult to properly compare their results to those generated in our study. In addition to the lack of restorative effects of anti-CD40 treatment on CTL activity, we also observed no beneficial effects of this treatment on cytotoxic alloantibody production in CD154-deficient mice. Furthermore, $CD154^{+/+}$ mice treated with similar doses of FGK45 displayed partially suppressed titers of alloantibodies when compared to the rat IgG controls. This negative effect of anti-CD40 mAb treatment is likely due to excessive signalling of B cells through this receptor. Although ligation of CD40 has been shown to be a critical step in the generation of humoral immunity, hyperstimulation can lead to a lack of antibody production due to an arrest of B cell terminal differentiation (Randall et al., 1998). Therefore, caution should accompany any attempt to boost cell-mediated immunity in human HIGM1 or AIDS patients with anti-CD40 antibody (or any other reagent capable of ligating CD40) as it may be at the expense of the other arm of acquired immunity, the antibody response.

We have previously reported that generation of CTL activity in the P815 tumor allograft model is CD4-dependent (Kerkvliet et al., 1996). However, this dependency can be circumvented by immunizing CD4-depleted mice with B7-transfected P815 cells as shown in Fig. 5B. We assume that these tumor transfectants are capable of driving $CD8^+$ CTLp directly to become $CTL_E$ because they possess both antigen ($H2\text{-}D^d$) and costimulatory molecules (CD86) on their cell surface. Therefore, it was totally unexpected to find that CD86-P815 immunized CD154-deficient mice, in contrast to CD4-depleted mice, did not generate comparable numbers of $CTL_E$ to those observed in $CD154^{+/+}$ mice. The only difference in the $CD8^+$ cells from each of these mice was the inability of the CD154 knockout mice to express functional CD154. However, this singular genetic defect negated the ability of the P815-CD86 to generate competent CTL activity from these mice, or properly prime their T cells as reflected in the suppressed production of Th1 cytokines. The data reflect that mice deficient for CD154 are clearly not equivalent to CD4-depleted mice, possibly explaining the differences in our inability to generate cell-mediated immunity in $CD154^{-/-}$ mice treated with anti-CD40 when compared to previous reports performed in CD4-depleted mice (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998).

The possible function of CD154 to transduce intracellular signals into the T cell remains to be proven. Several studies have provided some evidence that CD154 may serve as a receptor capable of transducing a costimulatory signal into the T cell. In these reports, it has been suggested that CD154 is a viable signal transducing molecule in T cells which when ligated is capable of activating a sphingomyelinase and releasing ceramide (Koppenhoefer et al., 1997), and/or activating the kinases JNK/p38-K and PKC (Brenner et al., 1997a; Brenner et al., 1997b). A study by Blotta et al. (1996) demonstrated that cross-linking of CD3 and CD154 on $CD4^+$ T cells enhances IL-4 production. Functional studies aimed at increasing tumor surveillance in mice suggested that CD40-transfection of poorly immunogenic tumors greatly enhances their clearance (R.J. Noelle, unpublished

observations), possibly due to enhanced ligation of CD154 on CTLp. Similar results from Cayabyab et al. (1994) suggested that the CD40-CD154 costimulation pathway may have allowed for expansion of T cells after their interaction with CD40-bearing APCs. Recently, a study by Suzuki et al. (1998) demonstrated that reverse signalling through CD95L, another member of the TNF superfamily to which CD154 belongs, is required for alloantigen-specific CTLs to achieve maximal proliferation. Taken together, these data suggest a possible role for CD154 in the generation of cell-mediated immunity by methods both indirect (i.e. induction of APC activity), and direct (i.e. transduction of essential priming signals into the T cell) following interaction with its receptor, CD40. Further studies directed at defining the potential signal transducing capabilities of CD154 or associated intracellular proteins should further our understanding of the mechanisms involved in T cell activation while also providing additional therapeutic possibilities for immunocompromised individuals.

# CHAPTER IV

## THE EFFECTS OF TCDD ON THE ACTIVATION OF OVA-SPECIFIC DO11.10 TRANSGENIC CD4+ T CELLS IN ADOPTIVELY-TRANSFERRED MICE

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## ABSTRACT

It has previously been shown that exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD) suppresses the generation of T-dependent immunity, both humoral and cell-mediated. However, the mechanism of TCDD-induced immune suppression remains to be defined. We hypothesized that exposure to TCDD suppresses the activation of naive CD4+ T cells and prevents their expansion and differentiation into effector T helper cells capable of driving T-dependent immune responses. To test this hypothesis, we adoptively-transferred DO11.10 transgenic T cells into syngeneic recipients allowing us to track the *in vivo* activation of naive, OVA-specific CD4+ T lymphocytes following exposure to antigen. Although TCDD exposure of adoptively-transferred mice did not affect the *in vivo* expansion or activation of OVA-specific CD4+ T cells as determined by flow cytometry, spleen cells from TCDD-treated mice produced reduced levels of the T cell-derived cytokines IL-2, IFN-γ, IL-4 and IL-10 following *in vitro* restimulation. These data suggest that TCDD interferes with the differentiation of OVA-specific T cells into effector T helper cells. Ultimately, the production of OVA-specific antibodies was dose-dependently suppressed in adoptively-transferred mice which had been exposed to TCDD. These results highlight the potential of the DO11.10 adoptive transfer system to assess possible immunotoxic effects of xenobiotics in an antigen-specific transgenic mouse model.


## INTRODUCTION

The activation of naive T lymphocytes is dependent on two distinct signals, ligation of the TCR by specific antigen complexed with MHC proteins and costimulation delivered via interactions of accessory molecules and/or cytokines (Croft, 1994; Mondino et al., 1996; Pape et al., 1997b). After receiving these signals, antigen-specific T cells will undergo clonal expansion and modulate their expression of membrane-bound activation

molecules such as CD69 (Very Early Activation Antigen), CD11a (LFA-1), CD44 (Pgp-1) and CD62L (L-selectin, LECAM-1) (Rogers et al., 1997). Ultimately, these lymphocytes will differentiate into cytokine-producing effector cells capable of promoting T-cell dependent immunity such as antibody production by B cells. Incomplete activation of T cells through insufficient TCR engagement, costimulation, or differentiation leads to antigen-specific T cell unresponsiveness or anergy (Kearney et al., 1994; Maier et al., 1998; Maier and Greene, 1998). Therefore, proper T cell activation is essential for the generation of effective T cell-mediated immunity and alteration/disruption of this process may compromise the capacity of an organism to evade pathogenic insult.

The immune system has been identified as a sensitive target of TCDD, and T cell - dependent functions have been shown to be particularly affected in laboratory animals following exposure to TCDD (Vos and Luster, 1989; Kerkvliet, 1995; Kerkvliet, 1998). Although immune suppression by TCDD has been demonstrated to be dependent on the Ah receptor (Kerkvliet et al., 1990a; Kerkvliet et al., 1990b), the specific mechanism of T cell suppression remains unknown. Previously, TCDD has been shown to inhibit T cell activation in several experimental models such as the humoral responses to SRBC and OVA, and the cell-mediated CTL response to allogeneic tumor cells (Kerkvliet et al., 1990a; Kerkvliet et al., 1990b; Lundberg et al., 1991; Matulka et al., 1997; Davis and Safe, 1988). In these reports, T cell function was assessed by *in vitro* restimulation of immune cells from mice previously exposed to TCDD and antigen. While these assays provide a functional assessment of secondary T cell responsiveness, they do not directly measure the fate of antigen-specific T cells following a primary exposure to antigen. Until recently, it has been impossible to directly monitor the activation of antigen-specific CD4+ T cells because they reside within the animal at undetectable frequencies. However, with the development of the DO11.10 adoptive transfer model by Kearney et al. (1994), we now have the technical means to overcome this obstacle. The key feature of this experimental system is the utilization of T cells from DO11.10 TCR transgenic mice (Pape et al., 1997a).

T helper cells from these mice express a transgene which encodes for a TCR specific for the $OVA_{323-339}$ peptide in the context of I-$A^d$ class II MHC. Following the adoptive transfer of small numbers of CD4+, OVA-specific T cells from DO11.10 mice into syngeneic Balb/c recipients, the Ag-specific CD4+ T cells are artificially elevated from 1 in 10,000 to a detectable frequency of approximately 1 in 100. In addition, the clonotype-specific monoclonal antibody KJ1-26 allows us to track the small population of OVA-specific T cells in the adoptive transfer recipients by flow cytometric methods. In this report, we have utilized the DO11.10 adoptive transfer system to directly monitor three phases of *in vivo* antigen-specific CD4+ T cell activation: (1) clonal expansion; (2) expression of activation markers; and (3) cytokine production. Using this system, we have tested the hypothesis that exposure to TCDD suppresses the activation of naive CD4+ T cells, and prevents their expansion and differentiation into effector T helper cells capable of driving T-dependent immune responses. The results presented in this report emphasize the potential of the DO11.10 adoptive transfer system to assess possible immunotoxic effects of xenobiotics on CD4+ T helper cell activation in an antigen-specific transgenic mouse model.

## MATERIALS AND METHODS

### Mice

Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used as recipients for the adoptive transfer experiments. DO11.10 TCR transgenic mice were generously provided by Dr. Marc Jenkins (University of Minnesota Medical School). DO11.10 mice were bred and maintained in our pathogen-free animal facility in accordance with National Research Council guidelines. These mice express a transgenic T cell receptor which recognizes the 323-339 peptide of chicken ovalbumin (OVA). DO11.10 mice have

been extensively backcrossed (>10 generations) onto the Balb/c background to express H-$2^d$ MHC molecules and serve as syngeneic donors of OVA-specific T cells.

## Reagents

2,3,7,8-Tetrachlorodibenzo-*p* -dioxin (Cambridge Isotope Laboratories, Inc., Woburn MA) was dissolved in anisole and diluted in peanut oil. A vehicle solution of anisole in peanut oil was prepared similarly. Mice were given a single dose of TCDD (15 μg/kg) or vehicle by gavage on day -3 relative to OVA/CFA immunization. For the dose response experiment, mice were given a single dose of 15, 5, 0.5 or 0 μg/kg TCDD on day -3. Chicken ovalbumin was purchased from Sigma (St. Louis, MO). The generation of the clonotypic mAb KJ1-26 which specifically recognizes the OVA-specific transgenic TCR has previously been described (Haskins et al., 1983). The biotinylated KJ1-26 mAb used in our studies was kindly provided by Dr. Marc Jenkins. Streptavidin-Red613 from Gibco BRL (Gaithersburg, MD) was used as a second step reagent to visualize staining with the biotinylated KJ1-26 mAb. T helper cell activation profiles were determined by flow cytometric analysis using the following mAbs from PharMingen (San Diego, CA): FITC-conj. anti-LFA-1 (2D7), FITC-conj. anti-CD44 (IM7), FITC-conj. anti-L-Selectin (MEL-14), PE-conj. anti-CD69 (H1.2F3), PE-conj. anti-CD25 (3C7), PE-conj. anti-CD154 (MR1), and PE-conj. anti-CD28 (37.51).

## Adoptive transfer and immunization

The protocol for the adoptive transfer of DO11.10 T cells was slightly modified from the previously described method (Kearney et al., 1994), and is shown in Figure IV-1. Briefly, splenocytes from DO11.10 donor mice were harvested, pooled and the percentage of CD4+KJ1-26+ cells was determined by flow cytometry. An aliquot of cells sufficient to achieve $5x10^6$ CD4+KJ1-26+ cells per injection was resuspended in HBSS and injected i.v. in a volume of 0.5 ml into age- and sex-matched Balb/c recipient mice. For our

studies, both donor DO11.10 and recipient Balb/c mice were treated with either vehicle or TCDD to provide exposure throughout the entire adoptive transfer procedure. Adoptively-transferred mice were rested for 2 days, and then immunized with 2 mg OVA (Sigma) emulsified in CFA (Difco Laboratories, Detroit, MI) by ip injection in a volume of 0.25 ml.

**Flow cytometric analysis of spleen cells**

Spleen cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Erythrocytes were removed by hypotonic lysis. Cells were washed once and resuspended in cold HBSS/5% FBS with 20 mM HEPES, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate. Non-specific binding was blocked with 10 μg of rat IgG, and then cells were stained with optimal concentrations of fluorochrome-conjugated mAb. Appropriately labeled, isotype-matched Igs were used as controls for non-specific fluorescence. Twenty thousand to 100,000 events were collected by listmode acquisition from freshly-stained cells using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WinList (Verity Software House, Topsham, ME). Detection of OVA-specific T helper cells was performed by initially gating on viable spleen cells followed by further gating on the CD4+/KJ1-26+ population. Subsequent measurement of activation markers and cell size (FALS) was performed on the antigen-specific T cells as well as the CD4+/KJ1-26- bystander T helper cell population. The expression of the activation markers CD69, CD44, CD62L and cell size was measured on days 0-4 but only the results from their peak expression/modulation are represented. CD11a expression was measured on days 0, 1, 3 and 5.

**ELISAs**

For detection of OVA-specific antibodies, enzyme immunoassay plates were coated overnight at 4°C with 1 mg/ml of chicken OVA in PBS, blocked with 3% BSA in PBS for

60 min at 37°C, washed, and incubated overnight at 4°C with fourfold serial dilutions of plasma samples. Plates were then washed and incubated with a 1/5000 dilution of biotinylated anti-mouse IgM, IgG1, or IgG2a (Southern Biotechnology, Birmingham, AL). The secondary biotinylated Abs were complexed with avidin-peroxidase and visualized with 2,2'-azino[3-ethylbenzthiazoline-6-sulfonic acid] as a substrate. Absorbance was read at 405 nm using a plate reader (Bio-Tek Instruments, Wincoski, VT). A standard curve was generated in each experiment using plasma collected from hyper-immunized mice to permit calculation of relative anti-OVA titers.

For detection of cytokines, spleen cells ($1x10^7$) were suspended in 1 ml of complete RPMI medium containing 10% FBS with or without OVA (10μM), and incubated for 24 h. Cytokines present in the culture supernatants were measured by sandwich ELISA based on noncompeting pairs of anti-IL-2 (JES6-1A12 and JES6-5H4), anti-IFN-γ (R4-6A2 and XMG1.2), anti-IL4 (11B11 and BVD6-24G2), or anti-IL-10 mAbs (JES5-2A5 and JES5-16E3). Capture and biotinylated detection antibody pairs and their respective standards were purchased from PharMingen, except for the IL-4 standard which was obtained from Genzyme (Cambridge, MA). ELISAs were performed according to manufacturer's directions, with known amounts of recombinant murine cytokines used to generate standard curves for comparison. Spleen cell cultures from vehicle- and TCDD-treated mice contained equivalent numbers of CD4+KJ1-26+ T cells at the start of the culture period.

## Statistical Analysis

Results are presented as the mean ± SE of 4-6 mice per group unless indicated otherwise. Most experiments were repeated at least once. Analysis of variance modeling was performed using Statview statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using the least significant difference multiple

comparison *t* test or Dunnet's *t* test for pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant.

## RESULTS

### The identification of antigen-specific CD4+ T helper cells

OVA-specific CD4+ T cells were identified in the spleen by co-staining with anti-CD4 and the clonotype-specific KJ1-26 monoclonal antibodies. As shown in Figures IV-1A and IV-1B, Balb/c mice do not normally possess detectable numbers of CD4+/KJ1-26+ T helper cells in their spleens, regardless of previous exposure to antigen. However, a small but defined population of CD4+/KJ1-26+ T helper cells is detectable in Balb/c mice which have received adoptively-transferred DO11.10 T cells (Figure IV-1C). Furthermore, three days after immunizing the adoptively-transferred mice with OVA in CFA, the population of antigen-specific T cells had clonally expanded to yield approximately 8-fold more CD4+/KJ1-26+ splenic T cells (Figure IV-1D).

### The effects of TCDD exposure on the clonal expansion of OVA-specific T helper cells in adoptively-transferred mice

A fundamental characteristic of a developing adaptive immune response is the clonal expansion of antigen-specific lymphocytes following encounter with antigen. Therefore, we measured the kinetics of the response of adoptively-transferred OVA-specific CD4+ T helper cells in vehicle- or TCDD-treated mice following injection with OVA/CFA (Figure IV-2). As previously reported, a small but detectable population of CD4+/KJ1-26+ T helper cells persists for approximately two weeks in adoptively-transferred mice which are not injected with OVA/CFA (Kearney et al., 1994). Exposure to TCDD did not alter the persistence of these resting T cells in adoptively-transferred mice. Following the

**Figure IV-1. Detection of OVA-specific CD4+ T helper cells in the DO11.10 adoptive transfer model.**

Spleen cell suspensions were stained with anti-CD4-Cy and biotinylated KJ1-26 followed by streptavidin-Red 613 and analyzed on a flow cytometer. The percentage of lymphocytes that were CD4+ and KJ1-26+ is indicated on the histogram. The stained spleen cells were from unimmunized Balb/c mice (A), Balb/c mice 3 days after injection of 2 mg OVA in CFA (B), unimmunized Balb/c mice injected with $5x10^6$ DO11.10 T cells (C), and Balb/c recipients of DO11.10 T cells 3 days after injection of 2 mg OVA in CFA (D).

**Figure IV-2. The effects of TCDD on the expansion and persistence of CD4+ KJ1-26+ T cells in the spleens of DO11.10 T cell recipients following injection with antigen.**

The percentage (A) and number (B) of CD4+ KJ1-26+ cells was measured at the indicated times from adoptively-transferred recipient mice which were treated with vehicle (squares) or TCDD (circles) following immunization with 2 mg in CFA (filled symbols) or unimmunized (open symbols). One representative experiment of 8 is shown. * indicates $p< 0.05$ for the comparison of vehicle- versus TCDD-treated mice which received antigen.

**Figure IV-1.**

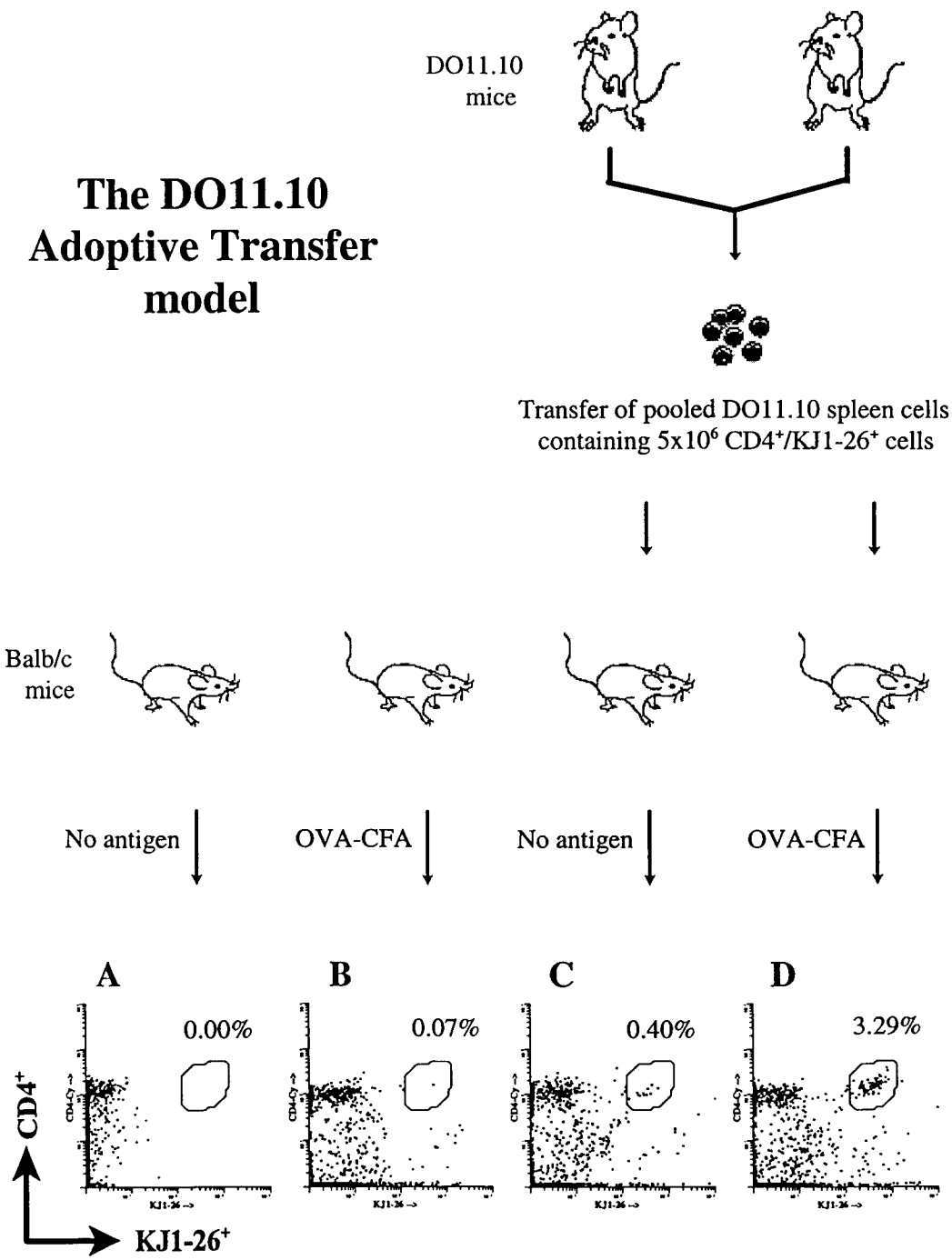
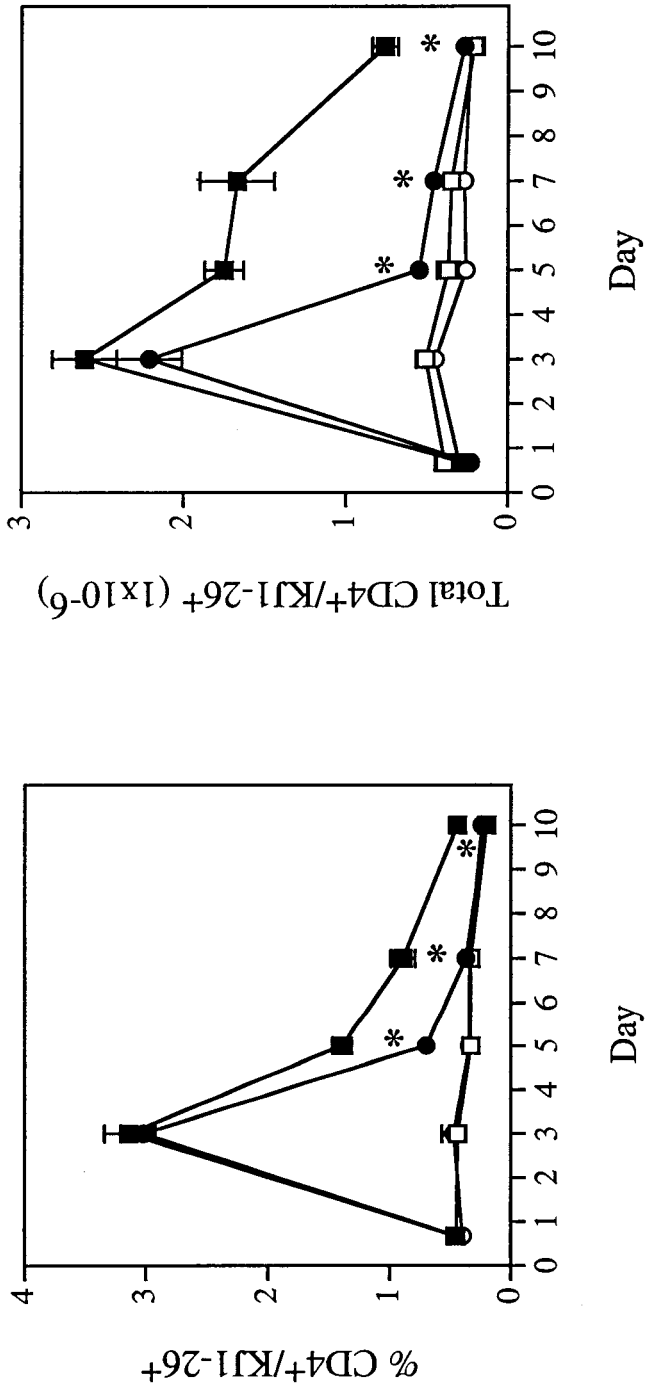

The DO11.10 Adoptive Transfer model
DO11.10 mice
Transfer of pooled DO11.10 spleen cells containing 5x10⁶ CD4⁺/KJ1-26⁺ cells
Balb/c mice
No antigen
OVA-CFA
No antigen
OVA-CFA
A
B
C
D
0.00%
0.07%
0.40%
3.29%
CD4⁺
KJ1-26⁺

**Figure IV-2.**



immunization of vehicle-treated mice with OVA-CFA, the accumulation of CD4+/KJ1-26+ T cells peaked on day 3 followed by a steady decrease in both the percentage and number of these cells through day 10. Surprisingly, TCDD exposure had no effect on the peak expansion of these OVA-specific T helper cells on day 3 post-immunization. In contrast, TCDD exposure significantly reduced the number of splenic CD4+/KJ1-26+ T helper cells from adoptively-transferred mice on days 5-10. These data suggest that while TCDD exposure does not interfere with the clonal expansion of antigen-specific T cells *in vivo*, it may enhance their deletion or interfere with their survival following antigen encounter.

**The effects of TCDD on the expression of activation markers on OVA-specific T helper cells in adoptively-transferred mice**

Following encounter with antigen, T lymphocytes modulate the expression of activation markers on their cell surface. As shown in Figure IV-3 and Table IV-1, resting CD4+ T helper cells expressed low levels of CD69, CD44 and CD11a but high levels of CD62L. This profile of activation marker expression was comparable for two separate populations of resting CD4+ T helper cells, the host CD4+/KJ1-26- T cells which were not specific for OVA (Figure IV-3 and Table IV-1) and the CD4+/KJ1-26+ adoptively-transferred cells that were not exposed to antigen (data not shown). The bystander CD4+ T cells residing within the adoptive transfer recipients served as internal controls for non-antigen-specific effects of our treatments. Likewise, no significant alterations in activation markers were observed on these cells over the duration of our studies. On the other hand, following injection of OVA/CFA, adoptively-transferred OVA-specific CD4+/KJ1-26+ T helper cells from vehicle-treated mice upregulated their expression of CD69 within 16 hours. By day 3 post-immunization, significantly increased expression of CD44 and CD11a was also detected on the CD4+/KJ1-26+ T cells whereas CD62L expression was reduced. Likewise, increased cell size was measured on the population of CD4+/KJ1-26+ cells on day 3 which is indicative of cellular blast

**Figure IV-3. The effects of TCDD exposure on the expression of phenotypic activation markers following immunization with antigen.**

Adoptive transfer recipients were immunized ip with 2 mg OVA in CFA. Spleen cells from each mouse were harvested and processed for flow cytometric analysis as described in *Materials and Methods*. For each activation marker, typical flow cytometric patterns are shown for the peak of response for the Ag-specific CD4+ KJ1-26+ population (black histograms) or the bystander CD4+ KJ1-26- population (white histograms) from vehicle- and TCDD-treated mice. The peak expression of CD69 was at 16 hr whereas the expression of CD44, CD62L, CD11a and cell size were found to peak on day 3 post-immunization.

**Figure IV-3.**

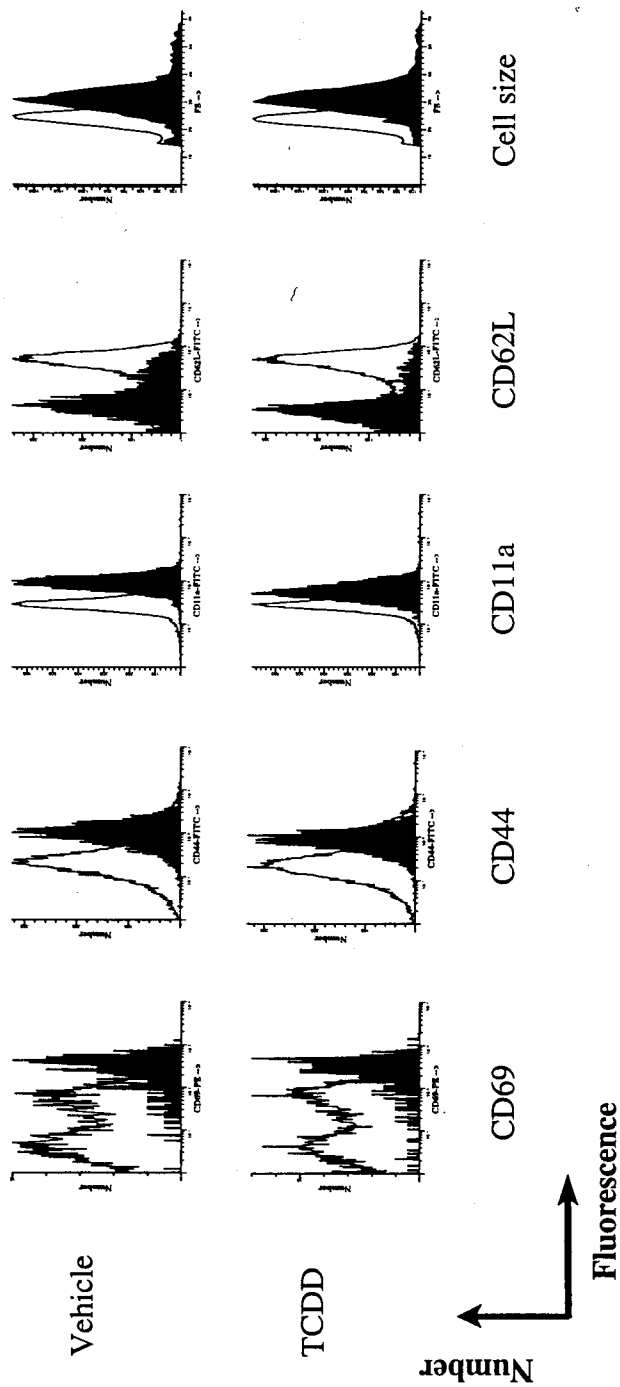

Vehicle
TCDD
CD69
CD44
CD11a
CD62L
Cell size
Number
Fluorescence

**Table IV-1.**

## Flow cytometric analysis of phenotypic activation markers in adoptive transfer recipients[a]

| | Vehicle | | TCDD | |
|---|---|---|---|---|
| | CD4+ | | CD4+ | |
| | KJ1-26+ | KJ1-26- | KJ1-26+ | KJ1-26- |
| % CD69+ | 73.2 (2.1) | 3.6 (0.1) | 69.4 (8.4) | 4.8 (0.4) |
| MCF CD69 | 250 (21) | 85 (6) | 230 (31) | 83 (9) |
| % CD44+ | 100 | 100 | 100 | 100 |
| MCF CD44 | 72 (1) | 43 (2) | 70 (2) | 43 (1) |
| Cell size (FALS) | 32.5 (0.3) | 26.3 (0.3) | 32 (0) | 26 (0) |
| % CD62L$^{high}$+ | 36 (2) | 80 (2) | 15 (1) [b] | 80 (2) |
| MCF CD62L | 22 (1) | 63 (4) | 22 (1) | 62 (2) |
| % CD11a+ | 100 | 100 | 100 | 100 |
| MCF CD11a | 92 (1) | 32 (0) | 56 (1) [b] | 31 (1) |

[a] Adoptive transfer recipients were immunized ip with 2 mg OVA in CFA. Spleen cells from each mouse were harvested and processed for flow cytometric analysis as described in Materials and Methods. For each activation marker, typical flow cytometric patterns are shown for the peak of response for the Ag-specific CD4+ KJ1-26+ population and the bystander CD4+ KJ1-26- population from vehicle- and TCDD-treated mice. Values are expressed as mean with standard error shown in parentheses.

b $p < 0.05$ for the comparison of CD4+ KJ1-26+ cells from vehicle- and TCDD-treated mice.

transformation. Interestingly, exposure of adoptively-transferred mice to TCDD had no effect on the OVA-induced expression of CD69 and CD44, or the increase in cell size of the OVA-specific T helper cells. However, TCDD exposure significantly reduced the expression of CD62L on the antigen-specific T cells of adoptively-tranferred mice when compared to the vehicle treatment. Since down-modulation of CD62L is associated with the activation of CD4+ T helper cells, these results would suggest that TCDD exposure was potentially augmenting the activation of the OVA-specific T cells in adoptively-transferred mice. In contrast, TCDD exposure significantly suppressed the OVA-induced expression of CD11a on the CD4+/KJ1-26+ cells on day 3. Minimal expression of the activation markers CD154 (CD40L), CD28 and CD25 (IL-2Rα) was detected on the CD4+/KJ1-26+ T cells from adoptively-transferred mice on days 0-4 post-immunization and no significant TCDD effect was observed (data not shown).

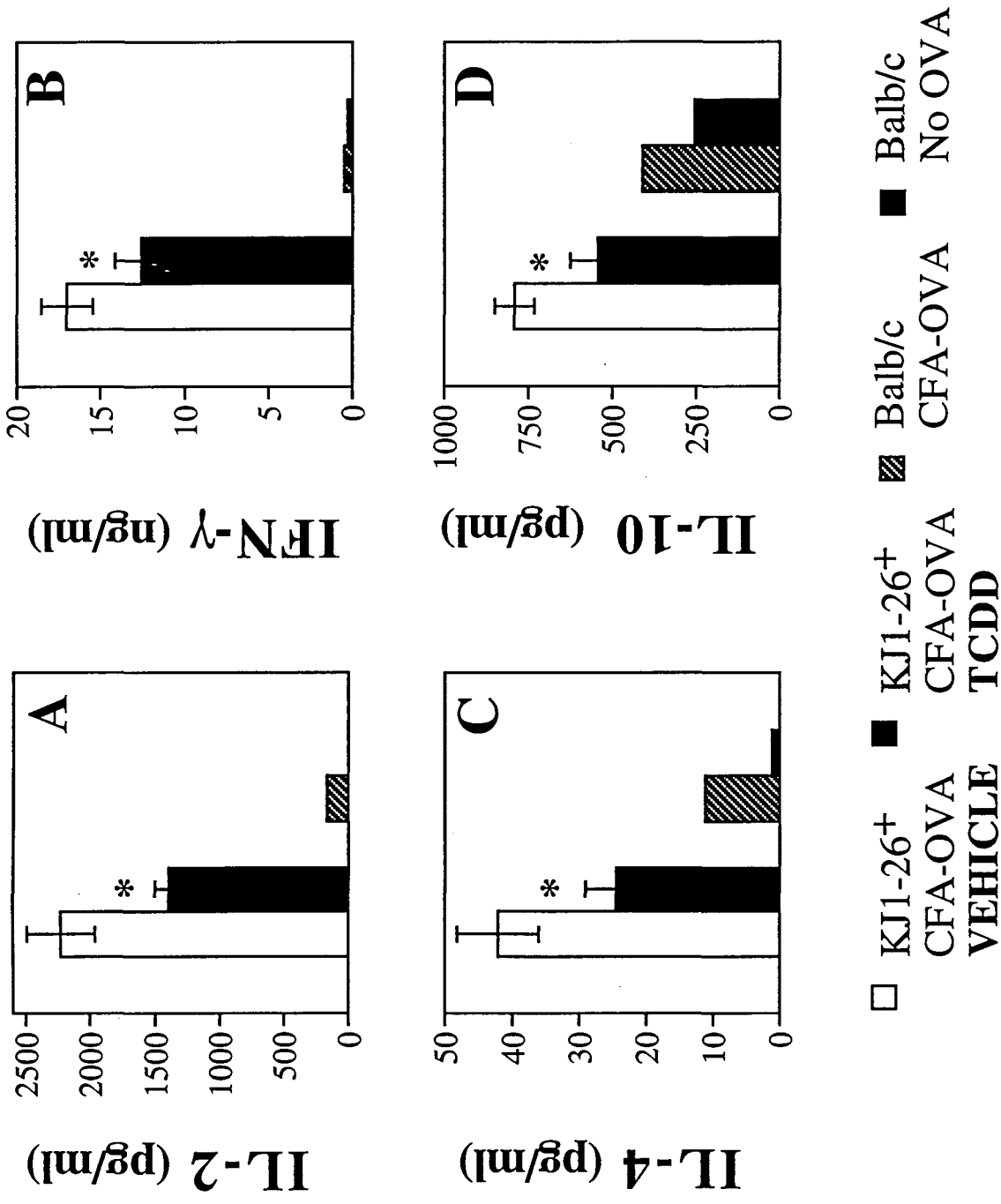
**The effects of TCDD exposure on cytokine production**

Another important function of activated T cells is the production of cytokines which contribute to the development of the antigen-specific response. Initially, IL-2 production by activated T cells drives their clonal expansion. Subsequently, activated T cells are believed to polarize their secretion of cytokines to reflect their differentiation into Th1 or Th2 effector cells capable of driving cell-mediated or humoral immune responses, respectively (O'Garra and Murphy, 1994). Th1 cells have been characterized by the production of IL-2 and IFN-γ while Th2 cells primarily produce IL-4, IL-5 and IL-10 (Mosmann et al., 1986; Cherwinski et al., 1987). Therefore, we measured cytokine production from restimulated OVA-specific spleen cells from adoptively-transferred mice to characterize the type of immune response generated to OVA/CFA. As shown in Figure IV-4, spleen cells from naive Balb/c mice secreted undetectable levels of IL-2 and IFN-γ, and nominal amounts of IL-4 and IL-10. Similarly, spleen cells from Balb/c mice

**Figure IV-4. The effects of TCDD exposure on *ex vivo* cytokine production in the DO11.10 adoptive transfer model.**

Spleen cells from normal and adoptive transfer recipient mice were restimulated *ex vivo* for 24 h with OVA (10 μM) as described in *Materials and Methods*. Culture supernatants from 2-6 mice per treatment were assayed for IL-2 (A), IFN-γ (B), IL-4 (C), and IL-10 (D) by cytokine-specific ELISAs. For each cytokine, supernatant levels are represented from the peak day of production (16 h for IL-2 and IFN-γ, and 3 days for IL-4 and IL-10). * indicates $p < 0.05$ for the comparison of vehicle- versus TCDD-treated mice.

**Figure IV-4.**


A
B
C
D
IL-2 (pg/ml)
IFN-γ (ng/ml)
IL-4 (pg/ml)
IL-10 (pg/ml)
2500
2000
1500
1000
500
0
20
15
10
5
0
50
40
30
20
10
0
1000
750
500
250
0
*
KJ1-26+ CFA-OVA VEHICLE
KJ1-26+ CFA-OVA TCDD
Balb/c CFA-OVA
Balb/c No OVA

immunized with OVA/CFA also produced little or no detectable cytokines following *in vitro* restimulation with OVA presumably because they possess very low frequencies of OVA-specific CD4+ T cells. However, spleen cells from vehicle-treated mice which had received adoptively-transferred OVA-specific CD4+ T helper cells produced increased levels of IL-2, IFN-γ, IL-4 and IL-10 following restimulation. In contrast, spleen cells from TCDD-treated, adoptively-transferred mice secreted significantly lower amounts of all cytokines tested. Subsequent analysis of IL-2 and IFN-γ production by intracellular staining confirmed that cytokine production from the restimulated spleen cells was derived exclusively from the CD4+/KJ1-26+ cells (data not shown).

**The effects of TCDD exposure on OVA-specific antibody production from adoptively-transferred mice**

Ultimately, the T-dependent immune response to OVA results in the production of OVA-specific antibodies (Lundberg et al., 1991). The measurement of these antibodies allows a readout of the effector portion of the immune response and an indirect assessment of T helper cell function. Thus, we measured OVA-specific antibody levels in adoptively-transferred mice which had been injected with OVA/CFA to determine the effects of TCDD exposure on this T-dependent humoral immune response. As measured by antigen-specific ELISA, adoptively-transferred vehicle-treated mice produced detectable amounts of OVA-specific IgM, IgG1 and IgG2a antibodies by day 5 post-immunization (Figures IV-5A-C). In contrast, adoptively-transferred mice which had been treated with TCDD produced significantly reduced amounts of these OVA-specific antibodies. Furthermore, TCDD-induced suppression of IgM and IgG OVA-specific antibodies occurred in a dose-dependent manner on day 5 (Figures IV-5D and IV-5E). Interestingly, while the levels of IgG1 and IgG2a antibodies specific for OVA were significantly reduced in TCDD-treated, adoptively-transferred mice on day 10, IgM was not (Figures IV-5F-H).

**Figure IV-5. OVA-specific antibody production in vehicle- and TCDD-treated adoptive transfer recipients.**

Mice were injected with 2 mg of OVA in CFA and plasma was collected on days 5 (A-C) and 10 (F-H) post-immunization. OVA-specific levels of IgM (A and F), IgG1 (B and G), and IgG2a (C and H) were determined by Ag-specific ELISAs as described in *Materials and Methods*. The dose-dependent effects of TCDD exposure on the production of OVA-specific IgM (D) and IgG (E) were subsequently determined from vehicle- and TCDD-treated adoptive transfer recipients on day 5 after injection with antigen.

**Figure IV-6. Schematic representation of the effects of TCDD (shown as gray lineouts) on the generation of the humoral response to ovalbumin (OVA).**

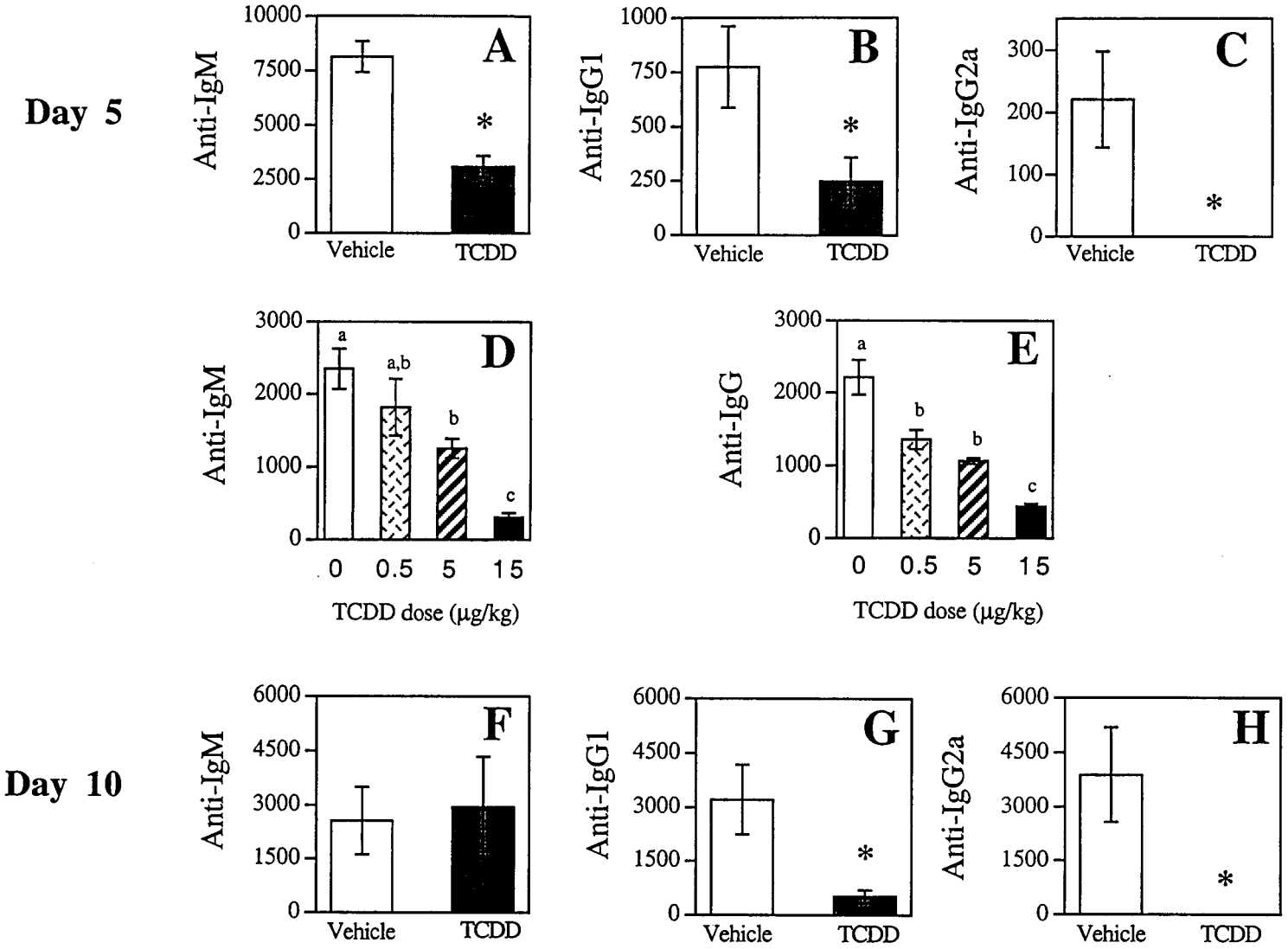
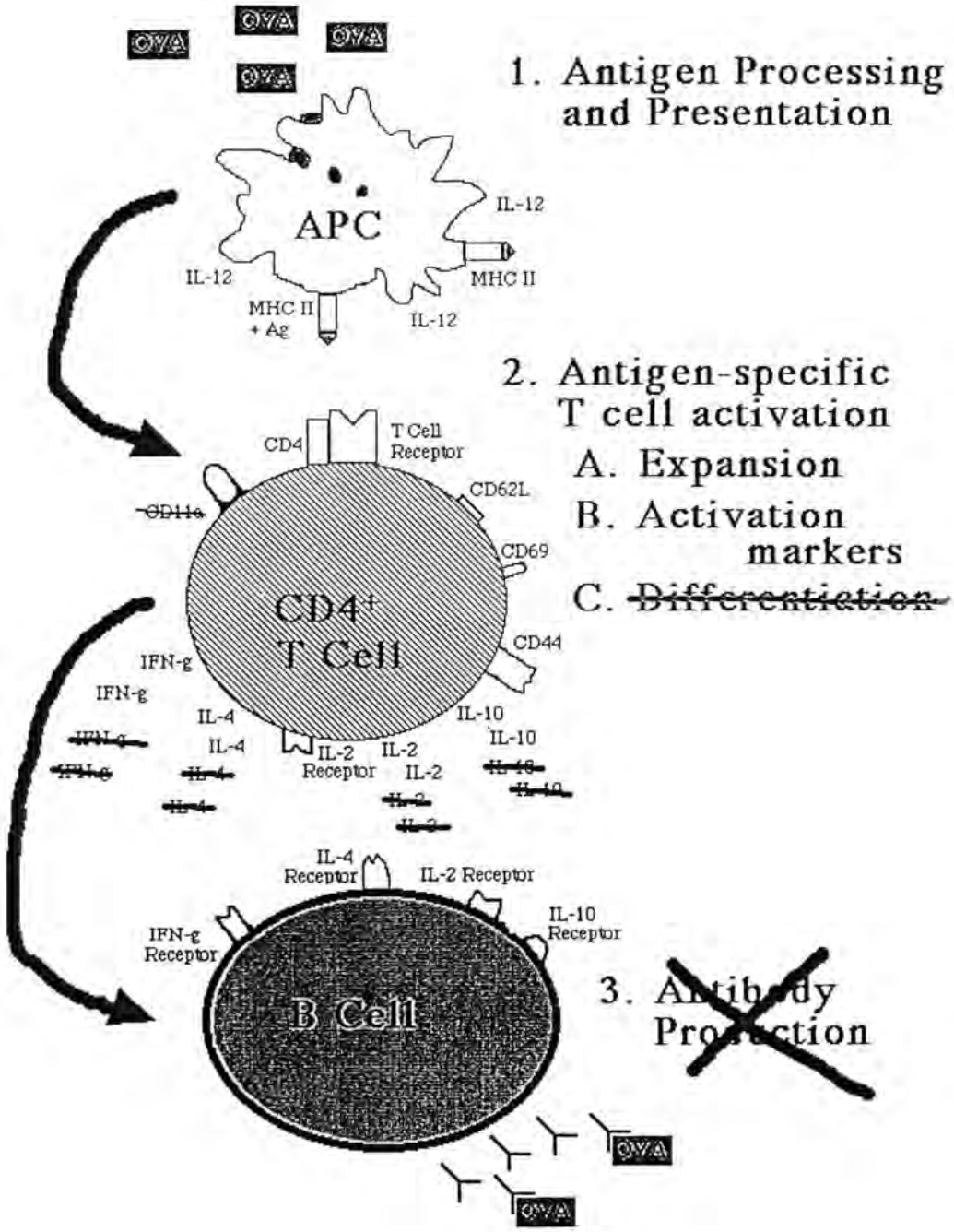

Day 5
Day 10
Anti-IgM
Anti-IgG1
Anti-IgG2a
Anti-IgG
A
B
C
D
E
F
G
H
Vehicle
TCDD
TCDD dose (µg/kg)
0
0.5
5
15
a
a,b
b
c
*

Figure IV-5.

**Figure IV-6.**


OVA
OVA
OVA
OVA
1. Antigen Processing and Presentation
APC
IL-12
IL-12
MHC II
MHC II + Ag
IL-12
2. Antigen-specific T cell activation
A. Expansion
B. Activation markers
C. Differentiation
CD4
T Cell Receptor
CD62L
CD11a
CD69
CD4+ T Cell
CD44
IFN-g
IFN-g
IL-4
IL-4
IL-2 Receptor
IL-2
IL-2
IL-10
IL-10
IL-4 Receptor
IL-2 Receptor
IL-10 Receptor
IFN-g Receptor
B Cell
3. Antibody Production
OVA
OVA

The suppression of plasma IgG1 and IgG2a OVA-specific antibodies in TCDD-exposed mice persisted through day 19 (data not shown), suggesting that the suppression of IgG antibody production in mice treated with TCDD was not simply due to a shift in kinetics.

## DISCUSSION

The immune system has been identified as a very sensitive target for the toxic effects of TCDD (Vos and Luster, 1989; Kerkvliet, 1998). The generation of adaptive immunity, both humoral and cell-mediated, has been demonstrated to be especially sensitive to the effects TCDD exposure in mice (Kerkvliet and Burleson, 1994). Specifically, the generation of T-dependent immune responses has been shown to be disrupted in laboratory animals exposed to TCDD. While several studies have attempted to evaluate the modulation of T cell function *in vivo*, none have directly monitored the effects of TCDD exposure on the activation of CD4+ T helper cells. Previously, in a study by Lundberg et al. (1992), the proliferation of OVA-specific T cells from TCDD-treated mice was shown to be suppressed following *in vitro* restimulation with OVA. These results differ from our results which showed that TCDD did not affect the clonal expansion of OVA-specific CD4+ T helper cells in adoptively-transferred mice immunized with antigen. In the study by Lundberg et al. (1992), however, mice were exposed to a significantly higher dose of TCDD (50 vs. 15 μg/kg) than in our studies, potentially explaining the differences in TCDD suppression of OVA-induced T cell expansion. Also, while our studies directly measured the effect of TCDD on the primary expansion of a clonal population of antigen-specific T cells, this other study measured the proliferative response of OVA-specific cells following a secondary challenge. Interestingly, several reports have shown a lack of TCDD suppression of T cell proliferation in mice injected with the polyclonal activator anti-CD3 (Lundberg et al., 1992; Neumann et al., 1993). In these reports, mice were exposed to doses of 20 to 50 μg/kg of TCDD which suggests that

strong or excessive signalling through the TCR may circumvent the suppressive effects of TCDD on T cell proliferation. Therefore, in our studies it is possible that the immunization of adoptively-transferred mice with 2 mg of OVA in CFA provides sufficient stimulation of the antigen-specific T cells and overrides any potential effects that TCDD might have had on their proliferation. It is also possible in our model that OVA-specific CD4+ T cells are accumulating in the spleens of adoptively-transferred mice by day 3 post-immunization due to immigration and not proliferation. However, the increase in relative cell size of the CD4+/KJ1-26+ T cells in both the vehicle- and TCDD-treated mice is representative of cellular blast transformation and suggests that these T cells are undergoing proliferation following exposure to OVA. Recently, it was demonstrated in a study using mice adoptively-transferred with CFSE-labelled DO11.10 T cells that proliferation and not immigration was responsible for the expansion of OVA-specific T cells following immunization with antigen (Gudmundsdottir et al., 1999). Therefore, although it remains to be definitively demonstrated in adoptively-transferred mice which have been exposed to TCDD, it seems likely that the expansion of CD4+/KJ1-26+ cells in our studies was due to proliferation and not an influx of OVA-specific cells. Another possible explanation for the lack of TCDD-induced suppression of proliferation of antigen-specific T helper cells hinges on the activation requirements of naive versus memory T cells. It is generally accepted that less stringent signalling is required for the activation of memory T lymphocytes (Vitetta et al., 1991). DO11.10 mice which are not on a RAG-/- or SCID background have been shown to possess a small percentage of CD4+/KJ1-26+ T cells which exhibit a memory phenotype ($CD45RB^{low}$) (Lee et al., 1996). The authors explained this observation by demonstrating that incomplete $\alpha$–chain allelic exclusion frequently occurs in DO11.10 mice resulting in the generation of dual TCR-bearing T cells which achieve memory phenotypes following exposure to environmental antigens. Therefore, it was possible that by adoptively-transferring memory CD4+/KJ1-26+ T cells into the TCDD-treated recipient

mice, we might be supplying a source of OVA-specific T helper cells which are refractory to the effects of TCDD. However, TCDD-treated mice receiving "naive" DO11.10 T cells purified by CD62L-bead enrichment demonstrated no measurable alterations in clonal expansion on day 3 post-immunization (data not shown) indicating that our results were not distorted by adoptively-transferring spleen cells from DO11.10 mice which were not on either the RAG-/- or SCID genetic background.

In contrast to the absence of TCDD effects on the expansion of OVA-specific T cells in our studies, the disappearance of CD4+/KJ1-26+ T cells was significantly enhanced in adoptively-transferred mice exposed to TCDD. Although TCDD-induced deletion of thymocytes has been extensively investigated (Silverstone et al., 1994; Staples et al., 1998a; Staples et al., 1998b), only a limited number of reports have described the effects of TCDD on peripheral T cell deletion (Prell et al., 1995; Pryputniewicz et al., 1998). In a study by Prell et al. (1995), TCDD enhanced the anti-CD3 induced deletion of CD4+ but not CD8+ T cells from TCDD-treated mice when compared to vehicle-treated mice. It was suggested that this deletion was an activation-induced cell death which was possibly mediated by increased apoptosis. Likewise, a study by Pryputniewicz et al. (1998) showed increased apoptosis of activated CD3+ T cells in TCDD-treated but not vehicle-treated mice which had been injected with anti-CD3. While future studies should help delineate the mechanism of enhanced deletion of CD4+/KJ1-26+ T helper cells in the spleens of TCDD-treated mice, it remains a possibility that these adoptively-transferred cells could be undergoing increased apoptosis, or they could simply be exiting the spleen following antigen-specific clonal expansion.

Delineation of resting and activated T cells can be facilitated by the phenotypic expression of specific cell-membrane proteins. Resting CD4+ T cells are typically defined by high expression of CD62L and low expression of CD44. Conversely, upon activation, helper T cells down-regulate their expression of CD62L and concomitantly induce higher expression of CD44. In addition, CD4+ T cells increase their expression of other

molecules such as CD69 and CD11a following antigenic stimulation. Surprisingly, few if any effects of TCDD exposure were observed on the activation profiles of OVA-specific CD4+ T cells from adoptively-transferred mice which had been injected with antigen. However, the induction of CD11a expression was found to be significantly suppressed on activated but not resting CD4+/KJ1-26+ cells. CD11a is an adhesion molecule which has been shown to participate in the homing of T cells in the periphery (Lub et al., 1995; Gahmberg, 1997). The alteration of CD11a expression on OVA-specific T helper cells following encounter with antigen could provide one possible explanation for the decrease in the number of CD4+/KJ1-26+ T cells in the spleen. Decreased adhesion of the Ag-specific CD4+ T cells could have permitted their emigration out of the spleen into the periphery, and this exodus may have been augmented in TCDD-treated mice. Current studies are underway in our laboratory to address the possibility of TCDD-induced modulation of cell trafficking via alteration of adhesion molecules such as CD11a.

The differentiation of T helper cells from naive Th0 cells into Th1 and Th2 effector cells is characterized by the production of specific patterns of cytokines (Mosmann et al., 1986). Th1 cells primarily produce IL-2 and IFN-γ and are associated with cell-mediated immunity while Th2 cells secrete IL-4 and IL-10 and primarily contribute to humoral immunity (O'Garra and Murphy, 1994). Few studies currently exist which have characterized the effects of TCDD on the differentiation of Th1 and Th2 cells. For example, while several studies have described the effects of TCDD exposure on IL-2 production, they did not measure the production of other cytokines which would have allowed for a more comprehensive assessment of the effects of TCDD on Th1/Th2 development. Previously, TCDD was shown to suppress the production of IL-2 but not TNF, IFN-γ or IL-6 in the anti-CD3 model (Prell et al., 1995). Additionally, the production of IL-2, IFN-γ and TNF was found to be suppressed by TCDD in the P815 tumor allograft model while no effect was observed on the production of IL-4 and IL-6

(Kerkvliet et al., 1996). However, since immunization of mice with P815 allogeneic cells produces a pronounced Th1 response, it was not clear from these studies if TCDD affected Th2 development. In the present study, we detected a significant production of both Th1 and Th2 cytokines from the OVA-specific spleen cells of adoptively-transferred mice. This observation was not completely unexpected since it has been previously reported that Balb/c mice are genetically predisposed towards producing Th2-mediated immunity while injecting CFA into mice induces Th1-mediated immunity. Therefore, it was possible to evaluate the concurrent development of both types of responses in the DO11.10 adoptive transfer model. In this model, we found TCDD exposure significantly suppressed the production of IL-2, IFN-γ, IL-4 and IL-10 indicating a lack of differentiation of CD4+ cells into either Th1 or Th2 effectors. Interestingly, in a study by Sad and Mosmann (1994), naive T helper cells were reported to undergo proliferation and alter their expression of cell surface markers to an activated phenotype (L-Selectin$^{low}$CD44$^{high}$) while maintaining an uncommitted Th0 state when provided an insufficient cytokine milieu. Stimulation of Th0 cells with the cytokines TGF-ß or IL-12 and IL-4 promoted their subsequent differentiation into Th1 or Th2 effector cells, respectively. TCDD produced similar effects on the development of effector T helper cells in our study suggesting that the administration of exogenous cytokines to TCDD-treated mice might have beneficial effects on the differentiation of adoptively-transferred OVA-specific CD4+ T cells into competent Th1/Th2 effector cells. Recent studies in our laboratory using the DO11.10 adoptive transfer model have revealed that splenic production of IL-12 is suppressed in TCDD-treated mice on days 1, 3 and 7 post-immunization (D.M.S., unpublished observations). Furthermore, the administration of exogenous IL-12 to TCDD-treated mice restores their production of anti-OVA IgG2a antibodies to levels measured from vehicle-treated mice. These results suggest that the administration of IL-12 to TCDD-treated mice may have provided a missing APC-derived differentiation factor which induced

otherwise defective CD4+ T cells to become competent Th1 effector cells. It is unclear at this time if the administration of IL-12 is affecting cytokine production from the CD4+/KJ1-26+ T cells, however, studies are currently being performed to address this possibility.

Numerous studies have demonstrated that antibody production is suppressed in laboratory animals exposed to TCDD (Kerkvliet, 1998). Most of these studies were performed in the SRBC model by measuring the effects of TCDD on the PFC response (Hinsdill et al., 1980; Vecchi et al., 1980; Clark et al., 1981; Holsapple et al., 1986; Kerkvliet et al., 1990b). For example, the T-dependent antibody response to SRBC was shown to have an $ID_{50}$ of only 0.6 μg TCDD/kg in C57Bl/6 mice (Kerkvliet et al., 1990b). Likewise, additional studies have measured the effects of TCDD exposure on the antibody production generated against antigens such as allogeneic tumor cells, OVA, tetanus toxoid, DNP-Ficoll and TNP-LPS (Hinsdill et al., 1980; Holsapple et al., 1986; Kerkvliet et al., 1990a; Lundberg et al., 1991; Kerkvliet et al., 1996). Although the humoral responses to these antigens were affected by TCDD exposure, they were found to be less sensitive to the suppressive effects of TCDD when compared to the anti-SRBC response. Collectively, these studies indicate that the nature of the antigenic stimulus and the dependence of antibody production on T helper cells may be important factors in determining sensitivity to TCDD exposure. Previously, it has been shown that the antibody response to OVA was suppressed in C57Bl/6 mice following exposure to 50 μg/kg TCDD, a result the authors attributed to disturbed T cell function (Lundberg et al., 1991). We have shown in this report that exposure of adoptively-transferred mice to lower doses of TCDD significantly suppressed the production of IgM, IgG1 and IgG2a OVA-specific antibodies. Although it is possible that TCDD is affecting the production of antibody by directly altering B cell function, the detection of comparable titers of IgM antibodies to OVA by vehicle- and TCDD-treated mice on day 10 post-immunization suggests otherwise. Also, it has been established that IgG antibody production is critically dependent on the interaction of

activated B cells with effector T helper cells (Parker, 1993). Specifically, the cognate interaction of CD154 (CD40L) on the activated CD4+ T helper cell with CD40 on the B cell provides an essential signal which promotes isotype switching and germinal center formation (Noelle et al., 1992). T cell-derived cytokines such as IL-4 and IFN-γ also contribute to the switching of antibodies from the IgM isotype to downstream isotypes such as IgG1 and IgG2a, respectively (Finkelman et al., 1990). Our data indicate that the production of both IgG1 and IgG2a OVA-specific antibodies was profoundly suppressed in adoptively-transferred mice which had been exposed to TCDD suggesting that disruption of T helper cell effector function might underlie this defect. Although we have demonstrated that the production of T cell-derived cytokines was significantly suppressed in TCDD-treated mice, we currently have no data which evaluates the possible effects of TCDD on CD4+ T cell-dependent cognate functions. However, studies in our laboratory are underway to investigate the role of CD154:CD40 in the production of OVA-specific antibodies in the DO11.10 adoptive transfer model and what effects, if any, TCDD exposure might have on this interaction. It is interesting to note that on days 5 and 10 post-immunization, suppression of OVA-specific antibody production corresponded with significantly decreased numbers of CD4+/KJ1-26+ cells in adoptively-transferred mice which had been treated with TCDD. Therefore, by effectively reducing both quantitative and qualitative contributions from the OVA-specific T helper cells, TCDD may be preventing proper B cell activation and subsequently suppressing the production of OVA-specific antibodies. However, this possibility also remains to be tested.

In this report, we present for the first time the effects of xenobiotic exposure on the *in vivo* activation of antigen-specific CD4+ T helper cells by evaluating the effects of TCDD in the DO11.10 adoptive transfer model. Although TCDD exposure of adoptively-transferred mice did not affect the *in vivo* expansion or activation of OVA-specific CD4+ T cells as determined by flow cytometry, spleen cells from

TCDD-treated mice produced reduced levels of several T cell-derived cytokines following *in vitro* restimulation (Figure IV-6). These data suggest that TCDD disrupts the differentiation of OVA-specific T cells into effector T helper cells which ultimately contributes to suppression of the humoral immune response to OVA. In addition, these results highlight the potential of the DO11.10 adoptive transfer system to serve as an antigen-specific transgenic mouse model to assess possible immunotoxic effects of xenobiotics on CD4+ T helper cell activation.

# CHAPTER V

## SUMMARY

Exposure to TCDD has been shown to suppress both cell-mediated and humoral acquired immune responses that are dependent on T lymphocytes. Although TCDD is known to exert its effects via the Ah receptor, specific underlying mechanisms and target cells remain undefined. Our laboratory has previously shown that TCDD suppressed the induction of the costimulatory molecule CD86 on splenic B220+ and Mac-1+ cells following the immunization of mice with P815 cells (Prell and Kerkvliet, 1997). Therefore, we hypothesized that TCDD affects T cell activation via effects on APC activation. To address this hypothesis, we further characterized the effects of TCDD on APC activation. TCDD suppressed the induction of not only CD86 but also the accessory molecules CD54 and MHC II on B220+, Mac-1+ and CD11c+ spleen cells. In addition, the production of the APC-derived cytokine IL-12 was also shown to be suppressed from the spleen cells of TCDD-treated mice when compared to vehicle-treated controls. These results suggest that TCDD suppressed the activation of APC in response to the P815 allograft. We postulated that if the APC were sufficiently activated in mice exposed to TCDD that they would be capable of overriding the TCDD-induced immunosuppression and generate effective T-dependent immunity. Although TCDD had only minimal effects on the *in vivo* activation of APC by anti-CD40 treatment, TCDD-treated mice receiving anti-CD40 were unable to generate allograft effector responses. These results suggest that defective APC activation may not be underlying the TCDD-induced suppression of the allograft response to P815 tumor cells. Alternatively, anti-CD40 therapy may not be stimulating APC to induce expression of additional accessory molecules such as CD44H, 4-1BBL , OX-40L and cytokines like IL-18 which might be necessary for T cell activation, differentiation and survival in this allogeneic response. Therefore, anti-CD40 treatment of

TCDD-exposed mice would not be expected to recover these costimulatory functions on the APC. In this thesis, the activation of APC was examined phenotypically but not functionally. Therefore, the additional experiments should be performed to evaluate if TCDD is altering the functional capacity of APC. In addition, future studies directed at determining the distribution and functionality of the AhR in various APC populations should provide useful information of potential TCDD targets in the immune system.

The production of IL-12 is considered to be important for the generation of cell-mediated immunity (Gately et al., 1998). TCDD significantly suppressed the production of IL-12 from spleen cells from P815-immunized mice which was presumably via reduction of IL-12 p40 mRNA. Treating mice with anti-CD40 induced large increases in the levels of IL-12 in the plasma and this effect was not altered by TCDD. This observation suggested that IL-12 production was selectively diminished in the spleen but not throughout the animal. More importantly, it suggested that TCDD-treated mice produced significant quantities of circulating IL-12 which should have been able to drive type-1 immunity. However, TCDD-treated mice failed to generate CTL effector cells and allograft immunity. Therefore, these data question the role of IL-12 in the P815 allograft response. Recent results from our laboratory using IL-12 p40-/- mice suggest that IL-12 is not necessary for the production of type-1 cytokines or the generation of allograft effector responses in the P815 model. Although IL-12 is needed for the generation of many type-1 immune responses, several groups have reported that it is not essential for all (Magram et al., 1996; Schijns et al., 1998). Therefore, future studies in the P815 model may help define alternate pathways which contribute to the generation of type-1 immunity.

In addition to IL-12, the interaction of CD154 with CD40 has been shown to be important for the generation of cell-mediated immunity (Grewal and Flavell, 1998). It has been reported that CD40 ligation is necessary for the activation of APC in several models (Mackey et al., 1998). Our studies using CD154-deficient mice suggest this may not be true for the generation of allograft immunity. Although P815-immunized CD154 -/- mice

were found to induce splenic costimulatory molecule expression and IL-12 production, they were unable to generate allograft effector responses. These results suggest that CD154:CD40 interactions may not be necessary for APC activation but they are for T cell activation. However, the splenic APC from the CD154-deficient mice need to be evaluated functionally to establish this possibility. On the other hand, our results suggest that CD154 may function as a costimulatory molecule for the activation of CD8+ T cells. Several groups have reported that CD154 is directly involved in T cell activation and that this molecule possesses signal transducing capabilities (Cayabyab et al., 1994; van Essen et al., 1995; Blotta et al., 1996; Brenner et al., 1997a; Brenner et al., 1997b; Koppenhoefer et al., 1997). However, this function remains to be proven. Interestingly, spleen cells from P815-immunized TCDD-treated mice displayed lower levels of CD154 mRNA which is consistent with CD154 playing a role in the activation of T cells in this model. It remains to be determined if this effect is a cause or result of the TCDD-induced suppression.

Our laboratory has previously shown that CD4+ T cells are essential for the generation of allograft effector functions in the P815 model (Kerkvliet et al., 1996). Likewise, CD4+ T helper cells have been identified as being integral for the generation of most acquired immune responses, yet no direct information exists describing the effects of TCDD on their *in vivo* activation. Therefore, we examined the effects of TCDD on CD4+ T cell activation by using the DO11.10 adoptive transfer system. We showed that TCDD exposure did not suppress the *in vivo* clonal expansion or acquisition of activation markers on the CD4+/KJ1-26+ T cells. However, spleen cells from adoptively-transferred mice exposed to TCDD produced significantly lower amounts of IL-2, IFN-$\gamma$ , IL-4 and IL-10 following *in vitro* restimulation. These results suggest that TCDD suppressed the differentiation of T helper cells into effector cells capable of driving T-dependent immunity. It remains to be demonstrated if TCDD acted directly on the antigen-specific CD4+ T cells to suppress their differentiation, or indirectly by suppressing the activation of the APC which promote T cell differentiation. Although previous studies from our laboratory and

others have suggested that TCDD does not act directly on T cells, it remains to be proven (Lawrence et al., 1996; Kerkvliet, 1998). It has been postulated by our laboratory and others that TCDD mediates its suppressive effects by altering the transcription of cytokine genes. DREs have been identified in several cytokine genes including IL-2, IL-5, IL-10, and IFN-γ (Lai et al., 1996). Since cytokine production was significantly suppressed in our studies, it remains a possibility to be tested that TCDD is affecting these genes through an AhR-dependent and DRE-mediated mechanism. Interestingly, the persistent suppression of anti-OVA IgG but not IgM antibody production in TCDD-treated mice is also consistent with altered T cell differentiation as effector CD4+ T cells provide essential help for B cells to undergo isotype class switching. However, it remains a possibility that the TCDD-induced suppression of the antibody response could be via direct effects on the B cells. TCDD has been reported to alter signal transduction pathways in B cells by increasing intracellular Ca2+ influx and protein phosphorylation (Snyder et al., 1993; Karras et al., 1996). Therefore, future studies should be directed at identifying which immune cells are being directly affected by TCDD in this immune response.

Another possible explanation for the suppressed antibody production might be found in the enhanced deletion of the CD4+/KJ1-26+ T cells in the TCDD-treated mice. If TCDD induces antigen-activated T helper cells to inappropriately undergo apoptosis or emigration out of peripheral immune tissue, it could affect the generation of humoral immunity. Current studies are underway in our laboratory to address these possibilities and the preliminary data suggests that CD4+/KJ1-26+ cells from TCDD-treated mice are undergoing increased apoptosis when compared to vehicle-treated controls. Recently, it has been reported that activated T cells may leave peripheral immune organs like the spleen and lymph nodes, go the liver, and die an apoptotic death (Huang et al., 1994). Since TCDD has been demonstrated to produce significant effects on hepatocytes and liver function, it is possible that TCDD is inducing the liver to become a death trap for activated T cells in the DO11.10 adoptive transfer model. This possibility is consistent with the

modulation of trafficking molecules like CD62L and CD11a on the surface of CD4+/KJ1-26+ T cells that occurs in TCDD-treated mice. Thus, these cells would become activated, clonally expand in the spleen, modulate CD62L and CD11a expression, immigrate to the liver and die. Future studies aimed at tracking the migration of activated antigen-specific T cells in vehicle- and TCDD-treated mice may provide useful information on the eventual fate of these cells.

The application of innovative models such as the DO11.10 adoptive transfer system should provide the tools to better assess the *in vivo* effects of TCDD and other xenobiotics on the activation of CD4+ T cells. Since T helper cells play a central role in the generation of most adaptive immune responses, the use of TCR transgenic technology in laboratory research should contribute greatly to our understanding of the effects of immunotoxicants.

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