AN ABSTRACT OF THE DISSERTATION OF

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are well-recognized for their immunosuppressive activity, which is mediated through an intracellular receptor and transcription factor, aryl hydrocarbon receptor (AhR). Laboratory animals exposed to TCDD are less resistant to infection and have severely impaired humoral and cell-mediated immune responses. This dissertation addressed the hypothesis that exposure to TCDD disrupts early events during the activation of $CD4^+$ T cells, leading to their premature loss from the spleen. Initially, ovalbumin (OVA)-specific $CD4^+$ T cells from transgenic DO11.10 mice were used to monitor the effects of TCDD on activated antigen-specific T cells. A graft-versus-host (GVH) model, in which T cells from C57Bl/6 (B6) mice are injected into C57Bl/6 x DBA/2 F1 (F1) mice, was used to study the role of AhR specifically in the T cells in response to TCDD. B6 donor T cells (from $AhR^{+/+}$ or $AhR^{-/-}$ mice) respond to DBA/2 antigens in F1 mice and a CD4-dependent CTL response is generated. In both models, exposure to TCDD significantly decreased the number of responding $CD4^+$ T cells in the spleen

beginning on day 4 after initiation of the response. Exposure to TCDD altered the phenotype of OVA-specific $CD4^+$ T cells beginning on day 2 after immunization with OVA. These studies also suggested that apoptosis was not the primary mechanism responsible for the loss of $CD4^+$ T cells from the spleen in TCDD-treated mice. Exposure to TCDD induced AhR-dependent changes in the phenotype of B6 donor $CD4^+$ T cells such that a subpopulation of $CD25^+$ cells was increased in TCDD-treated F1 mice, and these cells had *in vitro* functional characteristics consistent with regulatory T (Treg) cells. Exposure to TCDD increased the frequency of donor $CD4^+$ T cells producing interleukin (IL)-2. In addition, increased expression of CD25 in TCDD-treated mice was correlated with increased signaling through the IL-2 receptor. However, IL-2 alone was not sufficient to mimic the potent immunosuppressive effects of TCDD. These results suggest that TCDD suppresses T cell immunity in part by inducing and/or expanding a subpopulation of Treg cells by a mechanism that may involve IL-2.

The Influence of Aryl Hydrocarbon Receptor Activation on T Cell Fate

by

Castle J. Funatake

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

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Castle J. Funatake, Author

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

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Chapter 1

INTRODUCTION

The goal of this dissertation was to understand how signaling through the aryl hydrocarbon receptor (AhR) affects T cell activation and fate. These studies were accomplished using 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as the model ligand to activate the AhR during the initiation of immune responses to specific antigens. This chapter begins with a discussion of T cells and how immunotoxic chemicals can affect T cell function and immunity. In particular, it will discuss the immunosuppressive effects of halogenated aromatic hydrocarbons (HAH), ultimately focusing on the effects of TCDD in several murine models of T cell activation.

T cell immunology

The immune system is a complex network of cells and organs found throughout the body which are designed to work coordinately to protect the body from infection and disease. Long-term protection, or immunologic memory, is a hallmark of adaptive immunity in which T cells play a central role. All cells of the immune system originate from hematopoietic stem cells in the bone marrow, but T cell precursors

must migrate to the thymus in order to complete their development into mature, but still naïve, T cells. In the thymus, immature T cells undergo positive selection for those cells with T cell receptors (TCR) that can bind to self major histocompatibility complexes (MHC) with moderate affinity and negative selection for those cells with TCR that recognize self-peptides presented in the context of MHC or that bind to self-MHC with high affinity. Only a fraction of the immature T cells that enter the thymus survive the process of positive and negative selection, resulting in the generation of naïve T cells that, for the most part, will respond only to foreign antigens presented in the context of MHC and expressed on antigen presenting cells (APC). Naïve T cells leave the thymus and seed the peripheral organs of the immune system, including the spleen, lymph nodes, and Peyer's patches. It is in the periphery where T cells encounter antigen, become activated, differentiate into effector T cells (discussed below), and give rise to long-lived memory T cells.

A T cell becomes activated when the TCR binds to its cognate peptide presented in the context of MHC that is expressed on APC. However, the recognition of antigen alone is not sufficient to fully activate T cells and a second signal, in the form of costimulation, is required (reviewed in Chambers and Allison 1997). The most effective APC are the dendritic cells, which are sometimes referred to as "professional" APC due to their ability to effectively phagocytose foreign particles and load antigenic peptides onto MHC molecules, upregulate expression of costimulatory molecules, and migrate to the lymph nodes where they present antigen to naïve T cells (reviewed in Guermonprez *et al.* 2002). Signaling through both the

TCR and costimulatory receptors initiates a signaling cascade leading to the activation of transcription factors such as AP-1, NFAT and NFκB. Activated T cells undergo a period of proliferation and differentiation during which time expression of cell surface proteins change and cytokines are produced. Activated T cells develop different effector functions depending on the specific T cell subset, discussed below.

Helper T cells

Helper T (Th) cells, as their name suggests, help other cells of the immune system to respond to antigen. They do this primarily by secreting cytokines and chemokines to direct the immune cells to the site of antigen and to promote the development of appropriate effector mechanisms to eliminate foreign antigen. Th cells express CD4 on their cell surface ($CD4^+$ T cells) that facilitates optimal recognition of antigen in the context of class II MHC molecules, which are expressed primarily on APC. APC phagocytose/endocytose foreign material, process the proteins contained therein, and package the resulting peptides into class II MHC which is directed to the cell surface. When an appropriate Th cell recognizes the peptide-MHC complex an immunological synapse forms and the Th cell undergoes the process of activation.

There are two main types of Th cells, and they are distinguished based on the profile of cytokines they secrete. Th1 cells secrete interleukin (IL)-2 and interferon (IFN)-γ, and promote the development of cell-mediated immunity, as well as certain classes of antibodies, in response to viruses and tumors. Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, and promote antibody-mediated immunity, in response to parasites

and allergens (reviewed in Seder and Paul 1994). While we define these subpopulations based on the profile of cytokines they secrete, they are not necessarily mutually exclusive, although certain cytokines can inhibit production of the opposing type. For instance, IFN-γ and IL-12 have been shown to inhibit the generation of T cells capable of producing IL-4, thereby skewing the response toward a Th1-type of response, and conversely, IL-4 has been shown to inhibit the generation of IFN-γ-secreting cells (Scott 1991; Seder *et al.* 1993; Seder *et al.* 1992; Swain *et al.* 1990). In some cases, skewing the cytokine profiles to Th1 or Th2 is sufficient to impair the development of an immune response capable of clearing infection. The classic model for this is infection of Balb/c mice versus C57Bl/6 mice with *Leishmania major*. In Balb/c mice a Th2 response is dominant and leads to the development of severe lesions and a failure to resolve the infection, while in C57Bl/6 mice (and, in fact, most other strains of mice), a Th1 response predominates and the mice clear the pathogen without complications (Gumy *et al.* 2004).

Cytotoxic T cells

Cytotoxic T cells (or cytotoxic T lymphocytes [CTL]) are designed to kill virus-infected or altered self (tumor) cells. CTL express CD8 on their cell surface ($CD8^+$ T cells). The CD8 molecule aids the recognition of foreign antigen in the context of class I MHC which is expressed on the surface of nearly all cells in the body. As virus-infected or tumor cells die, they are phagocytosed by dendritic cells and then by using a non-conventional process known as cross-presentation these

peptides are loaded into class I MHC (Guermonprez *et al.* 2002). These non- or altered-self antigens are then presented by dendritic cells to $CD8^+$ T cells, and similar to the Th cells described above, an immunological synapse is formed and the cells undergo activation and differentiation into CTL. The development of a CTL response takes 7-10 days, during which time cytokines from Th cells are required until the CTL become self-sufficient around day 5-7. CTL effectors (CTLe) develop from a small pool of CTL precursors and both subpopulations can be identified based on co-expression of various surface proteins that are involved in cell adhesion, migration, and signaling (Kedl and Mescher 1997; Kerkvliet *et al.* 1996; Mobley and Dailey 1992; Oughton and Kerkvliet 1999; Walker *et al.* 1995). On day 10, CTLe can be identified phenotypically as $CD44^{hi}$ $CD62L^{low}$ (or $CD44^{hi}$ $CD45RB^{low}$) and by their ability to secrete IFN-γ (Harty *et al.* 2000; Kerkvliet *et al.* 1996; Mobley and Dailey 1992; Oughton and Kerkvliet 1999). Fully differentiated CTLe migrate to the site of infection where they recognize non- or altered-self antigen in the context of class I MHC expressed on the surface of virus-infected or tumor cells, so-called target cells. CTLe kill their targets by activating death receptors on the target cells (e.g., Fas-FasL interactions) or by secreting cytolytic products such as perforin and granzymes (Harty *et al.* 2000).

Regulatory T cells

Regulatory T (Treg) cells play an essential role in the maintenance of self-tolerance. In both humans and mice, the absence of Treg cells leads to the

development of lymphoproliferative and autoimmune diseases (reviewed in Chatila 2005). Although most self-reactive T cells are eliminated in the thymus during negative selection, a few can escape and find their way into the periphery. Treg cells, which can express CD4 or CD8, also originate in the thymus and are capable of inhibiting the activation of these self-reactive T cells. Much of the functional work has been carried out *in vitro* using $CD4^+$ Treg cells, however the exact behavior of Treg cells *in vitro* versus *in vivo* can be quite different, and relatively little is known about the function and phenotype of $CD8^+$ Treg cells altogether. For example, $CD4^+$ Treg cells are characterized by a state of nonproliferation in response to polyclonal stimulation with anti-CD3 antibodies when cultured *in vitro*, however, $CD4^+$ Treg cells transferred into syngeneic mice proliferate extensively when exposed to antigen *in vivo* (Takahashi *et al.* 1998; Walker *et al.* 2003). In contrast to divergent proliferative responses *in vivo* and *in vitro*, $CD4^+$ Treg cells are characterized by a cell number-dependent suppression of the response of naïve T cells to antigenic/mitogenic stimulation both *in vivo* and *in vitro* (Kuniyasu *et al.* 2000; Nishimura *et al.* 2004; Sakaguchi *et al.* 1995; Taylor *et al.* 2002).

Treg cells can be classified as natural or adaptive, depending on whether they develop in the thymus or are induced in the periphery, respectively (Bluestone and Abbas 2003). Natural Treg cells constitutively express CD25, CTLA-4, and GITR, as well as the transcription factor Foxp3 (Sakaguchi 2004). Natural Treg cells of the $CD4^+$ variety are the best characterized, although there are more recent studies that have identified a $CD8^+$ subpopulation of T cells with the same phenotype and function

as $CD4^+$ Treg cells (Bisikirska *et al.* 2005; Cosmi *et al.* 2003). Adaptive Treg cells do not have as well defined phenotypic markers, but have been shown to secrete IL-10 and TGF-β, and they do not necessarily express Foxp3 (Chen *et al.* 2003; Karim *et al.* 2004; Vieira *et al.* 2004; Vigouroux *et al.* 2004). The mechanism(s) by which Treg cells suppress the proliferation of naïve T cells is still under intense investigation, but based on studies conducted *in vitro*, it appears that natural Treg cells require cell contact while adaptive Treg cells may or may not depending on the strategy used to induce them (O'Garra and Vieira 2004; Takahashi *et al.* 1998; Takahashi and Sakaguchi 2003; Vieira *et al.* 2004). In addition, natural $CD4^+$ Treg cells have been shown to inhibit the production of interleukin (IL)-2 at the level of transcription in naïve T cells (Thornton and Shevach 1998). Since IL-2 is a cytokine that is essential for the sustained proliferation of T cells *in vitro* (reviewed in Nelson and Willerford 1998), the lack of necessary growth factors may also contribute to Treg cell-mediated inhibition of proliferation.

T cells as targets of immunotoxicants

Suppression of the immune response can occur by many different mechanisms. Many pathogens suppress immune responses in order to survive in their hosts; malnutrition, irradiation, drugs, and other chemicals can also cause various forms of acquired immunodeficiency. There are also several forms of inherited immunodeficiency. In all cases, impaired immune function leads to increased

susceptibility to infection and disease. Defects in antibody production are characterized by an inability to clear extracellular bacterial infections and can be caused by dysfunction of T cells, B cells or APC. Defects in T cell functions tend to be much more severe in nature, leading to multiple forms of, or combined, immunodeficiency, thus reflecting the central role that T cells play in all adaptive immune responses.

T cells have long been recognized as targets for immune modulating agents including chemotherapeutic drugs and immunotoxic industrial and environmental compounds. For example, cyclosporin A is a potent immunosuppressive drug discovered in the late 1970s that specifically suppresses T cell activation (Calne 1981). It is widely used today to treat patients receiving organ transplants as well as patients with autoimmune diseases such as inflammatory bowel disease (Calne 2004; Lichtenstein *et al.* 2006). Unintentional or environmental exposure to toxic chemicals can also have immunosuppressive effects. For instance, both lead and mercury have been shown to alter cytokine production in a way that favors Th2 responses (Heo *et al.* 1997; Heo *et al.* 1996; Iavicoli *et al.* 2006). Such a skewing of the Th1/Th2 cytokine profiles can result in the development of a non-protective immune response, as discussed above (see *Helper T cells*). Some compounds can also interfere with the signal transduction pathway from the TCR or costimulatory receptors, as is the case with nicotine which has been shown to block the upregulation of inositol triphosphate and the entry of T cells into S phase (Geng *et al.* 1995; Geng *et al.* 1996). Many compounds such as mercury, arsenic, atrazine, lindane, and malathion cause a

decrease in the numbers of T cells found in peripheral lymphoid tissues (Filipov *et al.* 2005; Neishabouri *et al.* 2004; Olgun *et al.* 2004). Decreased numbers of T cells either before or during an immune response can have many consequences, possibly resulting in more frequent infections due to decreased surveillance, more severe symptoms due to slower response time, or a complete failure to clear the infection resulting in permanent damage or death.

Halogenated aromatic hydrocarbons as immune suppressants

In the field of immunotoxicology, there is perhaps no better-studied group of chemicals than the halogenated aromatic hydrocarbons (HAH), which include the polychlorinated- and polybrominated-biphenyls (PCB, PBB), polychlorinated dibenzofurans (PCDF), and polychlorinated dibenzodioxins (PCDD). The US EPA regulates these compounds collectively as dioxin and dioxinlike compounds. As shown in Figure 1-1, these chemicals share some structural similarities, and the position and number of the halogen atoms can vary, giving rise to hundreds of possible

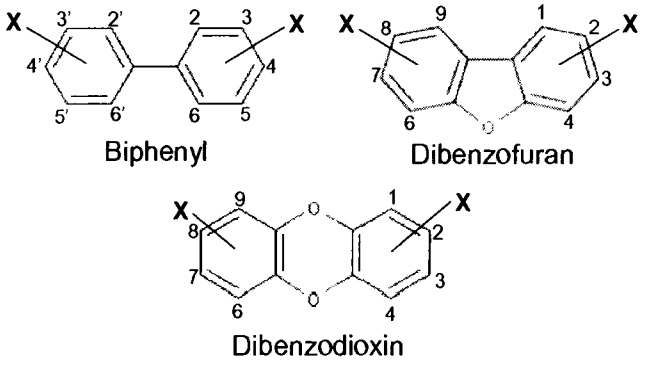

Figure 1-1. Basic chemical structures of halogenated aromatic hydrocarbons. *X indicates any halogen atom, typically chlorine or bromine.*

congeners. The most relevant congeners, from a regulatory and toxicologic point of view, are those that take on a planar conformation and bind the aryl hydrocarbon receptor (AhR), the intracellular receptor and transcription factor responsible for most, if not all of the toxic effects of these compounds. These compounds are of toxicologic interest because they are stable and persistent in the environment, are resistant to metabolic breakdown, and bioaccumulate in animals (Mukerjee 1998; Webster and Commoner 2003). TCDD, which is chlorinated in positions 2, 3, 7, and 8, is the most toxic congener due to its high affinity for AhR, with a K_d value in the range of 10^{-9} M (Ema *et al.* 1994).

Sources of HAH

PCB were produced extensively in the US beginning in 1929. Due to their thermal and chemical stability, they were used in various industrial applications such as transformers, capacitors, hydraulic fluids, and plasticizers. However, upon discovery that PCB are extremely persistent in the environment and fat-soluble, resulting in their bioaccumulation and biomagnification in the food chain, their production was halted in 1977. Nearly 1.5 million metric tons of PCB were produced worldwide and it is estimated that 20-30% of that has been deposited into the environment (Webster and Commoner 2003).

While PCB were produced for specific industrial applications, PCDD and PCDF (PCDD/F) were produced primarily as unwanted by-products during the manufacture of chlorinated herbicides, pentachlorophenol, and PCB. PCDD/F are also

produced during the incineration of municipal and hospital waste and during the bleaching of pulp and paper with chlorine, although modern practices have significantly reduced the generation of dioxins during this latter process. Once released into the environment, HAH are able to travel large distances, and as a result, they are found ubiquitously around the world, even in remote and otherwise pristine locations (Gilpin *et al.* 2003; Webster and Commoner 2003).

Exposure, absorption, distribution, metabolism, and excretion of PCDD/F

Most humans are exposed to PCDD/F through their diet, and it has been estimated that over 90% of the intake of dioxins is from food. Most dietary exposure occurs through ingestion of animal fat from meat, poultry, dairy and fish. The average daily intake of PCDD/F ranges between 1 and 3 pg/kg/day, which is near the tolerable daily intake recommended by the World Health Organization (Liem *et al.* 2000; Schecter *et al.* 2001; Startin and Rose 2003). However, since the early 1990s, there has been a decrease in the levels of PCDD/F in the environment and consequently in foods, and it is expected that these levels and thus human exposure to these compounds will continue to decrease.

Studies on the absorption, distribution, metabolism and excretion (ADME) of PCDD/F have been conducted primarily in laboratory rodents, although some tissue distribution data are available for humans and are consistent with the tissue distribution found in rats and mice (Mukerjee 1998; Schecter *et al.* 1994; Van den Berg *et al.* 1994). Absorption of PCDD/F following ingestion occurs readily by

diffusion across the gut epithelia. In the rat, oral gavage of TCDD diluted in corn oil resulted in 70-85% of the original dose being absorbed, with the highest concentrations found in the liver and fat; similar findings were seen in the hamster, mouse, and guinea pig (Gasiewicz *et al.* 1983; Neal *et al.* 1982; Van den Berg *et al.* 1994). TCDD was found to a lesser extent in the skeletal muscle, heart, testes, blood, and brain. In studies in different strains of mice, Gasciewicz *et al.* (1983) found that TCDD was present in tissues primarily as the parent compound, that excretion of TCDD occurs primarily through the feces and bile, and very little parent compound is found in these products. Together, these findings suggest that metabolism is primarily a detoxification and elimination process (reviewed in Neal *et al.* 1984), but because the metabolism of TCDD is quite slow there is significant accumulation of these compounds in adipose tissue. As a result TCDD has a long half-life in humans (7-10 years), rats (12-31 days), and mice (10-14 days) (Van den Berg *et al.* 1994).

Mechanism of action: activation of the AhR

Nearly all of the toxic effects of PCDD/F are mediated through the binding of these compounds to an intracellular receptor and transcription factor, AhR. AhR is a member of the basic helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of proteins, that are classified based on the presence of the PAS domain which is a region of 250-300 amino acids involved in homo- and heterotypic interactions between bHLH-PAS proteins and other cellular chaperones (Gu *et al.* 2000). Proteins in this family are involved in circadian rhythm, sensing oxidative stress, and, in the case of

AhR, coping with exposure to certain exogenous compounds. In the absence of ligand, AhR is sequestered in the cytosol as part of a complex including hsp90, c-Src, XAP2, and p23 (Gu *et al.* 2000; Schmidt and Bradfield 1996) Upon binding with ligand, AhR translocates to the nucleus, dissociates from the chaperone proteins and dimerizes with ARNT (Fig. 1-2). The ligand-AhR-ARNT complex is the active transcription factor

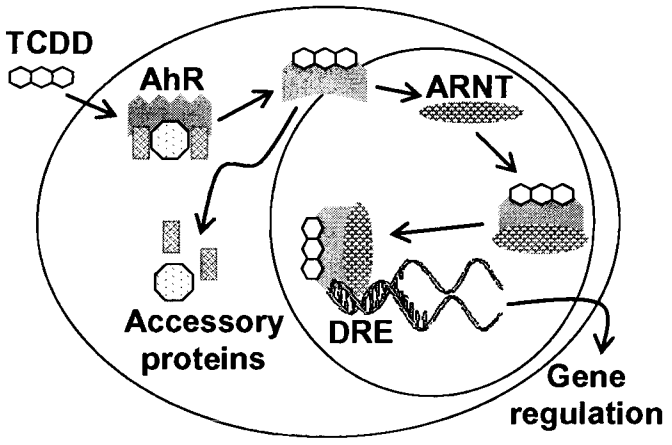

Figure 1-2. Schematic of the AhR signaling pathway. *Lipophilic ligands such as TCDD freely diffuse across the cell membrane and bind to the cytosolic AhR. Upon ligand-binding, the AhR translocates to the nucleus, dissociates from the accessory proteins, and dimerizes with ARNT. The AhR/ARNT heterodimer binds to DRE in the promoters of genes and upregulates transcription.*

that binds to dioxin response elements (DRE) found in the promoter regions of many genes. The DRE core sequence is 5'-GCGTG-3', and the flanking nucleic acids confer additional specificity (Lusska *et al.* 1993; Yao and Denison 1992). The best-characterized DRE-responsive genes include those genes involved in phase I and phase II metabolism: cytochrome P450 (CYP) 1A1, CYP1A2, CYP2B1, UDP-glucuronosyl transferase, and glutathione S-transferase (Whitlock 1999; Whitlock *et al.* 1997). Genes involved in cell cycle regulation, cell survival, proliferation, and

immune function are also modulated following exposure to TCDD, and can differ depending on the type of cells and exposure conditions (Frueh *et al.* 2001; Jeon and Esser 2000; Ma *et al.* 2001; Park and Lee 2002; Park *et al.* 2001; Puga *et al.* 2000b; Rininger *et al.* 1997; Zeytun *et al.* 2002).

Although dimerization with ARNT and subsequent gene regulation via the DRE has been the most studied mechanism of action for AhR, other transcription factors have been shown to associate with AhR. Binding of AhR with other transcription factors could lead to the formation of novel transcription factors, with promotional or inhibitory activity of their own, could result in competition between heterodimer partners, or could lead to the formation of non-functional heterodimers. For instance, activation of AhR has been shown to interact with retinoblastoma protein (Rb) and to induce G1 arrest in 5L hepatoma cells (Ge and Elferink 1998). Given the role of Rb in transcriptional repression, the authors hypothesize that TCDD-induced cell cycle arrest may involve the formation of a transcriptional repressor in the form of an AhR-Rb heterodimer. Other studies have shown that AhR can interact with RelA in a murine dendritic cell line, DC2.4, and a murine hepatoma cell line, Hepa1c1c7 (Ruby *et al.* 2002; Tian *et al.* 1999). RelA is one of five proteins in the NF-κB family of transcription factors. Using DC2.4 cells, Ruby *et al.* (2002) demonstrated that the AhR-RelA heterodimer did not bind to DNA and that overexpression of AhR blocked binding of RelA/p50 and RelB/p50 heterodimers. Overexpression of AhR did not block binding of the non-transcriptionally active p50 homodimer to DNA. These findings suggested that TCDD-mediated activation of AhR may skew the balance of

NF-κB family members leading to impaired NF-κB signaling in DC and thereby contribute to the suppression of adaptive immunity by altering DC activation.

In addition to transcriptional modification by AhR, several studies have also shown that ligand binding can lead to changes in protein phosphorylation. Increased protein tyrosine kinase activity may be mediated by c-Src, which is activated upon release from the cytosolic AhR complex following binding of TCDD (Enan and Matsumura 1996). Studies from the same laboratory demonstrated that c-Src-deficient mice (c-Src$^{+/-}$) were resistant to some of the toxic effects of TCDD, including decreased body, adipose tissue, and thymus weights, but a significant increase in liver weight due to lipid accumulation and induction of 7-ethoxyresorufin-O-deethylase (EROD) activity was still observed (Matsumura *et al.* 1997). Studies from other laboratories have also shown that exposure to TCDD is followed by an increase in kinase activity in Jurkat T cells (Kwon *et al.* 2003). Although these studies do not preclude the possibility that c-Src may be downstream of an AhR-regulated gene or is regulated by AhR itself, they do support the hypothesis that changes in protein phosphorylation contribute to TCDD-mediated immunotoxicity.

General immunotoxic effects of PCDD/F

PCDD/F are widely recognized for their potent immunotoxic effects. TCDD has been studied the most extensively because of its high affinity for AhR and because it is the most potent immunosuppressive member of this group of chemicals. Studies using various PCDD congeners with different affinity for the AhR demonstrated that

those congeners with the highest binding affinity had the most suppressive effects on the immune response (Harper *et al.* 1993; Kerkvliet *et al.* 1990). The same studies also used congenic strains of C57Bl/6 mice expressing either the high-affinity or low-affinity AhR allele and demonstrated that increased sensitivity to immune suppression by TCDD correlated with expression of the high-affinity AhR. Subsequent studies in which C57Bl/6 mice were challenged with allogeneic P815 tumor cells demonstrated that if the mice did not express AhR, they were completely resistant to the immune suppressive effects of TCDD (Vorderstrasse *et al.* 2001). These studies clearly showed that the AhR is responsible for the immunosuppressive effects of TCDD.

Human data on the immunotoxic effects of TCDD are limited and somewhat conflicting. The proliferation of peripheral blood lymphocytes in response to mitogenic stimulation from patients exposed to high levels of TCDD is either not affected or is sometimes increased compared to non-exposed individuals (Halperin *et al.* 1998; Tonn *et al.* 1996). Other studies have found either increased or decreased levels of IgG, IgA and/or IgM in plasma and there is also conflicting data on the effects of TCDD on the frequency of circulating lymphocyte subpopulations (Aoki 2001; Baccarelli *et al.* 2004; Halperin *et al.* 1998; Tonn *et al.* 1996). No overt effects on the severity or rate of infection have been noted in humans, although in a study of Dutch children exposed to relatively high levels of PCB and dioxins, there was a tendency toward reduced antibody titers to mumps and rubella vaccination (Weisglas-Kuperus *et al.* 2000). While the data from human epidemiological studies are

conflicting, they do indicate that there is at least a potential for altered immune function in people exposed to high levels of TCDD.

In contrast to the inconsistent results from human studies, TCDD has been shown to consistently and potently target the immune system of laboratory animals. One of the most consistent effects on immune-related tissues that has been observed in every species examined following exposure to TCDD is thymic atrophy (Bock 1994; Martinez *et al.* 2003). Furthermore, early studies in laboratory animals treated with TCDD demonstrated that host resistance to infectious diseases such as influenza (Burleson *et al.* 1996; House *et al.* 1990; Luebke *et al.* 2002; Warren *et al.* 2000) and samonella (Thigpen *et al.* 1975) were highly sensitive to TCDD. Indeed, doses of TCDD in the low μg/kg range have been shown to elicit profound suppression of both antibody- and cell-mediated immune responses in laboratory animals.

Effects of TCDD and the activation of AhR on T cells

While the immunotoxic effects of TCDD and related compounds have been recognized for many years, the precise mechanism of how TCDD and signaling through AhR mediates this suppression has been more difficult to determine. Although it was clear from prior studies that AhR was critical for the immunosuppressive effects of TCDD, these studies were not able to ascertain which cell or cells were the direct targets. The simplest explanation for the suppression of these T cell-dependent responses by TCDD would be that the T cells themselves were directly targeted,

however with few exceptions, no direct effects of TCDD on T cell responses *in vitro* could be demonstrated, and, if anything, there was a mild enhancement of proliferation in response to mitogenic stimulation (De Krey and Kerkvliet 1995; Halperin *et al.* 1998; Lang *et al.* 1996; Tonn *et al.* 1996). Additional studies examined possible indirect mechanisms for suppression, including altered levels of gluccocorticoids, arachidonic acid metabolites, and increased oxidative stress, all of which yielded, for the most part, inconclusive results (De Krey *et al.* 1993; De Krey *et al.* 1994; DeKrey and Kerkvliet 1995; Lawrence and Kerkvliet 1998; Lawrence *et al.* 1999). Subsequently, Kerkvliet *et al.* (2002) used T cells from $AhR^{-/-}$ mice in an acute graft-versus-host model and compared their response to donor T cells from $AhR^{+/+}$ mice. In this landmark study, they found that if the donor T cells were from $AhR^{-/-}$ mice, they were completely resistant to the effects of TCDD and developed a normal CTL response, demonstrating that T cells are in fact direct AhR-dependent targets of TCDD-mediated immunotoxicity (Kerkvliet *et al.* 2002).

Mechanisms underlying TCDD-mediated suppression of the CTL response to P815 tumor allograft

Studies had demonstrated that the CTL response to allogeneic P815 tumor cells was suppressed by TCDD in an AhR-dependent manner *in vivo* (Kerkvliet *et al.* 1990; Vorderstrasse *et al.* 2001). In order to more fully characterize the effects of TCDD on the P815 tumor allograft response, Kerkvliet *et al.* (1996) conducted a detailed analysis of the time course of the developing immune response to P815 tumor cells. Th1-dependent (type 1) cytokines (IL-2, IFN-γ, and TNF) were suppressed after day 5,

relative to injection with P815, while Th2-dependent (type 2) cytokines (IL-4, IL-6, IL-10) were largely unaffected. Corresponding effects on alloantibody isotypes were seen—type 1 dependent isotypes (IgM and IgG2a) were suppressed, while levels of IgG1, a type 2 isotype, appeared similar to vehicle controls. The authors concluded that while initiation of the anti-allo response appeared to proceed normally in TCDD-treated mice, it was terminated prematurely. In these same studies, depletion of either $CD4^+$ or $CD8^+$ T cells *in vivo* revealed that the cytokines produced in response to P815 tumor cells on days 4-10 were due primarily to $CD8^+$ T cells. However, depletion of $CD4^+$ T cells during the first three days of the response severely inhibited development of the CTL response on day 10, indicating that $CD4^+$ T cells are essential during the early stages of the immune response to P815 (Kerkvliet *et al.* 1996). Interestingly, suppression of the CTL response was induced by TCDD during the first three days of the response; if treatment with TCDD was delayed until day 4 or later, the CTL response developed normally and was indistinguishable from the vehicle response (Kerkvliet *et al.* 1996). Together, these data suggested that $CD4^+$ T cells might be the proximal cellular targets for the immunotoxic effects of TCDD. To address this, Prell and Kerkvliet (1997) used P815 cells transfected with the costimulatory molecules B7.1 or B7.2 to allow $CD8^+$ T cells to receive both TCR-signaling and costimulation directly from the tumor cells, thus bypassing the need for $CD4^+$ T cell help. If the $CD4^+$ T cell was the proximal target, then bypassing the requirement for $CD4^+$ T cells in the response to P815 should make the CTL response resistant to the immunosuppressive effects of TCDD. Indeed, when B7-transfected

P815 tumor cells were injected into mice that had been depleted of $CD4^+$ T cells, the CTL response developed normally, and the CTL response to B7-transfected P815 was completely resistant to the suppressive effects of TCDD in the presence or absence of $CD4^+$ T cells (Prell and Kerkvliet 1997).

One of the major roles for $CD4^+$ T cells during the initiation of an immune response is the production of cytokines, such as IL-2, which are important for driving the expansion of $CD8^+$ T cells and the development of CTL effectors (CTLe). Interestingly, Oughton and Kerkvliet (1999) had demonstrated that the development of CTLe from CTL precursors, *per se*, was not inhibited but rather that the expansion of activated CTL precursors was impaired in a dose-dependent manner. This suggested that TCDD might suppress development of the CTL response by decreasing cytokine production from $CD4^+$ T cells. Therefore, Prell *et al.* (2000) examined whether or not treatment with exogenous IL-2 at various time points would restore the CTL response to P815. Exogenous IL-2 administered on days 7-9 after injection of P815 dose-dependently restored the CTL response in TCDD-treated mice to the same levels seen in control mice that were not treated with IL-2. However, treatment with IL-2 on days 1-3 did not restore CTL activity on day 10 and actually suppressed the control response (Prell *et al.* 2000). Thus, while exogenous IL-2 given later in the response appeared to make up for the lack of IL-2 produced by $CD8^+$ T cells by expanding the population of CTLe, IL-2 administered early in the response had a detrimental effect on the development of CTL activity on day 10. This particular finding, while perplexing, was consistent with other studies demonstrating that administration of

high-dose IL-2 on days 0 – 2.5 following allogeneic bone marrow transplantation in mice prevented the development of graft-versus-host (GVH) disease, a CTL-mediated response against host tissues of the recipient mice (Abraham *et al.* 1992); although the dose of IL-2 required to suppress the P815 response was 50-500 times lower than the doses used to suppress the GVH response in those studies. Interestingly, work done in the Esser laboratory showed that the *IL-2* gene contains three DRE in its distal promoter (Lai *et al.* 1996). They also demonstrated that these DRE can be bound by the ligand-activated AhR/ARNT dimer. In addition, when splenocytes were stimulated *in vitro* with anti-CD3 in the presence of TCDD, this lead to increases in both IL-2 mRNA and secreted IL-2 compared to splenocytes stimulated with anti-CD3 alone (Jeon and Esser 2000). Collectively, studies using the P815 model identified the $CD4^+$ T cell as a possible proximal target of TCDD, possibly involving excess production of IL-2, but exactly how or what TCDD was doing to the $CD4^+$ T cells remained to be determined.

Effects of TCDD on the function and fate of $CD4^+$ T cells

Studies using anti-CD3 polyclonal activation of T cells. While the response to P815 tumor cells serves as an excellent model in which to study the CD4-dependent CTL response, it does not lend itself to studying $CD4^+$ T cells in particular, due to the low frequency of allo-specific $CD4^+$ T cells found in a normal C57Bl/6 mouse. One approach for increasing the frequency of responding T cells is to use a polyclonal activator such as anti-CD3 antibody that crosslinks the TCR and initiates the signaling

cascade for T cell activation. Using the anti-CD3 model, early studies revealed that exposure to TCDD increased incorporation of ^{3}H-TdR in lymph node cells isolated 24 hours after immunization with anti-CD3 and this corresponded with an increase in the percentage of T cells in both S and G_2/M phases of the cell cycle (Neumann *et al.* 1993). In these same studies, a small and inconsistent increase in the frequency of T cells expressing CD25 was observed 4 hours following anti-CD3. In no instances did TCDD alter incorporation of ^{3}H-TdR in naïve T cells, suggesting that TCDD specifically targets T cells undergoing activation rather than naïve T cells (Neumann *et al.* 1993; Pryputniewicz *et al.* 1998). Prell *et al.* (1995) followed up these studies to further characterize TCDD-mediated alterations in T cell subsets and production of cytokines in response to stimulation with anti-CD3. They found that as early as 48 hours after immunization with anti-CD3, exposure to TCDD resulted in a significant decrease in the percentage of $CD4^+$ T cells in the lymph nodes that persisted through day 6. Minimal effects were observed on cytokine levels in the plasma, but exposure to TCDD did cause a significant decrease in the amount of IL-2 and GM-CSF secreted from spleen cells isolated 1.5 hours after injection of anti-CD3 (Prell *et al.* 1995). Interestingly, the light scatter properties of lymph node cells isolated 48 hours after injection with anti-CD3 revealed a subpopulation that was consistent with apoptotic or dying cells and this was enhanced by TCDD (Prell *et al.* 1995). Thus, TCDD appeared to selectively target $CD4^+$ T cells as revealed by an enhanced loss of $CD4^+$ T cells from the lymph nodes. It is possible that this loss was the result of increased cell death given the alterations in light scatter properties of the lymph node cells. To determine if

increased apoptosis was responsible for the loss of $CD4^+$ T cells, Dearstyne and Kerkvliet (2002) examined the apoptosis of $CD4^+$ T cells following the immunization of mice with anti-CD3 and exposure to TCDD. Anti-CD3 alone caused an increase in apoptotic $CD4^+$ T cells as early as 12 hours after injection. Exposure to TCDD did not increase the percentage of $CD4^+$ T cells undergoing apoptosis, but did increase the frequency of cells with a late apoptotic/necrotic phenotype (Dearstyne and Kerkvliet 2002). This was consistent with earlier studies in which histological examination of lymph nodes revealed extensive necrosis that was more severe in TCDD-treated mice (Prell *et al.* 1995). Other studies suggested that Fas/FasL may be involved in the decreased cellularity of lymph nodes from TCDD-treated mice because Fas- or FasL-deficient mice appeared to be partially resistant to the effects of TCDD (Camacho *et al.* 2001; Pryputniewicz *et al.* 1998). However, when Dearstyne and Kerkvliet (2002) treated Fas- or FasL-deficient mice with anti-CD3 and TCDD and examined $CD4^+$ T cells specifically, exposure to TCDD still resulted in a 40% decrease in the number of $CD4^+$ T cells. While this decrease was not statistically significant, it did suggest that signaling through Fas/FasL alone was not responsible for the effects of TCDD on activated $CD4^+$ T cells. Collectively, studies using anti-CD3 stimulated T cells revealed a selective effect of TCDD on $CD4^+$ T cells that appeared to result in increased cell death.

Studies using antigen-specific activation of transgenic $CD4^+$ T cells. As *in vivo* treatment with high doses of anti-CD3 is known to induce significant cell death (Ferran *et al.* 1990), it was not known if TCDD would affect an antigen-specific

response in a similar manner. To this end, T cells from mice expressing a transgenic TCR specific for chicken ovalbumin (OVA) were used to study the effects of TCDD on the response of antigen-specific $CD4^+$ T cells following their transfer into syngeneic hosts (Shepherd *et al.* 2000). Following immunization with OVA, the antigen-specific $CD4^+$ T cells expanded through day 3 and this was unaffected by TCDD. However, beginning on day 5, TCDD caused a significant decrease in the number of antigen-specific $CD4^+$ T cells in the spleen, similar to the decrease of $CD4^+$ T cells in the lymph nodes of mice immunized with anti-CD3. Additionally, a significant decrease in the secretion of IL-2 was observed on day 1, with similar trends observed for IL-10, IL-4 and IFN-γ (although the decreases for the latter three cytokines on day 1 were not significant) (Shepherd *et al.* 2000). Surprisingly, minimal changes in the expression of activation markers were observed with the following exceptions. Exposure to TCDD led to a significant decrease in the up-regulation of CD11a on day 3 post-immunization, suggesting that TCDD might be inhibiting T cell activation. However, TCDD also augmented the activation-induced down-regulation of CD62L on day 3, indicating that TCDD has differential effects on the expression of surface proteins on activated T cells (Shepherd *et al.* 2000). Thus, similar to earlier findings, the initiation of the antigen-specific T cell response appears to proceed normally but is prematurely terminated, and this is reflected in a premature loss of $CD4^+$ T cells from the lymph nodes and/or spleen.

Hypothesis

Several important questions remained to be answered regarding the effects of TCDD on $CD4^+$ T cells. First, the underlying cause for the premature decline in the number of $CD4^+$ T cells, following what appeared to be essentially normal expansion, was unclear. Based on data from the P815 tumor allograft model that TCDD targets the first three days of the response, the initial hypothesis was that TCDD would alter the activation of $CD4^+$ T cells during the first three days following activation and that these alterations would sensitize the $CD4^+$ T cells to activation-induced cell death, possibly through alterations in the migration, adhesion, and/or the proliferation of antigen-specific $CD4^+$ T cells. This hypothesis was tested using OVA-specific transgenic T cells from DO11.10 mice and then extended to the GVH model. During the analysis of the responding $CD4^+$ T cells, it became apparent that TCDD was increasing the frequency of a subpopulation of $CD4^+$ T cells that expressed high levels of CD25, as well as GITR and CTLA-4, a phenotype that is consistent with natural Treg cells (Takahashi and Sakaguchi 2003). This new phenotypic data, together with previous data demonstrating that excess IL-2 administered early in the response is detrimental to the CTL response, led to the development of an alternate hypothesis: rather than augmenting T cell activation leading to increased activation-induced cell death, TCDD alters the differentiation of $CD4^+$ T cells to induce Treg cells. The generation of these Treg-like $CD4^+$ T cells and subsequent suppression of the CTL response is mediated at least in part by increased production of IL-2.

Chapter 2

EARLY CONSEQUENCES OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN EXPOSURE ON THE ACTIVATION AND SURVIVAL OF ANTIGEN-SPECIFIC T CELLS

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Abstract

TCDD is a potent immunotoxicant that suppresses adaptive immunity by mechanisms that are not well defined. To gain insight at the level of the T cell, we used the DO11.10 transgenic T cell receptor (TCR) mouse model in an adoptive transfer approach to characterize the influence of TCDD on the responsiveness of antigen-specific $CD4^+$ T cells *in vivo*. Flow cytometry was used to track the response of the OVA-specific transgenic $CD4^+$ T cells in syngeneic recipients using an antibody specific for the transgenic TCR (KJ1-26 [KJ]). Consistent with a previous report, exposure of the recipient mice to TCDD (15 μg/kg po) did not alter the initial expansion of the $CD4^+KJ^+$ T cells in the spleen following immunization with OVA but resulted in a significant decline in the number of cells present on and after day 4. The degree of decline was dependent on the dose of TCDD. On day 3 after OVA injection, a higher percentage of the $CD4^+KJ^+$ T cells in the spleen of TCDD-treated mice had down-regulated the expression of CD62L, a phenotype associated with T cell activation. Also on day 3, an increased number of $CD4^+KJ^+$ T cells were found in the blood of TCDD-treated mice. However, as in the spleen, the number of $CD4^+KJ^+$ T cells in the blood rapidly declined on day 4. $CD4^+KJ^+$ T cells in both the spleen and blood of TCDD-treated mice failed to up-regulate CD11a, an adhesion molecule important for sustained interaction between T cells and DC whereas the up-regulation of the adhesion molecule CD49d was not altered. Based on analysis of cell division history, $CD4^+KJ^+$ T cells in vehicle-treated mice continued to divide through day 4

whereas $CD4^+KJ^+$ T cells in TCDD-treated mice showed no further division after day 3. Increased annexin V staining on $CD4^+KJ^+$ T cells in TCDD-treated mice was also observed but not until days 5 and 6. Fas-deficient $CD4^+KJ^+$ T cells were depleted from the spleen of TCDD-treated mice in a manner similar to wild-type $CD4^+KJ^+$ T cells, suggesting that Fas signaling does not play a critical role in this model. On the other hand, gene array analysis of purified $CD4^+KJ^+$ T cells on day 3 showed that the expression of several genes associated with cell survival/death were altered by TCDD. Taken together, the results are consistent with our hypothesis that TCDD provides an early but inappropriate activation signal to the antigen-specific T cells that allows, and possibly enhances, the initial activation and proliferation of the T cells, yet at the same time, interferes with the vital expression of certain adhesion/costimulatory molecules that serve to enhance the survival of the T cells. These changes result in truncated proliferation, increased T cell death and suppression of the adaptive immune response.

Introduction

T helper cells ($CD4^+$ T cells) are a central component in the adaptive immune response to foreign antigen. When $CD4^+$ T cells recognize and bind antigenic peptide presented with major histocompatibility complex class II molecules on dendritic cells (DC), they become activated, clonally expand, and differentiate into effector T cells. The primary effector function of $CD4^+$ T cells is to support the survival and conditioning of dendritic cells, the differentiation and survival of cytotoxic T

lymphocytes (CTL), and the development of antibody-producing B cells. Without sufficient expansion and prolonged survival of $CD4^+$ T cells, adaptive immune responses are severely impaired.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a widespread environmental contaminant that possesses a profound capacity to suppress adaptive immune responses (Kerkvliet 2002; Kerkvliet and Burleson 1994). The immunotoxic effects of TCDD are mediated through binding to the aryl hydrocarbon receptor (AhR), which then translocates to the nucleus where it dimerizes with the AhR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003; Schmidt and Bradfield 1996; Vorderstrasse *et al.* 2001). The AhR-ARNT heterodimer functions as a transcription factor, binding to specific sequences of DNA called dioxin response elements (DRE). Alterations in DRE-regulated gene transcription are thought to underlie most, if not all, of the toxic responses induced by TCDD. Recent studies using a graft-vs-host model indicate that AhR expression in both $CD4^+$ and $CD8^+$ T cells is required for full suppression of T cell-mediated immunity by TCDD (Kerkvliet *et al.* 2002). However, the specific changes induced in T cells by activation of the AhR that result in suppression of their responsiveness have not been established.

Several animal models have been developed for the exclusive study of antigen-specific T cells. One such model is the DO11.10 adoptive transfer model that was developed by Kearney *et al.* (1994) and is used to study antigen-specific $CD4^+$ T cells *in vivo*. DO11.10 mice express a transgenic T cell receptor (TCR) that is specific for chicken ovalbumin (OVA) peptide 323-339 in the context of $I\text{-}A^d$ (Murphy *et al.*

1990). Since nearly all $CD4^+$ T cells in the DO11.10 mice express the transgenic TCR, a more physiologic frequency of antigen-specific T cells is achieved by adoptively transferring a small number of OVA-specific T cells into syngeneic Balb/c mice. The OVA-specific $CD4^+$ T cells are identified after adoptive transfer using the KJ1-26 (KJ) antibody, which recognizes the transgenic TCR. In addition, the majority of the $CD4^+$ T cells in the Balb/c mice that do not respond to OVA can be identified as the $CD4^+KJ^-$ population, allowing for the differentiation between antigen-specific and non-specific effects within the same animal.

Previous studies using the DO11.10 adoptive transfer model have shown that activated antigen-specific $CD4^+$ T cells expand normally in the spleen during the first three days of the response but then prematurely decline in mice treated with 15 μg TCDD/kg body weight (Shepherd *et al.* 2000). This premature loss of $CD4^+$ T cells was associated with a decrease in the production of anti-OVA IgM and IgG antibodies. However, phenotypic analysis of the activated $CD4^+$ T cells revealed only small changes in the expression of several early activation markers, providing little insight into how TCDD was causing the premature contraction of the T cell response.

In the studies reported here, we have examined in greater detail the effects of TCDD on the activation, proliferation, and survival of antigen-specific T cells. Multi-color flow cytometry was used to compare the OVA-induced activation of adoptively transferred $CD4^+KJ^+$ T cells in the spleen and blood of vehicle- and TCDD-treated mice. Cell division of the adoptively transferred T cells was measured by labeling the DO11.10 spleen cells with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester

(CFSE) prior to adoptive transfer. Altered T cell survival was examined by annexin V/SytoxGreen staining, and the specific role of Fas-mediated signaling in the loss of T cells was addressed by adoptively transferring OVA-specific T cells from Fas-deficient D011.10-lpr/lpr (DO11-lpr) mice. To identify other signaling pathways that may be altered in T cells by TCDD, changes in the expression profile of several genes associated with cell death or survival were analyzed in purified $CD4^+KJ^+$ T cells from vehicle- and TCDD-treated mice.

Materials and Methods

Animals

Balb/c mice (male and female) were purchased from either the Jackson Laboratory (Bar Harbor, ME) or ATL (Kent, WA) at 6-8 weeks of age. DO11.10 transgenic mice (originally provided by Dr. Marc Jenkins, University of Minnesota Medical School, Minneapolis, MN) were bred and maintained in the Laboratory Animal Resources Facility, Oregon State University (Corvallis, OR). DO11-lpr mice were generated by crossing DO11.10 mice with Balb/c-lpr/lpr mice (provided by Dr. Jan Erikson, Wistar Institute, Philadelphia, PA), and were bred and maintained in the Laboratory Animal Science Center, Boston University Medical Center (Boston, MA). All mice were housed in front of laminar flow units and were fed standard rodent chow and tap water *ad libitum*. All mice were maintained according to the National Research Council guidelines.

Reagents

All cell culture reagents were purchased from GibcoBRL (Grand Island, NY) except for fetal bovine serum (FBS), which was purchased from Hyclone (Ogden, UT). Phycoerythrin (PE)-anti-CD4 (clone RM4-5), CyChrome (CY)-anti-CD4 (clone H129.19), PE-anti-CD95 (Fas; clone Jo2), PE-KJ1-26 (DO11.10 hybridoma), fluorescein isothiocyanate (FITC)-anti-CD11a (clone M17/4), PE-anti-CD62L (clone MEL-14), PE-anti-CD49d (clone R1-2), CY-anti-CD45 (clone 30-F11), and PE-streptavidin were purchased from BD Biosciences Pharmingen (San Jose, CA). FITC-KJ1-26 and biotinylated KJ1-26 were purchased from Caltag (Burlingame, CA). Red613-streptavidin was purchased from GibcoBRL. ECD-streptavidin was purchased from Immunotech (Marseille, France).

TCDD Exposure

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1.5 μg/mL. Balb/c recipient mice were given a single oral dose of 15 μg TCDD/kg body weight or a similarly prepared vehicle solution. For the dose-response experiments, both donor DO11.10 and Balb/c recipient mice were given a single oral dose of 0, 0.5, 5, or 15 μg TCDD/kg body weight.

Adoptive transfer of DO11.10 cells

One day after treatment with TCDD or vehicle, Balb/c recipient mice (sex- and age-matched) were injected intravenously with DO11.10 spleen cells containing 3-5 x

10^6 $CD4^+KJ^+$ T cells as previously described (Shepherd *et al.* 2000). In each experiment, a single pool of DO11.10 spleen cells was used to inject both vehicle- and TCDD-treated mice, thereby eliminating any experimental variation due to different preparations of antigen-specific $CD4^+$ T cells.

For the experiments in which the role of Fas signaling was examined, spleens from both DO11-lpr and DO11.10-wildtype littermate (DO11-wt) mice were collected at Boston University Medical Center and shipped overnight on ice to Oregon State University. The spleens were received the following day and processed into single-cell suspensions. The viability of the spleen cells was similar to freshly collected spleen cells (> 90%), based on the exclusion of trypan blue and by flow cytometric analysis of forward and side scatter. Because there is an excessive proliferation of $CD4^-CD8^-$ T cells in lpr mice beginning at 6 weeks of age, $CD4^+$ spleen cells were enriched from both DO11-lpr and DO11-wt mice by magnetic cell-sorting prior to adoptive transfer. Spleen cells were labeled with anti-CD4 MicroBeads (Miltenyi Biotech, Auburn, CA) and then sorted using an autoMACS™ automated magnetic cell-sorter (Miltenyi Biotech) according to the manufacturer's recommendations. The purity of $CD4^+$ T cells was 85-90%, as determined by flow cytometry.

Immunization with OVA

Balb/c recipient mice were immunized with 2 mg OVA emulsified in complete Freund's adjuvant by intraperitoneal injection two days after the adoptive transfer of

DO11.10 cells. At various times after immunization the mice were sacrificed and the spleens were collected. In some experiments peripheral blood was also collected.

CFSE-labeling of DO11.10 cells

CFSE (Molecular Probes, Eugene, OR) was used to monitor the proliferation of $CD4^+KJ^+$ T cells in response to OVA *in vivo* (Lyons and Parish 1994). Spleen cells from DO11.10 mice were labeled with 10 μM CFSE at room temperature for 8 minutes. After washing, the cells were adoptively transferred into Balb/c recipients as described above.

Flow cytometry

Spleens were processed into single-cell suspensions using the frosted ends of glass slides. For most experiments, splenic red blood cells were removed by hypotonic lysis. For studies in which apoptosis was being assessed, the red blood cells were lysed using ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2). Blood samples were collected by heart puncture (500 μL/mouse) using a syringe coated with heparin and stored on ice. All cell preparations were enumerated using a Coulter Counter (Coulter Electronics, Hialeah, FL).

Aliquots of spleen or blood cells were washed and resuspended in phosphate buffered saline containing 1.0% bovine serum albumin and 0.1% sodium azide. Binding of the antibodies to Fc receptors was blocked with rat IgG, hamster IgG, or normal mouse serum (Jackson Immunoresearch Labs, Inc., West Grove, PA). The

cells were stained with anti-CD4 and KJ antibodies along with antibodies to one or two of the following markers: CD11a, CD49d, CD62L, and Fas. Separate aliquots of the cells were stained with isotype-matched immunoglobulin to determine non-specific antibody binding. For analysis of peripheral blood lymphocytes (PBL), the lymphocyte population was identified by side scatter and staining with anti-CD45. The majority of the red blood cells were removed after staining using FACS lysing solution (BD Biosciences, San Jose, CA).

For some experiments, after surface staining, the cells were stained for apoptosis and viability using the Vybrant apoptosis assay kit from Molecular Probes (Eugene, OR) according to the manufacturer's instructions. The kit contains Alexa Fluor 488-labeled-annexin V, which binds to phosphatidylserine residues in the outer leaflet of the cell membrane, and SytoxGreen, a fluorescent viability stain. Apoptotic cells are then differentiated from dead cells by the intensity of the fluorescence, with dead cells exhibiting higher fluorescence than apoptotic cells.

Data were collected on freshly stained cells using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL). The total number of events analyzed for each sample was determined by first gating on the live cells and then monitoring the $CD4^+KJ^+$ population until 3000-5000 $CD4^+KJ^+$ cells had been collected. The data were analyzed using Winlist software (Verity Software House, Topsham, ME).

Analysis of cell division history by fluorescence of CFSE

Forward scatter and CFSE-fluorescence associated with the $CD4^+KJ^+$ population were displayed in a two-parameter density plot. Electronic regions were drawn to identify each cell division based on the 50% decrease in the mean channel fluorescence of CFSE which results when the cells divide. The mean number of cell divisions for each animal was calculated by taking the weighted average of the cell divisions: $\sum$ ([percentage of cells within division$_N$/100] x N), where N is the number of cell divisions from 0 to 7.

Isolation of $CD4^+KJ^+$ T cells for gene array analysis

Spleens from vehicle- or TCDD-treated Balb/c recipient mice (n = 6-7/group) that had been previously injected with D011.10 spleen cells were pooled and processed three days after immunization with OVA as described above. T cells were labeled with biotinylated KJ antibody followed by anti-biotin MicroBeads (Miltenyi Biotech) and enriched using an autoMACS™ automated magnetic cell-sorter according to the manufacturer's recommendations. The enriched KJ^+ cells were then labeled with PE-streptavidin and CY-anti-CD4 antibody and sorted on a MoFlo® high-performance cell-sorter (DakoCytomation, Fort Collins, CO) to a purity of $\geq$ 95% $CD4^+KJ^+$ T cells.

RNA preparation and gene array

Total RNA was isolated from purified $CD4^{+}KJ^{+}$ T cells using RNeasy Mini columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA was quantified and checked for purity by measuring the absorbance at 260 nm and 280 nm. In addition, a 1.5% agarose gel was run to ensure little or no DNA contamination.

The RNA samples were analyzed using the mouse apoptosis Q series GEArray (SuperArray, Inc., Bethesda, MD) following the manufacturer's protocol. The 96 genes represented on the array are shown in Table 1. Chemiluminescence was measured using a Kodak ImageStation 440CF (Eastman Kodak Company, Rochester, NY). The digital images were then quantitated using ImageQuant software (Molecular Dynamics, Piscataway, NJ).

Statistical analysis

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

Results

Premature loss of $CD4^+KJ^+$ T cells from TCDD-treated mice is dose-dependent

Previous studies documented the expansion and decline of $CD4^+KJ^+$ spleen cells in vehicle- and TCDD-treated mice on days 1, 3, 5, 7, and 10 after immunization with OVA. In mice treated with an immunosuppressive dose of TCDD (15 μg/kg), normal expansion was observed on day 3 followed by a significant reduction in the number of $CD4^+KJ^+$ spleen cells beginning on day 5 and persisting through day 10 (Shepherd *et al.* 2000). In order to establish the early time-course of the effects of TCDD on $CD4^+$ T cells, adoptively transferred $CD4^+KJ^+$ T cells in the spleen were

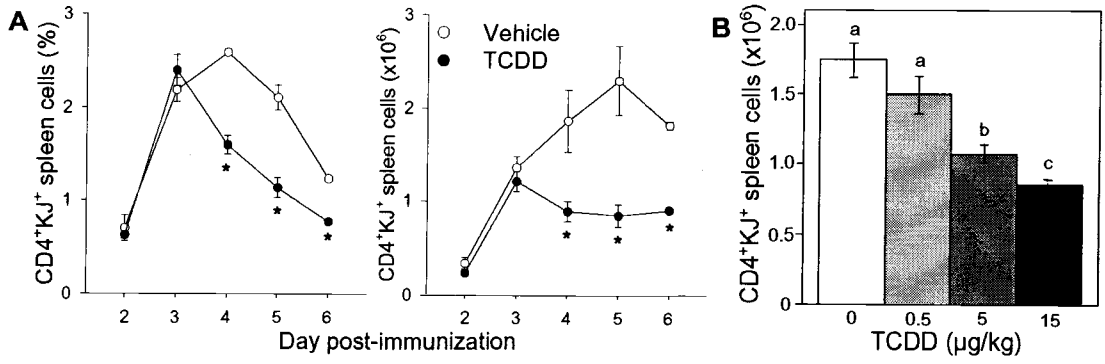

Figure 2-1. TCDD decreases the percentage and number of $CD4^+KJ^+$ spleen cells beginning on day 4 after immunization with OVA. *(A) Mice were dosed with 15 μg TCDD/kg body weight (filled circles) or vehicle (open circles) three days prior to immunization with OVA. Spleen cells were collected daily beginning on day 2 after immunization with OVA. The spleen cells were then stained with anti-CD4 and KJ antibodies as described in Materials and Methods and analyzed by flow cytometry. Each data point is the mean ± SEM of 4 mice, except the vehicle group on day 2 and the TCDD group on day 4, where n = 3. * $p \leq 0.05$ compared to vehicle on the same day. (B) Mice were dosed with 0, 0.5, 5, or 15 μg TCDD/kg body weight and the number of $CD4^+KJ^+$ spleen cells was determined on day 5 after immunization with OVA. Each bar represents the mean ± SEM (n = 6/group). Statistically significant differences ($p \leq 0.05$) between groups are indicated by different letters above each bar.*

monitored daily on days 2 through 6 following immunization with OVA. As shown in Figure 2-1A, the percentage and number of $CD4^+KJ^+$ T cells in the spleen of vehicle-treated mice increased following immunization with OVA, peaking on day 4 and day 5, respectively. Exposure to TCDD (15 μg/kg) significantly reduced both the percentage and number of $CD4^+KJ^+$ T cells in the spleen beginning on day 4 and continuing through day 6. These data confirm and extend the results of Shepherd *et al.* (2000).

To determine if the depletion of $CD4^+KJ^+$ spleen cells was dose-dependent, mice were treated with 0, 0.5, 5, or 15 μg TCDD/kg body weight and the number of $CD4^+KJ^+$ T cells in the spleen was determined on day 5. As shown in Figure 2-1B, the number of $CD4^+KJ^+$ spleen cells decreased in a dose-dependent manner and was statistically significant at doses of TCDD ≥ 5 μg/kg. A 15 μg/kg dose of TCDD was used in all subsequent experiments.

TCDD influences the number of $CD4^+KJ^+$ T cells in the blood

Activated T cells can be found circulating in the blood following exposure to antigen. To determine if the decline in splenic $CD4^+KJ^+$ T cells induced by TCDD reflected a systemic effect, blood samples were taken from vehicle- and TCDD-treated mice on days 2 through 5 post-immunization and examined for the presence of $CD4^+KJ^+$ T cells. As shown in Figure 2-2, the percentage and number of $CD4^+KJ^+$ T cells in the blood increased in vehicle-treated mice between days 2 and 3, followed by a modest decline on days 4 and 5. In TCDD-treated mice, a similar pattern was

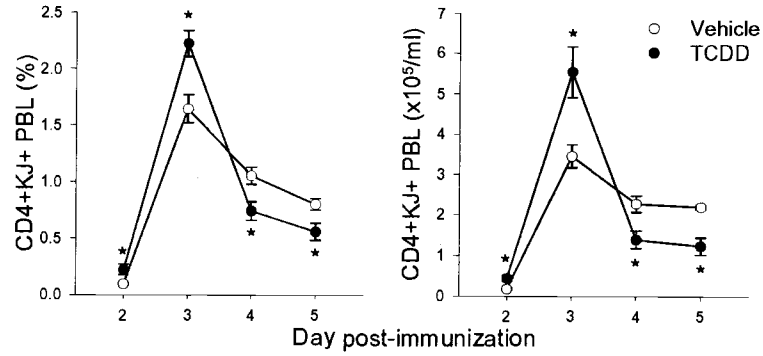

Figure 2-2. TCDD increases, and then decreases, the percentage and number of $CD4^+KJ^+$ T cells in the blood after immunization with OVA. *Mice were dosed with TCDD (filled circles) or vehicle (open circles) as described in Figure 2-1A. Blood lymphocytes were stained on days 2-5 as described in the Materials and Methods. Each data point is the mean ± SEM of 5 mice. * $p \leq 0.05$ compared to vehicle on the same day.*

observed, however both the increase and decrease of $CD4^+KJ^+$ T cells was more pronounced. In fact, the total number of $CD4^+KJ^+$ T cells in the blood on day 3 was 36% higher in TCDD-treated mice while on day 4 there were 39% fewer $CD4^+KJ^+$ T cells in the blood (Fig. 2-2). The significant increase in $CD4^+KJ^+$ T cells in the blood of TCDD-treated mice on day 3 was an unexpected observation. However, similar results have been observed in two additional experiments.

TCDD selectively alters the expression of activation molecules on $CD4^+KJ^+$ T cells

The activation of T cells is characterized by changes in the expression of numerous co-stimulatory and adhesion molecules on the cell surface. We examined the kinetics of expression of three important activation molecules, CD62L, CD11a, and CD49d, on $CD4^+KJ^+$ T cells in the spleen after OVA injection. We also examined expression of CD11a and CD49d on $CD4^+KJ^+$ T cells in the blood of the same animals

(CD62L expression was not detected on $CD4^+KJ^+$ or $CD4^+KJ^-$ cells in the blood, which was likely related to technical problems).

CD62L is a surface protein that is expressed at a high level on naïve T cells and is gradually shed from the surface of activated T cells as they divide. As shown in Figure 2-3A, CD62L expression was reduced on a majority of the antigen-specific $CD4^+KJ^+$ T cells in the spleen of vehicle-treated mice on day 3 after OVA injection when compared to $CD4^+KJ^-$ T cells. Over time, the percentage of $CD4^+KJ^+$ T cells that had fully down-regulated CD62L expression increased from approximately 50% on day 3 to 70% on day 6 in vehicle-treated mice while no change was seen in the $CD4^+KJ^-$ T cells. Interestingly, the percentage of $CD4^+KJ^+$ T cells that had fully down-regulated CD62L was significantly increased by TCDD exposure and was already at 72% on day 3. The higher percentage of $CD62L^{neg}$ cells in TCDD-treated

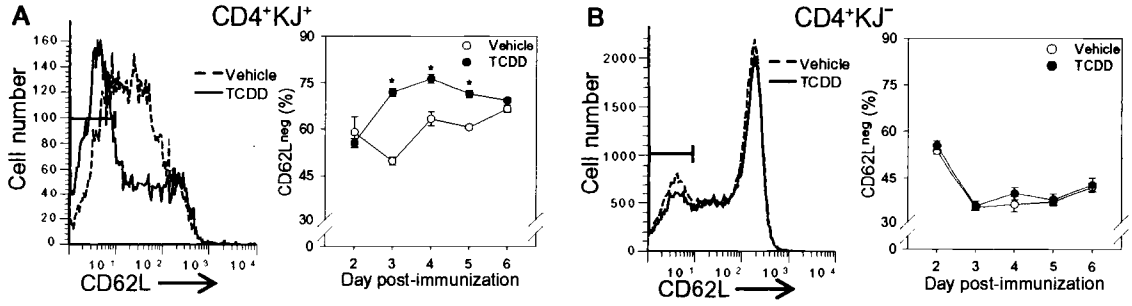

Figure 2-3. TCDD enhances the down-regulation of CD62L on $CD4^+KJ^+$ spleen cells but not on $CD4^+KJ^-$ T cells. *Mice were dosed with TCDD (solid line and filled circles) or vehicle (dotted line and open circles) as described in Figure 2-1A. The expression of CD62L on $CD4^+KJ^+$ T cells (A) or $CD4^+KJ^-$ T cells (B) was determined by flow cytometry as described in the Materials and Methods. Representative histograms from day 3 are shown (left-hand panels). The bars on the histograms indicate the region used to identify the $CD62L^{neg}$ population. The expression of CD62L was also measured on days 2-6 (right-hand panels; same animals as in Figure 2-1A), where each data point is the mean ± SEM of 4 mice, except the vehicle group on day 2 and the TCDD group on day 4, where n = 3. * $p \leq 0.05$ compared to vehicle on the same day.*

mice was maintained through day 6 despite the fact that the total number of $CD4^+KJ^+$ T cells had significantly decreased during this time. TCDD did not alter the percentage of $CD4^+KJ^-$ T cells that were $CD62L^{neg}$ over the same time period (Fig. 2-3B), indicating that the influence of TCDD on CD62L expression was related to antigenic activation. These data suggest that more $CD4^+KJ^+$ T cells are being activated earlier in TCDD-treated mice.

Increased expression of CD11a on the cell surface is another characteristic of T cell activation. As shown in Figure 2-4A, the mean channel fluorescence (MCF) of

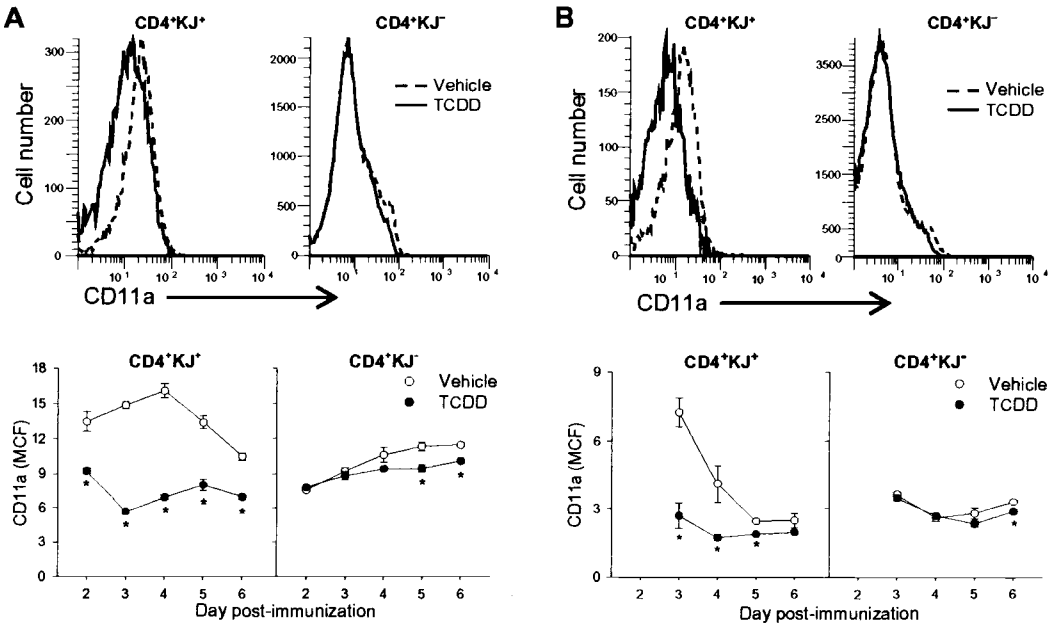

Figure 2-4. The OVA-induced up-regulation of CD11a on $CD4^+KJ^+$ T cells in the spleen and blood is blocked by TCDD. *Mice were dosed with TCDD or vehicle as described in Figure 2-1A. The expression of CD11a on $CD4^+KJ^+$ T cells or $CD4^+KJ^-$ T cells in the spleen (A) or blood (B) was determined by flow cytometry as described in the Materials and Methods. Representative histograms from day 3 are shown (upper panels), where vehicle = dotted line, TCDD = solid line. Isotype staining was on the baseline. In the lower panels, the time course of the expression of CD11a is shown (vehicle = open circle, TCDD = filled circle; same animals as in Figure 2-1A), where each data point is the mean ± SEM of 4 mice, except the vehicle group on day 2 and the TCDD group on day 4, where n = 3. * $p \leq 0.05$ compared to vehicle on the same day.*

CD11a was already increased on $CD4^+KJ^+$ T cells in the spleen of vehicle-treated mice on day 2 compared to $CD4^+KJ^-$ T cells, and it remained elevated through day 5. Unexpectedly, exposure to TCDD appeared to prevent the up-regulation of CD11a on $CD4^+KJ^+$ T cells in the spleen and expression remained at the level found on $CD4^+KJ^-$ T cells throughout the time-course (Fig. 2-4A). The expression of CD11a on $CD4^+KJ^-$ spleen cells increased slightly over time, probably as a result of bystander activation via cytokines. This antigen-nonspecific increase in CD11a expression was also significantly decreased in TCDD-treated mice on days 5 and 6 of the response (Fig. 2-4A).

CD11a expression on $CD4^+KJ^+$ T cells in the blood is shown in Figure 2-4B. In vehicle-treated mice, CD11a expression was increased on days 3 and 4 after immunization with OVA and then returned to the baseline level expressed on $CD4^+KJ^-$ T cells. As in the spleen, no up-regulation of CD11a on the $CD4^+KJ^+$ T cells in the blood of TCDD-treated mice was observed throughout the time-course and expression levels remained similar to the level expressed on $CD4^+KJ^-$ T cells (Fig. 2-4B).

The expression of CD49d on $CD4^+KJ^+$ T cells in both the spleen (Fig. 2-5A) and blood (Fig. 2-5B) was elevated on all days examined when compared to the level expressed by $CD4^+KJ^-$ T cells. In contrast to the other activation molecules, TCDD had no effect on the expression of CD49d on $CD4^+KJ^+$ or $CD4^+KJ^-$ T cells (Fig. 2-5).

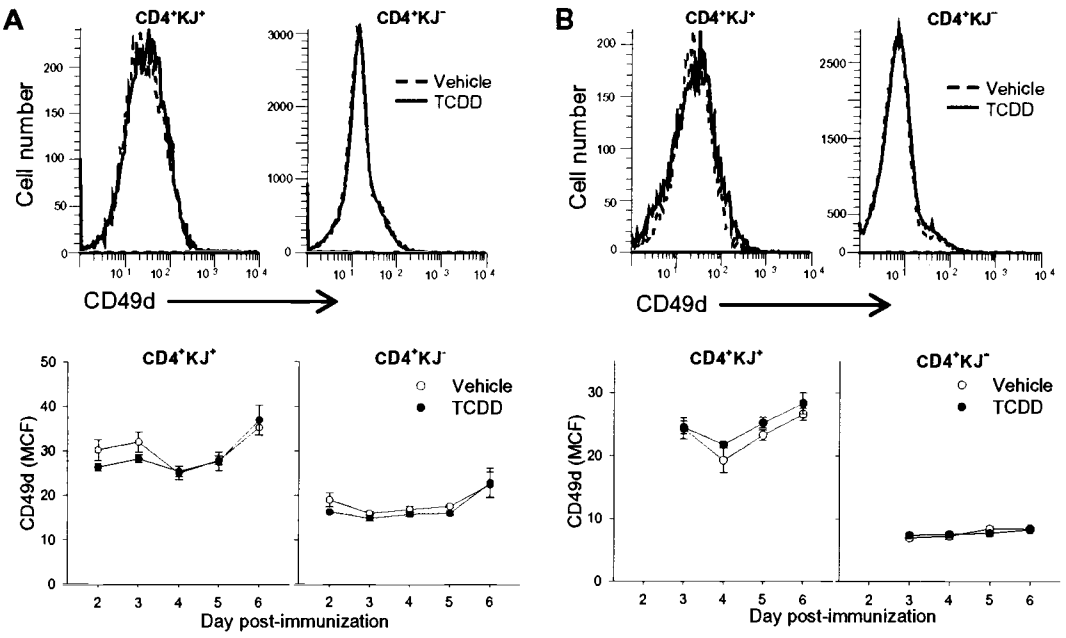

Figure 2-5. TCDD does not affect the expression of CD49d on $CD4^+KJ^+$ T cells in the spleen or blood. *Mice were dosed with TCDD or vehicle as described in Figure 2-1A. The expression of CD49d on $CD4^+KJ^+$ T cells or $CD4^+KJ^-$ T cells in the spleen (A) or blood (B) was determined by flow cytometry as described in the Materials and Methods. Representative histograms from day 3 are shown (upper panels), where vehicle = dotted line, and TCDD = solid line. Isotype staining was on the baseline. In the lower panels, the time course of the expression of CD49d is shown (vehicle = open circle, TCDD = filled circle; same animals as in Figure 2-1A), where each data point is the mean ± SEM of 4 mice, except the vehicle group on day 2 and the TCDD group on day 4, where n = 3. * $p \leq 0.05$ compared to vehicle on the same day.*

Taken together, the enhanced down-regulation of CD62L expression and the lack of up-regulation of CD11a expression on $CD4^+KJ^+$ T cells in TCDD-treated mice suggest that TCDD is selectively altering early activation events in $CD4^+$ T cells.

TCDD prematurely terminates the proliferation of $CD4^+KJ^+$ T cells

Sufficiently prolonged clonal expansion of the antigen-specific $CD4^+$ T cell population is necessary for the generation of an adaptive immune response. The

systemic decline in the antigen-specific $CD4^+KJ^+$ T cell population between day 3 and day 4 in TCDD-treated mice suggested that exposure to TCDD may be inducing premature termination of clonal expansion. To test this hypothesis, D011.10 T cells were labeled with CFSE prior to adoptive transfer into Balb/c mice, and spleen cells were collected on days 3 and 4 after immunization with OVA for analysis of cell division history. As shown in Figure 2-6, naïve $CD4^+KJ^+$ T cells from non-immunized mice did not divide, whereas nearly all of the $CD4^+KJ^+$ T cells from both TCDD- and vehicle-treated mice immunized with OVA had divided one or more times by day 3. By day 4 the $CD4^+KJ^+$ spleen cells from vehicle-treated mice had divided further, as indicated by the additional decrease in the fluorescence intensity of CFSE compared to

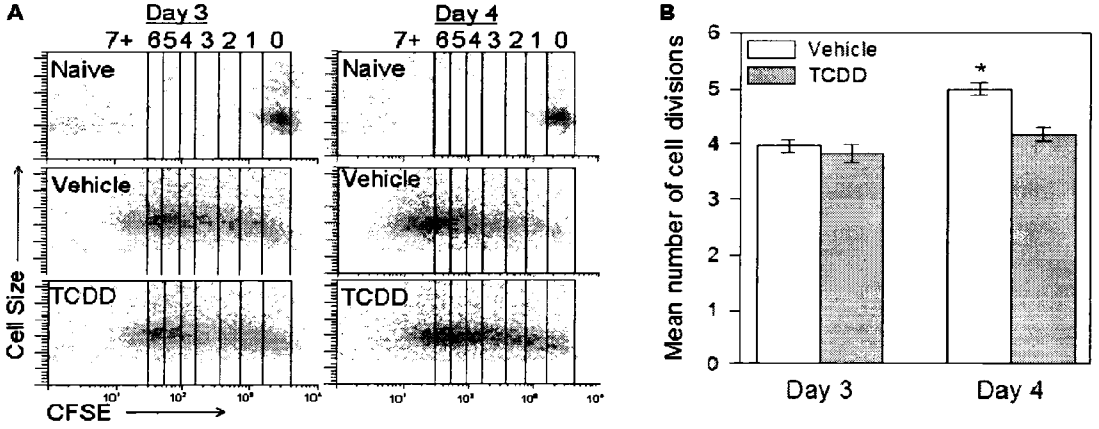

Figure 2-6. TCDD inhibits the continued cycling of $CD4^+KJ^+$ spleen cells after day 3. *Mice were dosed with TCDD or vehicle as described in Figure 2-1A. Spleen cells from DO11.10 mice were labeled with CFSE prior to adoptive transfer into Balb/c mice. On days 3 and 4 post-immunization, spleen cells were stained with anti-CD4 and KJ antibodies as described in the Materials and Methods. A, Representative density plots of naïve, vehicle- or TCDD-treated CFSE-labeled $CD4^+KJ^+$ spleen cells from day 3 and day 4 are shown. Numbers above the histograms indicate the number of division cycles. B, The mean number of cell divisions was calculated on day 3 and day 4 for vehicle- (open bars) and TCDD-treated (filled bars) mice as described in the Materials and Methods. * $p \leq 0.05$ compared to all other groups ($n = 4$ mice/group/day).*

day 3. In contrast, in TCDD-treated mice the CFSE fluorescence distribution on day 4 was similar to day 3, suggesting that no additional cell division had occurred. When the mean number of divisions for the $CD4^+KJ^+$ spleen cells was calculated (Fig. 2-6B), the $CD4^+KJ^+$ cells from both vehicle- and TCDD-treated mice had undergone an average of 4 divisions by day 3. By day 4, the average number of cell divisions had increased to 5 for vehicle-treated mice but was unchanged in TCDD-treated mice. These results suggest that premature termination of cell division may contribute to the reduced number of activated $CD4^+KJ^+$ T cells in the spleen of TCDD-treated mice.

TCDD decreases the survival of $CD4^+KJ^+$ T cells

The decline in the number of $CD4^+KJ^+$ T cells in TCDD-treated mice could also be due to enhanced cell death. In order to address this possibility, the percentage of cells expressing an apoptotic or dead phenotype was determined using annexin V and SytoxGreen, respectively. Figure 2-7A shows the typical staining pattern of annexin V and SytoxGreen on spleen cells. Annexin V^+ cells typically fall within the third log-decade, while the $SytoxGreen^+$ cells can be found in the fourth log-decade. As shown in Figure 2-7C, the percentage of vehicle-treated $CD4^+KJ^+$ T cells expressing an apoptotic phenotype appeared to increase slightly over time but remained within the same low range as seen in the $CD4^+KJ^-$ T cells. Treatment with TCDD significantly increased the percentage of apoptotic $CD4^+KJ^+$ T cells on days 5 and 6 after immunization with OVA, without affecting the $CD4^+KJ^-$ T cells. Interestingly, in this study, annexin V^+ cells were detected after gating on the

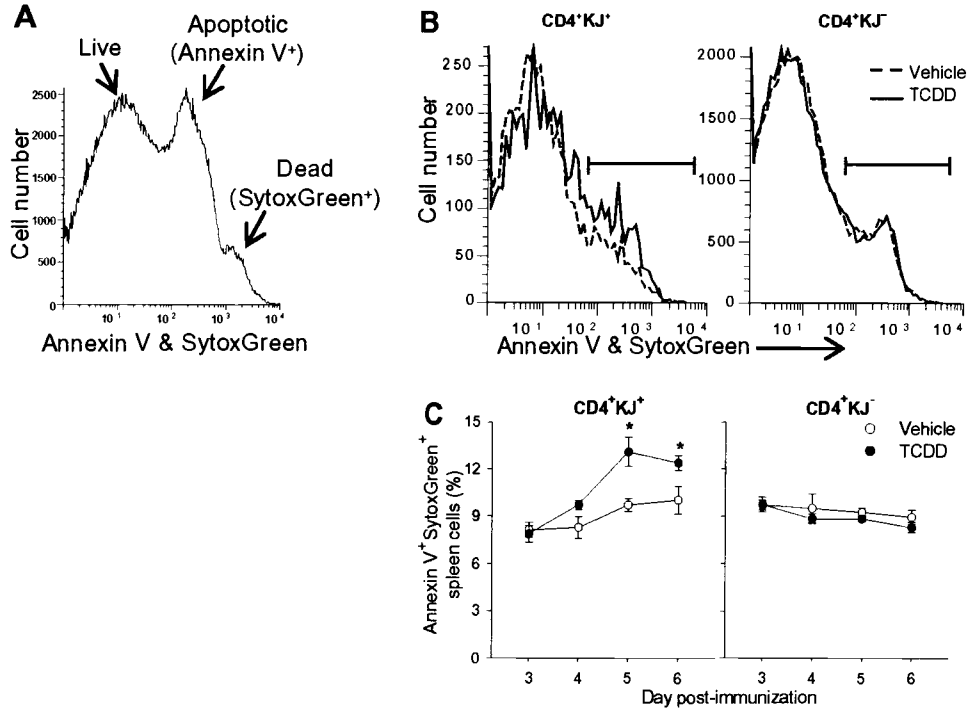

Figure 2-7. TCDD exposure increases the percentage of CD4$^+$KJ$^-$ spleen cells with an apoptotic phenotype. *Mice were treated with TCDD or vehicle as described in Figure 2-1A. The percentage of CD4$^+$KJ$^+$ T cells or CD4$^+$KJ$^-$ T cells displaying an apoptotic phenotype and their viability was determined by flow cytometry as described in the Materials and Methods. A, The typical staining pattern for annexin V and SytoxGreen on ungated spleen cells is shown. B, Representative histograms from day 5 are shown, where vehicle = dotted line and TCDD = solid line. The bars on the histograms indicate the region used to identify the AnnexinV$^+$SytoxGreen$^+$ population. C, The apoptotic phenotype of CD4$^+$KJ$^+$ T cells over time is shown (vehicle = open circles, TCDD = filled circles; same animals as in Figure 2-1A), where each data point is the mean ± SEM of 4 mice, except the TCDD group on day 4, where n = 3. * $p \leq 0.05$ compared to vehicle on the same day.*

CD4$^+$KJ$^+$ T cells but few, if any SytoxGreen$^+$ cells were seen (Fig. 2-7B). This contrasts with the results of a previous study using 7-amino actinomycin D (7-AAD) as a viability stain, wherein TCDD significantly increased the percentage of CD4$^+$KJ$^+$ T cells that stained with both annexin V and 7-AAD rather than annexin V alone (data not shown). The reason for these different results is not known. In either case, increased apoptosis or death was not apparent until days 5 and 6, suggesting that the

major decline in T cells between day 3 and day 4 may be precipitated by a cause other than enhanced cell death.

Fas is not required for the loss of $CD4^+KJ^+$ T cells

Previously published studies have implicated Fas/FasL (CD95/CD178) in the effects of TCDD on T cells (Camacho *et al.* 2001; Camacho *et al.* 2002; Dearstyne and Kerkvliet 2002). To determine if Fas signaling in the T cell was necessary for the decreased number of $CD4^+KJ^+$ T cells, splenocytes from DO11-lpr mice, which lack functional Fas, were used for adoptive transfer. As shown in Figure 2-8, the expansion

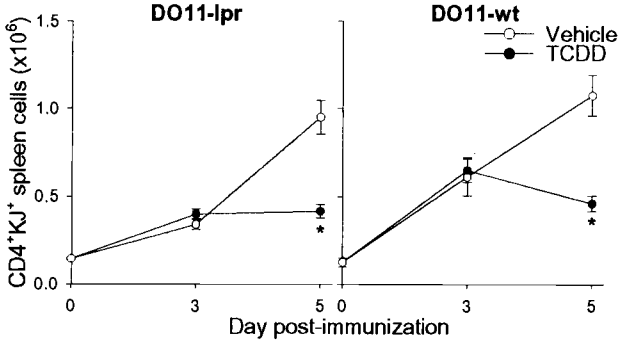

Figure 2-8. Fas expression on $CD4^+KJ^+$ T cells is not required for the deletion of $CD4^+KJ^+$ cells in the spleen induced by TCDD. *Mice were dosed with TCDD (filled circles) or vehicle (open circles) as described in Figure 2-1A. Spleen cells from DO11-lpr or DO11-wt mice were adoptively transferred as described in the materials and methods. The number of $CD4^+KJ^+$ spleen cells was determined by flow cytometry as described in the Materials and Methods. Each data point is the mean ± SEM of 5 mice. * $p \leq 0.05$ compared to vehicle on the same day.*

of $CD4^+KJ^+$ T cells from DO11-lpr mice and DO11-wt mice was similar in vehicle-treated mice through day 5 following immunization with OVA. Treatment with TCDD induced the same level of depletion of DO11-lpr and DO11-wt $CD4^+KJ^+$ spleen cells

on day 5 (56% and 57% decrease, respectively, Fig. 2-8), suggesting that Fas signaling in the T cells was not required for the decreased number of $CD4^+KJ^+$ T cells.

To further address a possible effect of TCDD on Fas signaling, the expression of Fas on $CD4^+KJ^+$ T cells in the spleen was assessed. As shown in Figure 2-9, the median channel fluorescence (MedianCF) of Fas was increased on $CD4^+KJ^+$ T cells as early as one day after immunization with OVA, and continued to increase through day 4 in vehicle-treated mice. Interestingly, the expression of Fas was increased to a lesser degree in mice treated with TCDD, starting on day 3 (Fig. 2-9), and this reduction was evident through day 10 (data not shown). Minimal changes in the surface expression of Fas were seen on $CD4^+KJ^-$ T cells following immunization with OVA and TCDD had no effect on the expression of Fas on these cells (Fig. 2-9). Taken together, these data support the conclusion that Fas signaling in the T cell is not involved in the loss of the $CD4^+KJ^+$ T cells in mice treated with TCDD.

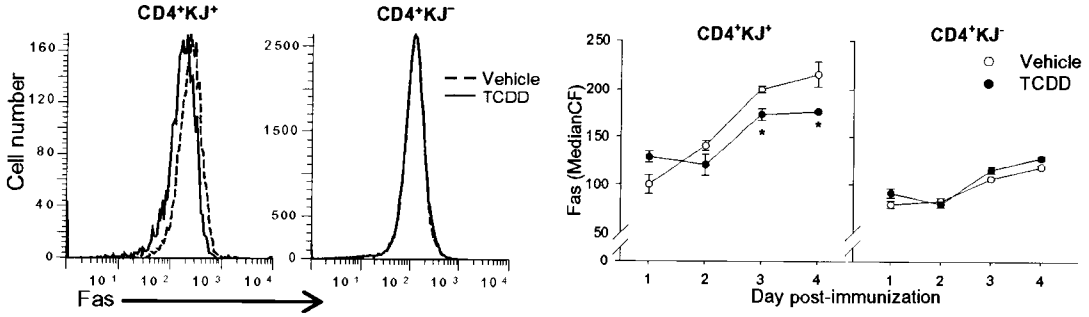

Figure 2-9. TCDD decreases the expression of Fas on $CD4^+KJ^+$ spleen cells. *The expression of Fas was determined by flow cytometry on the $CD4^+KJ^+$ T cells and $CD4^+KJ^-$ T cells from mice treated with vehicle (open circles) or TCDD (filled circles) as described in the Materials and Methods and Figure 2-1A. Each data point is the mean ± SEM of 3 mice. * $p \leq 0.05$ compared to vehicle on the same day.*

TCDD alters the expression profile of genes involved in cell death and survival

Because Fas did not appear to play a significant role in the depletion of the $CD4^+KJ^+$ T cells in the spleen, a gene array was used to determine if TCDD influenced other pathways of cell survival (Table 2-1). Day 3 post-immunization was selected for analysis based on the rationale that changes on day 3 would best predict the precipitating cause of the decline in the number of $CD4^+KJ^+$ T cells that occurs during the next 24 hours. In addition, analysis on day 3 avoided the complication of T

Table 2-1. Genes represented on the mouse apoptosis Q series array.[a]

Gene/Domain Family	Genes
Bcl-2	Bad, Bak, Bar-like, Bax, Bcl-2, Bcl-w, Bcl-x, Bfl-1, Bid, Bik, Bim, Blk, Bok, Hrk, Mcl-1, Nip3
CARD	Apaf-1, Arc, ASC, Bcl-10, Bcl-2-L-10, Nop30-like
Caspase	caspase 1, caspase 2, caspase 3, caspase 6, caspase 7, caspase 8, caspase 9, caspase 11, caspase 12, caspase 14
CIDE	CIDE-A, CIDE-B, DFF40, DFFA
DD/DED	Casper, CRADD, DAPkinase, FADD, MyD88, RIP, Flash
IAP	Bruce, IAP1, IAP2, NAIP1, NAIP2, NAIP5, survivin, XIAP
ATM/p53	ATM, chk1, chk2, gadd45, Hus1, Mdm2, p21, p53, RPA
TNF	4-1BBL, April, CD27L, CD30L, CD40L, DR3L, FasL, HVEM-L, LTβ, OX40-L, TNFα, TNFβ, TRAIL, TRANCE
TNFR	4-1BB, CD27, CD30, CD40, DR3, DR5, DR6, Fas, LTαR, OPG, OX40, RANK, TNFR1, TNFR2
TRAF	TANK, TRAF1, TRAF2, TRAF3, TRAF4, TRAF5, TRAF6, TRIP

[a] For a full description of each gene, see http://www.ncbi.nlm.nih.gov/omim/ (Online Mendelian Inheritance in Man, OMIM™. McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), 2000.)

cell depletion having already occurred in TCDD-treated mice. Two independent experiments were conducted using $CD4^+KJ^+$ T cells purified from the spleens of 6-7 vehicle- or TCDD-treated mice. For each experiment, the expression of each gene was calculated as a percentage of the housekeeping gene, GAPDH. The results of the two experiments were then averaged (n = 2). As shown in Figure 2-10, the expression of 21 genes was increased at least 2-fold in the $CD4^+KJ^+$ T cells from the spleens of TCDD-treated mice. 4-1BBL was not detected in the vehicle-treated samples so the expression relative to GAPDH was zero. Of the 21 genes shown in Figure 2-10, all but four (caspase 1, caspase 8, cide b, and rpa) had non-overlapping standard deviations (SD). The remaining 75 genes were unaffected by TCDD or were increased in one experiment and decreased in the other.

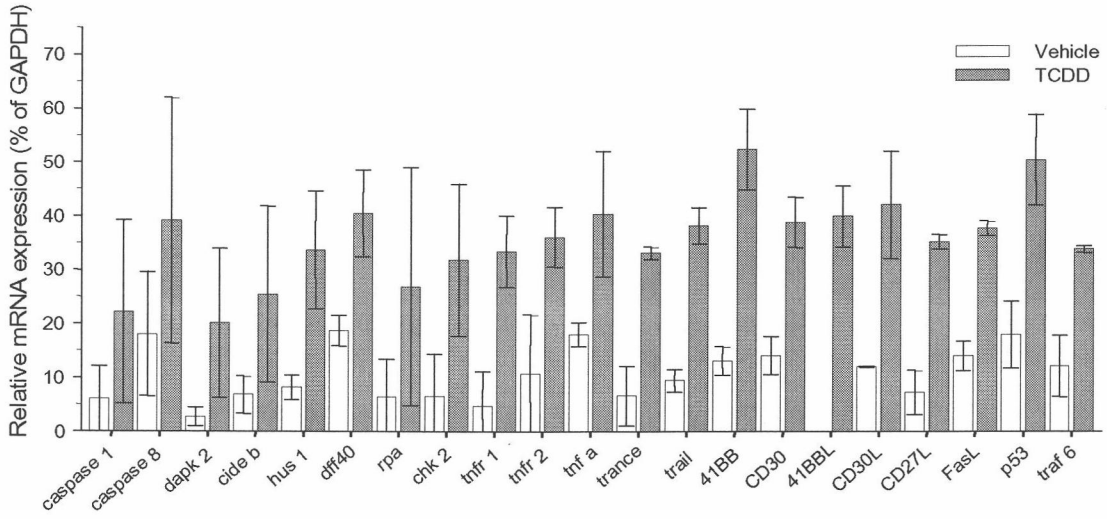

Figure 2-10. Apoptosis- and survival-related genes are differentially expressed in purified $CD4^+KJ^+$ spleen cells from TCDD- (filled bars) and vehicle-treated (open bars) mice on day 3 post-immunization. *Mice were dosed with TCDD or vehicle as described in Figure 2-1A. The array was prepared using RNA from purified $CD4^+KJ^+$ T cells pooled from 6-7 mice as described in the Materials and Methods. Data shown represent the mean mRNA expression (± SD) as a percentage of the housekeeping gene, GAPDH, from two independent experiments.*

Discussion

TCDD is a highly potent immunosuppressive chemical that impairs adaptive immune responses by mechanisms that are not understood. Although engagement of the AhR in both $CD4^+$ and $CD8^+$ T cells was recently shown to be required for suppression of the $CD4^+$-dependent $CD8^+$-mediated CTL response in a graft-vs-host model (Kerkvliet *et al.* 2002), the cellular changes that underlie altered T cell responsiveness have not been defined. Previous studies using the DO11.10 adoptive transfer model have shown that the antigen-specific $CD4^+KJ^+$ T cells expand normally in TCDD-treated mice following immunization with OVA but are prematurely lost from the spleen (Shepherd *et al.* 2000). In the present studies, we have tracked the antigen-specific response of adoptively transferred $CD4^+KJ^+$ T cells in the spleen and blood of vehicle- and TCDD-treated mice in order to identify changes in the T cells that precede the loss of activated $CD4^+KJ^+$ T cells.

One of the earliest changes induced by TCDD was a block in the up-regulation of CD11a, which could have several ramifications in terms of suppressed immune responses. CD11a is a β2 integrin that is expressed on activated T cells and is critical for maintaining contact between T cells and antigen presenting cells, thus allowing the T cells to become fully activated (Bachmann *et al.* 1997; Bleijs *et al.* 2000; Cai *et al.* 1997). A failure to sufficiently up-regulate CD11a in TCDD-treated mice could result in a reduced ability to maintain contact with dendritic cells (DC) leading to a loss of adequate co-stimulation or sufficiently prolonged survival signals. The loss of these

DC-derived signals could result in the passive death of the activated T cells. It is important to note that basal expression of CD11a was not reduced by TCDD. Thus, the initial interaction between the DC and T cells did not appear to be impeded, consistent with the seemingly normal OVA-induced proliferation of the $CD4^+KJ^+$ T cells in the first three days of the response.

As in the spleen, CD11a expression was elevated on $CD4^+KJ^+$ T cells in the blood of vehicle-treated mice on day 3 after immunization and this up-regulation was similarly blocked in TCDD-treated mice. In studies where CD11a was blocked with monoclonal antibodies, the localization of T cells at the site of antigen was impaired (Hamann *et al.* 2000; Issekutz 1993), suggesting that CD11a is involved in the emigration of activated T cells from the blood into sites of inflammation. The lack of increased CD11a expression on $CD4^+KJ^+$ T cells in the blood of TCDD-treated mice could impede the emigration of the cells out of the blood and into the tissues, resulting in increased numbers of $CD4^+KJ^+$ T cells in the blood. This would be consistent with the finding of significantly more $CD4^+KJ^+$ T cells in the blood of TCDD-treated mice on day 3 after immunization. However, it is also possible that the unimpeded up-regulation of CD49d, the ligand for endothelial VCAM-1, on T cells from TCDD-treated mice was sufficient for extravasation. Further studies are necessary to resolve the fate of activated T cells in the blood of TCDD-treated mice.

The possibility that TCDD exposure enhanced the early activation of $CD4^+KJ^+$ T cells was suggested by the increased percentage of T cells that down-regulated expression of CD62L. CD62L is a selectin that is involved in the homing of naïve T

cells to the lymph nodes (Arbones *et al.* 1994; Bradley *et al.* 1994). During activation, CD62L expression decreases as a result of both altered transcriptional rates and proteolytic cleavage from the T cell surface, thus preventing activated T cells from homing to the lymph nodes (Borland *et al.* 1999; Preece *et al.* 1996). Following immunization with OVA, CD62L expression on $CD4^+KJ^+$ T cells was decreased on the majority of cells from both vehicle- and TCDD-treated mice. However, the degree of down-regulation was significantly greater on cells from TCDD-treated mice on all days examined except day 2 with a higher percentage showing a complete loss of CD62L expression. Data from day 2 were difficult to interpret owing to the transient down-regulation of CD62L on the $CD4^+KJ^-$ cells as well as the $CD4^+KJ^+$ cells. This likely reflected a transient non-specific response to the Freund's adjuvant used in the immunization. The down-regulation of CD62L has been shown to occur progressively during the initial rounds of T cell division following exposure to antigen (Gudmundsdottir *et al.* 1999). Since TCDD promoted CD62L down-regulation, it suggests that TCDD was promoting $CD4^+KJ^+$ T cell division. However, cell division history was similar in CFSE-labeled $CD4^+KJ^+$ T cells from vehicle- and TCDD-treated mice on day 3, suggesting that CD62L expression may be uncoupled from cell division in TCDD-treated mice. In other studies in the laboratory, we have looked at the association between CD62L expression and cell division using an acute graft-vs-host model of T cell function. We found that CD62L expression on $CD4^+$ T cells from TCDD-treated mice was, in fact, correlated with cell division but the degree of decrease in expression was greater with each cell division (unpublished observations).

This enhanced down-regulation resulted in no expression of CD62L on the $CD4^+$ T cells from TCDD-treated mice after division 4 whereas T cells from vehicle-treated mice still expressed low levels of CD62L (unpublished observations). The mechanism underlying this effect of TCDD is not currently known.

Another possible mechanism for the loss of T cells in TCDD-treated mice is premature termination of T cell proliferation. Consistent with this hypothesis, the proliferation of activated $CD4^+KJ^+$ T cells from TCDD-treated mice appeared to terminate on day 3, whereas cells from vehicle-treated mice continued to divide through day 4. Similar to our results, studies by Mitchell and Lawrence (2003) showed that treatment with TCDD did not inhibit the proliferation of influenza virus-specific $CD8^+$ T cells early in the response but significantly reduced the proliferation after day 5. Since the early cycling of activated T cells is not reduced by TCDD, the delay in cell cycle arrest could be dependent on the induction of proteins regulated by the AhR. TCDD has been shown to alter the expression of several cell cycle regulators, including $p27^{kip1}$ and retinoblastoma protein, in other cell types (Ge and Elferink 1998; Kolluri *et al.* 1999; Puga *et al.* 2000a; Puga *et al.* 2002; Rininger *et al.* 1997). However, it should be noted that interpretation of the cell cycling data is complicated by the fact that the $CD4^+KJ^+$ T cell population analyzed on day 4 was already decreased in TCDD-treated mice. Thus, it is possible that TCDD selectively depleted the cells that had divided 4 or more times. Further studies will be necessary to determine if TCDD induces cell-cycle arrest in antigen-specific T cells and the mechanism underlying this effect.

The finding that $CD4^+KJ^+$ T cells from TCDD-treated mice exhibited increased annexin V staining on day 5 and 6 post-immunization suggests that T cell survival was also impaired by TCDD. Reduced T cell survival could result from ligation of death receptors or reduction in survival signals. Many of the survival signals for $CD4^+$ T cells are derived from activated DC that express ligands for co-stimulatory and cytokine receptors on the T cells. Based on previous studies showing that TCDD exposure reduces the number of DC in the spleen (Vorderstrasse and Kerkvliet 2001), it is possible that the loss of DC plays a role in decreased T cell survival. It has also been reported that TCDD induces apoptosis of activated T cells by a process involving Fas signaling (Camacho *et al.* 2001; Camacho *et al.* 2002). In these reports, lymph node T cells were obtained from mice that had been injected in the footpad with anti-CD3 antibody or Staphylococcal enterotoxin A (SEA) along with Freund's adjuvant. Apoptosis was evaluated by terminal dUTP nick-end labeling (TUNEL), and was apparent only after culturing the lymph node cells *in vitro* for 24 hours. To determine if Fas signaling contributed to the loss of antigen-specific $CD4^+KJ^+$ T cells following OVA injection, $CD4^+KJ^+$ T cells from DO11.10 mice that had been backcrossed to Fas-deficient Balb/c-lpr/lpr mice were used for adoptive transfer. The results of this study showed that TCDD caused the depletion of both Fas-deficient and Fas-expressing $CD4^+KJ^+$ T cells in a similar manner, indicating that depletion was independent of Fas signaling. This conclusion was supported by the finding that TCDD decreased, rather than increased, cell surface expression of Fas on wildtype $CD4^+KJ^+$ T cells beginning on day 3 post-immunization. The apparent discrepancy in

the role of Fas in T cell depletion mediated by TCDD in the D011.10 model compared to previous studies may be due to differences in how the T cells were activated, or to possible confounding effects of the lymphoproliferation that occurs with the lpr mutation. These mice undergo uncontrolled proliferation of $CD4^-CD8^-$ T cells that could confound the effects of TCDD by disturbing the normal frequency and number of single-positive $CD4^+$ and $CD8^+$ T cells in the secondary lymphoid organs. In our study, by adoptively transferring only the $CD4^+$ T cells from the DO11-lpr mice into normal Balb/c mice, any confounding effect of the $CD4^-CD8^-$ T cells was avoided.

Since Fas expression on the T cells did not appear to be involved in the loss of $CD4^+KJ^+$ T cells from the spleens of TCDD-treated mice, we used a gene array approach to determine if TCDD changed the expression pattern of other apoptosis- or survival-related genes in $CD4^+KJ^+$ T cells. The majority of the 21 genes that increased in expression in the $CD4^+KJ^+$ T cells from TCDD-exposed mice were pro-apoptotic, although no members of the Bcl-2 family were affected by TCDD. Several members of the TNF/TNF receptor superfamilies, such as TNFα, TNFR1, TNFR2, TRAIL, and FasL were increased, which agreed with previous studies that analyzed mRNA preparations from whole spleens (Zeytun *et al.* 2002). The mRNA level of Fas on day 3 was not affected by exposure to TCDD which is consistent with the minimal change in the expression of Fas on the cell surface, as measured by flow cytometry. Although TNF receptor expression was altered, TNF-signaling did not appear to be involved in the TCDD-dependent depletion of T cells from anti-CD3 treated mice (Dearstyne and Kerkvliet 2002). Several genes whose products are involved in the signal transduction

of apoptosis were also increased, including caspases 1 and 8, as well as genes whose products are involved in cell cycle arrest, such as DAP kinase, Hus 1, CHK 2, RPA 3, and p53. CIDE-B and DFF40, whose gene products are responsible for DNA fragmentation, were also increased in the $CD4^+KJ^+$ T cells from TCDD-treated mice. Paradoxically, there were also increases in several genes generally associated with increased survival of activated T cells. These pro-survival genes were all members of the TNF/TNF receptor superfamilies: TRANCE, CD27L, 4-1BB, 4-1BBL, CD30, and CD30L. The up-regulation of these pro-survival genes could reflect an attempt by the $CD4^+KJ^+$ T cells to enhance their interaction with dendritic cells. The results of the gene array experiments need to be confirmed at the level of protein expression before specific conclusions can be made about the importance of any changes in gene expression, particularly because many of these gene products are not regulated at the level of transcription. However, it is interesting to note that analysis of the same RNA samples in a separate array showed CYP1A1 expression in $CD4^+KJ^+$ T cells from TCDD-treated but not vehicle-treated mice (data not shown), demonstrating direct effects of TCDD on the $CD4^+KJ^+$ T cells.

In summary, the studies presented here addressed several questions about the fate of antigen-specific T cells responding to antigen in the presence of TCDD. Figure 2-11 summarizes a potential pathway for the effects of TCDD on activated antigen-specific T cells and illustrates the hypothesis that TCDD-dependent changes in activation early during the response lead to premature loss of adhesion between the antigen-specific T cells and DC, which in turn leads to a loss of survival signals for

both cells, and contracted proliferation and increased death of activated T cells. The specific lack of CD11a up-regulation on activated T cells in the blood could also result in decreased emigration of T helper cells into sites of antigen deposition. Current studies are aimed at identifying the specific cascade of events that is disrupted by TCDD during T cell activation that lead to altered activation and survival of antigen-specific T cells.

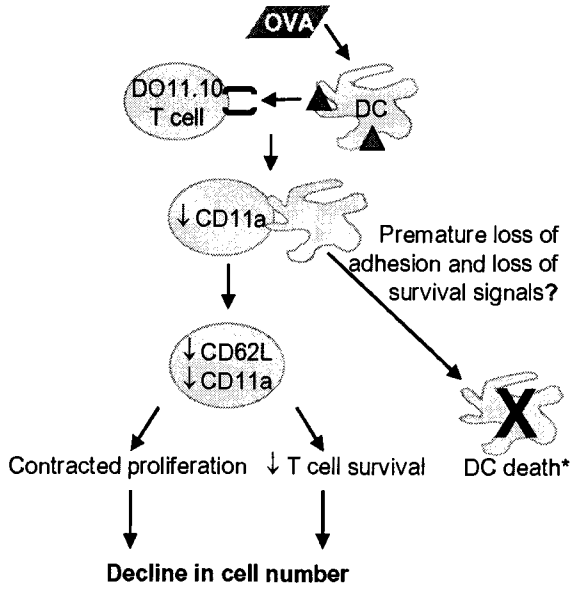

Figure 2-11. A possible model for the effects of TCDD on the activation and survival of antigen-specific T cells. *Exposure to TCDD enhances the activation of T cells and dendritic cells following exposure to antigen. Subsequently, the expression of several activation markers is changed, possibly resulting in a premature loss of adhesion between the T cell and dendritic cell, a premature cessation of proliferation, and increased cell death. Ultimately, the effects of TCDD on T cell proliferation and survival both contribute to the reduced number of activated antigen-specific T cells in the spleen. *A significant decrease in the number of splenic DC has been shown previously (Vorderstrasse and Kerkvliet 2001), and we have also observed a TCDD-dependent increase in the death of DC in vitro (unpublished observations).*

Chapter 3

ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN GENERATES A POPULATION OF $CD4^+CD25^+$ CELLS WITH CHARACTERISTICS OF REGULATORY T CELLS

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Abstract

Activation of the AhR by its most potent ligand, TCDD, leads to immune suppression in mice. While the underlying mechanisms responsible for AhR-mediated immune suppression are not known, previous studies have shown that activation of the AhR must occur within the first three days of an immune response and that $CD4^+$ T cells are primary targets. Using the B6-into-B6D2F1 model of an acute graft-versus-host response, we show that activation of AhR in donor T cells leads to the generation of a subpopulation of $CD4^+$ T cells that expresses high levels of CD25, together with $CD62L^{low}$, CTLA-4 and GITR. These donor-derived $CD4^+CD25^+$ cells also display functional characteristics of regulatory T (T_{reg}) cells *in vitro*. These findings suggest a novel role for AhR in the induction of T_{reg} cells and provide a new perspective on the mechanisms that underlie the profound immune suppression induced by exposure to TCDD.

Introduction

The AhR, together with its nuclear binding partner, ARNT, is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of transcriptional regulators (Schmidt and Bradfield 1996). PAS domain-containing proteins play a role in sensing and responding to changes induced by environmental stimuli, such as oxygen partial pressure, redox potential,

light intensity, and xenobiotic chemicals (Gu *et al.* 2000). Ligands for AhR are diverse and include products of cellular metabolism such as tryptophan derivatives and arachidonic acid metabolites, as well as dietary components such as indole-3-carbinol found in cruciferous vegetables and quercetin found in green and black teas (Ciolino *et al.* 1999; Denison and Nagy 2003). However, the functional effects of AhR activation have been elucidated using ligands of toxicological concern, such as the polycyclic aromatic hydrocarbon benzo[*a*]pyrene, found in cigarette smoke and broiled meats, and a variety of halogenated aromatic hydrocarbons, noted for their widespread contamination of the environment. Of the latter group of chemicals, TCDD is the most potent AhR agonist due to its high binding affinity (Kd 10^{-10}-10^{-9} M) and resistance to metabolism (Ema *et al.* 1994).

Extensive studies in laboratory rodents have shown that a single treatment with TCDD in the low μg/kg range induces profound suppression of antibody- and cell-mediated immune responses and alters host resistance to many diseases (Kerkvliet 2002). Human exposure to TCDD and other dioxin-like compounds has also been linked to altered immune function, particularly when exposure occurs during fetal/neonatal development (Tonn *et al.* 1996; Weisglas-Kuperus *et al.* 2000). Although most cells of the immune system express AhR, adult $AhR^{-/-}$ mice have no reported defects in immune development or immune responsiveness (Vorderstrasse *et al.* 2001). On the other hand, $AhR^{-/-}$ mice are highly resistant to the immune suppression associated with exposure to TCDD.

The underlying cellular mechanisms that drive AhR-dependent immune suppression have not been elucidated. Recent studies from our laboratory have shown that expression of AhR in both $CD4^+$ and $CD8^+$ T cells is required for TCDD to fully suppress an allospecific CTL response generated in an acute graft versus host (GVH) model (Kerkvliet *et al.* 2002). In this study, we used the same model to determine if TCDD alters the activation of donor $CD4^+$ cells and to assess the dependence of the effects observed on the presence of AhR in the donor T cells. Treatment of F1 hosts with TCDD resulted in a significant increase in the percentage of donor $CD4^+$ cells that expressed high levels of CD25, low levels of CD62L, as well as GITR and CTLA-4, a phenotype associated with some types of T_{reg} cells (Bluestone and Abbas 2003). Donor $CD4^+CD25^+$ cells purified from the spleen of F1 mice expressed functional characteristics associated with T_{reg} cells, namely unresponsiveness to stimulation with anti-CD3 unless exogenous IL-2 was also provided and a potent ability to suppress the proliferation of $CD4^+CD25^-$ cells. The development of the $CD4^+CD25^+$ population was dependent on the presence of AhR in the donor T cells, but not on the presence of pre-existing $CD4^+CD25^+$ cells. Taken together, these results suggest a novel role for AhR in the generation of T_{reg} cells and provide a new perspective on the mechanisms that underlie the profound immune suppression induced by exposure to TCDD.

Materials and Methods

Mice and treatment with TCDD

C57Bl/6J (B6) mice (H-2^b, Thy1.2) and B6D2F1/J (F1) mice (H-$2^{b/d}$, Thy1.2) were purchased from The Jackson Laboratory. B6.PL-Thy1^a/CyJ (Thy1.1) mice and B6.129-AhR^{tm1Bra}/J (AhR$^{-/-}$) mice (originally purchased from The Jackson Laboratory) were bred and maintained in our specific pathogen-free animal facility at Oregon State University. B6 mice purchased from The Jackson Laboratory were used as wildtype controls for AhR$^{-/-}$ mice. All animal procedures were approved by the Institutional Animal Care and Use Committee. F1 mice were dosed orally with vehicle or 15 μg TCDD/kg body weight one day prior to the injection of donor B6 T cells as previously described (Kerkvliet *et al.* 2002). This dose of TCDD is not overtly toxic but effectively suppresses the GVH CTL response.

Preparation of donor T cells

T cells were purified from pooled B6 spleens by magnetic bead sorting (Pan T isolation kit, Miltenyi Biotec). The purity of the T cells was ≥90% and viability was >95%. F1 host mice were injected i.v. with $2x10^7$ donor T cells. In some experiments, cell division was assessed by labeling the donor T cells with 5 μM CFSE (Molecular Probes) prior to injection into F1 hosts (Funatake *et al.* 2004).

Flow cytometry

Spleen cells were stained with anti-H-2D^d and anti-CD4 Ab to identify the donor CD4$^+$ T cells (Fig. 3-1A) along with Ab to the following markers: CD62L, CD25, CD28, (BD Pharmingen) and GITR (R&D Systems). Following surface-staining, the cells were fixed and permeabilized (Cytofix/Cytoperm Plus Kit™; BD Pharmingen) and stained with anti-CTLA-4 (BD Pharmingen). Isotype-matched fluorochrome-conjugated Ab were used as controls for nonspecific fluorescence. After gating on live spleen cells, listmode data on 5000-10,000 donor CD4$^+$ T cells were collected using either a Coulter XL or FC500 flow cytometer (Beckman Coulter). All data analyses, including software compensation, were performed using WinList software (Verity Software House).

In vitro suppression and anergy assays

The ability of donor-derived or naïve CD4$^+$CD25$^+$ cells to proliferate and to suppress the proliferation of CFSE-labeled naïve CD4$^+$CD25$^-$ cells was assessed as described (Kruisbeek *et al.* 2004). Donor Thy1.1$^+$ cells were enriched from pooled spleens of 6-7 TCDD-treated F1 mice on day 2 by magnetic bead sorting; the CD4$^+$CD25$^+$ fraction was further enriched using a MoFlo® high speed cell sorter (DakoCytomation). Pooled spleen cells from 3 naïve B6 mice were sorted into CD4$^-$, CD4$^+$CD25$^-$, and CD4$^+$CD25$^+$ fractions using a CD4$^+$CD25$^+$ regulatory T cell isolation kit (Miltenyi Biotec). The CD4$^-$ cells were irradiated (3000 rad) and used as accessory cells. The CD4$^+$CD25$^-$ cells were labeled with 2 μM CFSE prior to

culturing with anti-CD3 (BD Pharmingen) and accessory cells; donor-derived or naïve $CD4^+CD25^+$ cells were added to some wells. After 72 hr, CFSE dilution was measured by flow cytometry.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Inc.). Comparisons between means were made using the least significance difference multiple comparison *t*-test, with $p < 0.05$ considered statistically significant.

Results

Loss of donor $CD4^+$ T cells in the spleen of TCDD-treated F1 hosts is preceded by an increase in their activation phenotype

Previous studies have shown that treatment with TCDD does not alter the initial expansion of activated $CD4^+$ T cells in the spleen of antigen-challenged mice but promotes a premature decline in their number prior to effector cell development (Camacho *et al.* 2002; Funatake *et al.* 2004; Shepherd *et al.* 2000). TCDD produced a similar effect on the expansion and contraction of donor $CD4^+$ cells following their transfer into F1 mice (Fig. 3-1D). When the phenotype of donor $CD4^+$ cells was examined, treatment with TCDD was associated with a significant increase in the percentage of $CD62L^{low}$ cells (Fig. 3-1B). By day 2, 80% of the donor $CD4^+$ cells were $CD62L^{low}$, and this phenotype was maintained through day 5 (Fig. 3-1E). At the same time, treatment with TCDD led to a transient increase in the percentage of donor

$CD4^+$ cells that expressed CD25 (Fig. 3-1C, F). On day 2, the percentage of donor $CD4^+CD25^+$ cells in TCDD-treated mice was consistently 2-fold greater than the percentage in vehicle-treated mice. The percentage of $CD4^+CD25^+$ cells declined on day 3 and thereafter in both treatment groups.

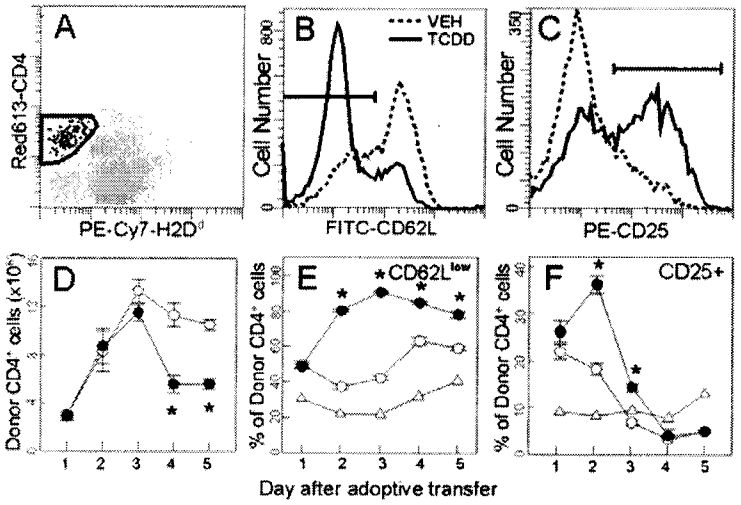

Figure 3-1. The premature loss of donor $CD4^+$ cells from the spleen of TCDD-treated mice is preceded by increased expression of an activated phenotype. *F1 mice were dosed with vehicle (○) or TCDD (●) one day prior to the i.v. injection of B6 donor T cells. As a syngeneic control (△), B6 donor T cells were injected into B6-Thy1.1 congenic mice. A, After gating on live spleen cells, the donor $CD4^+$ T cells were identified as $H2D^d{}^-CD4^+$. The region identifying $CD62L^{low}$ (B) or $CD25^+$ (C) cells is indicated by the black bar, as determined by syngeneic controls and isotype staining, respectively. The number of donor $CD4^+$ T cells in the spleen (D) and the percentage of $CD62L^{low}$ (E) and $CD25^+$ (F) donor $CD4^+$ cells was determined on days 1-5. The data are combined from two time course experiments (days 1-3 and days 3-5, n = 4-5 mice per group per day) except for CD25 on days 1 and 2 where n = 7 and 20 per group, respectively. * $p \leq 0.001$, compared to vehicle.*

Division-dependent changes in expression of CD62L and CD25 are enhanced by TCDD

The expression levels of CD62L and CD25 on newly activated $CD4^+$ T cells decreases and increases, respectively, with progressive rounds of cell division

(Demirci *et al.* 2002; Gudmundsdottir *et al.* 1999). We injected CFSE-labeled donor T cells to determine if treatment with TCDD altered this relationship. As previously observed, on day 2, TCDD induced a significant increase in the percentage of donor $CD4^+$ cells that were $CD62L^{low}$ (vehicle = 32.3±1.3%; TCDD = 56.5±1.6%; $p <$ 0.0001) and $CD25^+$ (vehicle = 16.6±0.9%; TCDD = 45.7±1.1%; $p < 0.0001$). Based on dilution of CFSE, no division of the donor $CD4^+$ cells was apparent one day after transfer into vehicle- or TCDD-treated F1 mice (data not shown). On day 2, more than

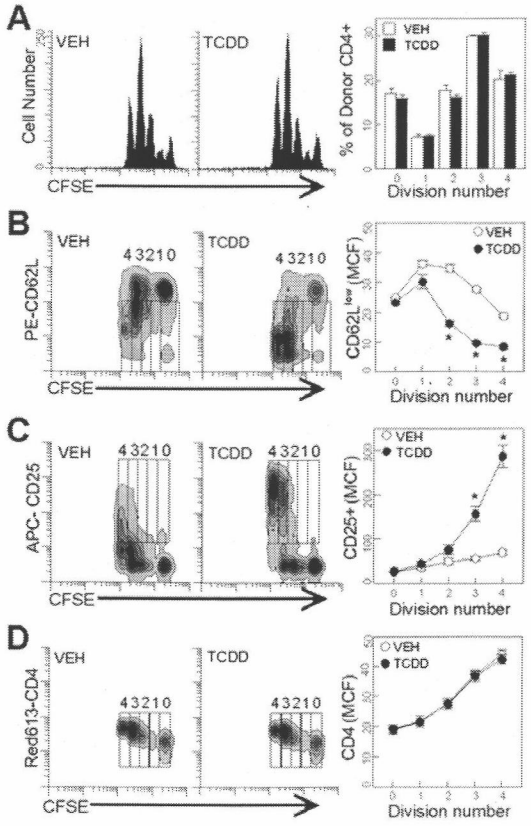

Figure 3-2. TCDD alters the intensity of CD25 and CD62L expression on donor $CD4^+$ cells but preserves the relationship to cell division. *B6 donor T cells were labeled with CFSE prior to injection into F1 mice. On day 2, spleen cells were collected and the $H2D^{d-}CD4^+$ cells were analyzed for cell division (A) and expression of $CD62L^{low}$ (B), $CD25^+$ (C), and CD4 (D). Data are representative of 5 independent experiments with n = 4-6 mice per group. For B-D, the boxes correspond to each cell division and indicate the regions from which the MCF data for $CD62L^{low}$, $CD25^+$, and CD4 are derived. * $p \leq 0.01$, compared to vehicle.*

80% of the donor $CD4^+$ cells from both treatment groups had undergone 1-4 cell divisions, with no observable effect of TCDD (Fig. 3-2A). Figures 3-2B and 3-2C show the expression of $CD62L^{low}$ and $CD25^+$ on donor $CD4^+$ cells in relation to the number of cell divisions. For both treatment groups, at least two rounds of cell division were required before changes in expression level of CD62L or CD25 were observed. TCDD did not influence the overall kinetics, but augmented the degree of downregulation of CD62L and upregulation of CD25 within each cell division. As early as the second cell division, the MCF of $CD62L^{low}$ was significantly lower on donor $CD4^+$ cells from TCDD-treated mice as compared to vehicle-treated mice (Fig. 3-2B, right panel). Likewise, after three divisions, the MCF of $CD25^+$ on donor $CD4^+$ cells from TCDD-treated mice was several-fold higher (Fig. 3-2C, right panel). Similar to the findings of Maury *et al.* (2001), the expression of CD4 increased on donor T cells with progressive rounds of cell division, however, this increase was unaffected by TCDD (Fig. 3-2D).

Expression of AhR in the donor T cells is required for induction of the $CD25^+CD62L^{low}$ phenotype

In the GVH model, both the donor T cells and many types of F1 host cells express AhR. To determine if AhR expression in the donor T cells was required for the altered phenotype induced by TCDD, T cells from $AhR^{-/-}$ mice were examined. Figure 3-3A shows the co-expression of CD25 and CD62L on $AhR^{+/+}$ or $AhR^{-/-}$ donor $CD4^+$ cells. As expected, when donor T cells expressed AhR, TCDD induced a significant increase in the percentage of donor $CD4^+$ cells expressing $CD25^+CD62L^{low}$

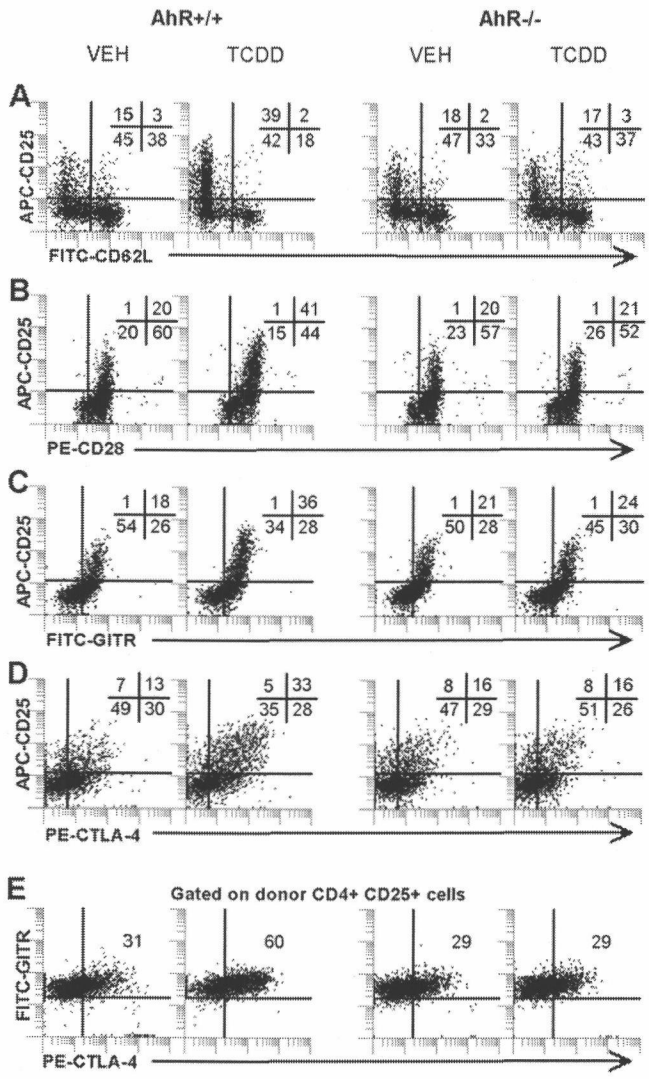

Figure 3-3. Activation of AhR increases the subpopulation of donor CD4$^+$ cells that co-expresses CD25 with CD62L^{low}, CD28, GITR, and CTLA-4. *AhR$^{+/+}$ or AhR$^{-/-}$ donor T cells were injected into vehicle- or TCDD-treated F1 mice. On day 2, spleen cells were collected and stained for H2D^d, CD4 and CD25 together with CD62L (A), CD28 (B), GITR (C), or CTLA-4 (D). E, The co-expression of GITR and CTLA-4 was examined on the H2D^{d-}CD4$^+$CD25$^+$ cells from AhR$^{+/+}$ or AhR$^{-/-}$ mice. Data show the percentage of cells within each quadrant for a single animal representative of 8-10 mice from 2 independent experiments. For quadrant means above 5, the standard errors averaged 12% of the mean. Quadrants for CD62L were set based on syngeneic controls; all others were set based on isotype.*

(vehicle = 16.9±1.6; TCDD = 33.0±1.3, $p = 0.0001$; Fig. 3-3A, left panels). A concomitant decrease occurred in the CD25$^-$CD62L^{hi} population, while the

$CD25^{-}CD62L^{low}$ population was unchanged. When the donor T cells did not express AhR, TCDD did not alter the co-expression of CD62L and CD25 (Fig. 3-3A, right panels).

Donor $CD4^{+}CD25^{+}$ cells from TCDD-treated mice also express high levels of CD28, GITR and CTLA-4

The $CD25^{+}CD62L^{low}$ phenotype has generally been attributed to activated $CD4^{+}$ T cells, suggesting that ligation of AhR by TCDD could be promoting CD25-mediated activation-induced cell death (Refaeli *et al.* 1998; Schmitz *et al.* 2003). However, the same phenotype defines some $CD4^{+}CD25^{+}$ T_{reg} cells that have a potent ability to suppress allograft responses (Cohen *et al.* 2002; Ermann *et al.* 2005). Therefore, we examined additional markers of activated and regulatory T cells to characterize the donor $CD4^{+}$ cells. The histograms in Figures 3-3B-D show the co-expression of CD25 and CD28, GITR, or CTLA-4 on donor $CD4^{+}$ cells from $AhR^{+/+}$ or $AhR^{-/-}$ mice. Treatment with TCDD led to a 2-fold increase in the percentage of double positive cells for all three markers. For CD28, a concomitant decrease occurred in the $CD25^{-}CD28^{+}$ cells (Fig. 3-3B). For GITR and CTLA-4, a concomitant decrease occurred in the double negative population (Fig. 3-3C and D). These changes in donor T cell phenotype occurred only if the donor T cells expressed AhR. Figure 3-3E shows that AhR activation doubled the population of $CD4^{+}CD25^{+}$ cells that expressed GITR and CTLA-4 from 31.6±1.7% in vehicle-treated mice to 58.0±2.0% in TCDD-treated mice ($p < 0.0001$). This doubling was dependent on the AhR in the donor T cells as no increase was observed following treatment with TCDD if they did not express AhR.

Taken together, these results suggest that activation of AhR in T cells may be promoting the development of $CD4^+CD25^+$ T_{reg} cells from unactivated donor $CD4^+$ cells ($CD25^-CD62L^{hi}CD28^+GITR^-CTLA\text{-}4^-$).

Depletion of $CD25^+$ cells from the donor T cell inoculum does not influence the TCDD-dependent increase of donor $CD4^+CD25^+$ cells in F1 mice

The increase in donor $CD4^+CD25^+$ cells in TCDD-treated mice could reflect the expansion of natural $CD4^+CD25^+$ T_{reg} cells, which are present in the donor T cell inoculum at a frequency of ~10%. We used magnetic beads to deplete the $CD25^+$ cells from the purified donor T cells prior to injection into F1 hosts. Figure 3-4A shows the TCDD-induced increase in donor $CD4^+CD25^+$ cells on day 2 when undepleted donor T cells were injected (vehicle = 10.7±0.6%, TCDD = 36.8±1.2%, $p < 0.0001$). Figure 3-4B shows that depletion of $CD4^+CD25^+$ cells from the donor inoculum did not impair the TCDD-induced increase in $CD25^+$ cells (vehicle = 10.5±0.6%, TCDD = 33.4±1.3%, $p < 0.0001$). In addition, depletion of $CD25^+$ cells did not affect the

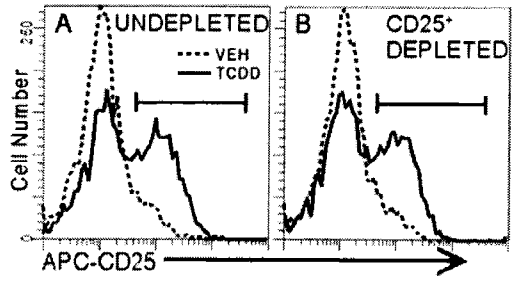

Figure 3-4. The presence of $CD4^+CD25^+$ cells in the donor T cell inoculum is not required for the increase in donor $CD4^+CD25^+$ cells in TCDD-treated F1 mice. *Vehicle- or TCDD-treated F1 mice were injected with undepleted donor T cells (9.4% $CD4^+CD25^+$ cells) (A) or CD25-depleted donor T cells (0.1% $CD4^+CD25^+$ cells) (B). On day 2 after injection, the expression of CD25 gated on donor $CD4^+$ cells was determined. Representative data of 4-6 mice per group are shown.*

TCDD-dependent increase in expression of $CD62L^{low}$, GITR, and CTLA-4 on the donor $CD4^+CD25^+$ cells (data not shown).

Alloresponsive donor $CD4^+CD25^+$ cells from TCDD-treated F1 mice are anergic and suppressive in vitro

T_{reg} cells are characterized by two functional attributes in *in vitro* assays: 1) anergy to stimulation with anti-CD3 and accessory cells that can be overcome by addition of exogenous IL-2, and 2) the ability to suppress the proliferative response of non-regulatory T cells stimulated with anti-CD3 and accessory cells (Takahashi and Sakaguchi 2003). Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F1 host mice on day 2 after injection failed to proliferate in response to stimulation with anti-CD3 and accessory cells (Fig. 3-5A). Likewise, $CD4^+CD25^+$ cells from naïve mice (a

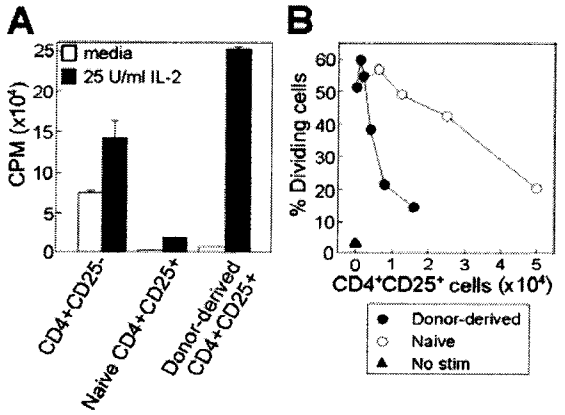

Figure 3-5. Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F1 mice on day 2 are anergic and suppress the proliferation of $CD4^+CD25^-$ cells in vitro. *A, The indicated subsets of cells were cultured in triplicate with anti-CD3 and irradiated accessory cells with or without IL-2, and the incorporation of [^{3}H]-TdR was measured on day 3. B, CFSE-labeled $CD4^+CD25^-$ cells were cultured with anti-CD3, irradiated accessory cells, and increasing numbers of donor or naïve $CD4^+CD25^+$ cells. Six wells from a 96-well plate were pooled and the percentage of $CD4^+CD25^-$ cells that had divided was determined on day 3 by flow cytometry. Data are representative of 2 independent experiments.*

natural T_{reg} population) failed to proliferate, whereas $CD4^+CD25^-$ cells from the same naïve mice proliferated extensively. Addition of IL-2 led to increased proliferation in all the cultures. As shown in Figure 3-5B, donor $CD4^+CD25^+$ cells isolated from TCDD-treated F1 mice inhibited the division of CFSE-labeled $CD4^+CD25^-$ cells stimulated with anti-CD3 and accessory cells. The suppressive ability of the $CD4^+CD25^+$ cells from TCDD-treated F1 mice was greater than equivalent numbers of $CD4^+CD25^+$ cells from naïve mice.

Discussion

The initial goal of these studies was to characterize changes in the activation of $CD4^+$ T cells induced by ligation of the AhR with TCDD that might explain the premature loss of alloresponsive $CD4^+$ T cells and subsequent suppression of the GVH CTL response (Kerkvliet *et al.* 2002). While several AhR-dependent changes in the phenotype of activated $CD4^+$ cells were observed, further analysis revealed that the changes occurred on a distinct subpopulation of donor $CD4^+$ cells. This subpopulation of $CD4^+$ cells expressed high levels of CD25, low levels of CD62L, as well as GITR and CTLA-4. Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F1 mice were anergic and suppressed the proliferative response of naïve T cells *in vitro*, demonstrating that these cells possessed what is currently the best functional definition of T_{reg} cells (Takahashi and Sakaguchi 2003). If the donor T cells did not express AhR, treatment with TCDD did not induce the $CD4^+CD25^+$ population. These results

suggest that signaling through the AhR plays a role in the generation of adaptive $CD4^+CD25^+$ T_{reg} cells. Whether this occurs with ligands other than TCDD remains to be determined.

AhR-dependent generation of $CD4^+CD25^+$ T_{reg} cells early in the immune response is consistent with the potent immunosuppressive effects of TCDD. Previous studies have shown that TCDD suppresses the development of allospecific $CD8^+$ CTL activity to P815 tumor cells by reducing the number of CTL precursors that are activated early in the response (Oughton and Kerkvliet 1999; Prell *et al.* 2000). This effect on CTL activation was lost if treatment with TCDD was delayed more than 3 days after the injection of P815 cells (Kerkvliet *et al.* 1996) and required the presence of $CD4^+$ T cells (Prell and Kerkvliet 1997). Furthermore, in an acute GVH response, suppression of allospecific $CD8^+$ CTL activity by TCDD was dependent on the presence of $AhR^{+/+}$ donor $CD4^+$ T cells (Kerkvliet *et al.* 2002). This CD4-dependent suppression could reflect the development of T_{reg} cells, since several studies have shown that $CD4^+CD25^+$ T_{reg} cells suppress pathogenic T cell responses in GVH disease (Cohen *et al.* 2002; Ermann *et al.* 2005; Taylor *et al.* 2002).

In recent years, several different types of regulatory T cells have been described that fall broadly into natural and adaptive categories (Bluestone and Abbas 2003). Natural T_{reg} cells that derive from the thymus constitutively express CD25, CTLA-4, GITR and CD62L, as well as the transcription factor Foxp3. In our model, depletion of the $CD25^+$ cells from the donor inoculum prior to injection into TCDD-treated F1 hosts did not alter the development of the $CD4^+CD25^+$ subpopulation,

suggesting that ligation of the AhR is not simply expanding the population of natural T_{reg} cells. Furthermore, the level of foxp3 mRNA was lower in donor T cells isolated from TCDD- versus vehicle-treated F1 mice on day 2, despite the fact that there were twice as many cells expressing the T_{reg} phenotype in the TCDD group (unpublished observations). Consistent with the low expression of CD62L on the $CD4^+CD25^+$ cells, it is likely that activation of the AhR is inducing an adaptive T_{reg} population that may not depend on expression of foxp3 (Bluestone and Abbas 2003; Vieira *et al.* 2004). In addition, the transient nature of the increase in expression of CD25 is not contradictory with a T_{reg} hypothesis, since studies have shown that T_{reg} cells can downregulate CD25 while still retaining their suppressive activity (Nishimura *et al.* 2004).

One mechanism by which activation of the AhR could promote the development of T_{reg} cells is by enhancing expression of the *IL-2* gene. The generation and expansion of $CD4^+CD25^+$ T_{reg} cells have been shown to depend on IL-2 (Furtado *et al.* 2002; Nelson 2004). Interestingly, Jeon and Esser (Jeon and Esser 2000) reported that the mouse *IL-2* promoter contains three AhR/ARNT-response elements that bind the ligand-activated AhR and induce reporter-gene expression. Increased expression of the *IL-2* gene was also observed in thymocytes after *in vivo* exposure to TCDD and in mitogen-activated spleen cells. Studies are underway to delineate the potential role of AhR-induced production of IL-2 in the development of T_{reg} cells.

Chapter 4

THE ROLE OF INTERLEUKIN-2 IN ARYL HYDROCARBON RECEPTOR-MEDIATED SUPPRESSION OF THE GRAFT-VERSUS-HOST RESPONSE

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Abstract

Recent studies from our laboratory using a model of the graft-versus-host (GVH) response have shown that exposure of F1 mice to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) doubles the frequency of donor $CD4^+$ T cells expressing high levels of CD25 compared to vehicle-treated controls and this effect is dependent on expression of the aryl hydrocarbon receptor (AhR) in the donor T cells. In the present studies, we have examined the role of interleukin (IL)-2 in this process. The results show that up-regulation of CD25 on donor $CD4^+$ T cells is dependent on the expression of functional AhR in donor $CD4^+$ T cells, themselves. In contrast, the up-regulation of CD25 on donor $CD8^+$ T cells did not depend on AhR expression in $CD8^+$ T cells, but was instead dependent on AhR expressed in the donor $CD4^+$ T cells. These data are consistent with the hypothesis that TCDD enhances IL-2 production by donor $CD4^+$ T cells in an AhR-dependent manner that acts in an autocrine and paracrine manner to increase CD25 expression on donor $CD4^+$ and $CD8^+$ T cells, respectively. Subsequent studies revealed that exposure to TCDD induced a small, but significant increase in the frequency of donor $CD4^+$ T cells that secreted IL-2. Furthermore, the higher levels of CD25 expressed on donor $CD4^+$ T cells from TCDD-treated mice correlated with increased signaling through the IL-2 receptor (IL-2R), as measured by increased phosphorylation of STAT5 following a short *ex vivo* stimulation with IL-2. In contrast, when exogenous IL-2 alone was administered to mice on days 0 – 3 of the GVH response, $CD4^+$ T cells showed only a small increase in CD25 expression, without

changes in CD62L or CTLA-4. Much higher doses of exogenous IL-2 on days 0 – 3 of the GVH response were required to suppress CTL activity as well as decrease the number of CTL effectors (CTLe) on day 10. These results indicate that IL-2 alone is not sufficient to mimic the effects of TCDD. Whether or not IL-2 is required for TCDD-mediated effects remains to be determined.

Introduction

Activation of the AhR following exposure to TCDD has long been recognized as a potent immunosuppressive signal (reviewed in Kerkvliet 2002). Previous studies using the GVH response and $AhR^{-/-}$ mice as a source of donor T cells revealed that T cells are direct AhR-dependent targets of TCDD *in vivo* (Kerkvliet *et al.* 2002). More recently, we found that exposure to TCDD leads to the AhR-dependent expansion of a $CD4^+CD25^+$ subpopulation of responding T cells with phenotypic and functional characteristics of regulatory T (Treg) cells (Funatake *et al.* 2005). Although constitutive expression of CD25 defines a subpopulation of natural Treg cells which also express the transcription factor Foxp3, TCDD did not affect host $CD4^+CD25^+$ natural Treg cells nor expression of Foxp3 in the donor $CD4^+$ T cells (unpublished observations), and exposure to TCDD still expanded the donor $CD4^+CD25^+$ subpopulation even when natural Treg cells were depleted from the donor T cell inoculum (Funatake *et al.* 2005). These data suggest that TCDD induces $CD4^+CD25^+$

adaptive Treg cells in the context of activation signals. These studies were the first to suggest that AhR may influence the development of adaptive Treg cells.

The development and function of Treg cells has been under intense investigation during the past several years. With the identification of a population of natural Treg cells that express CD25 constitutively, much of the attention has focused on the role of IL-2 and signaling through the high-affinity IL-2 receptor (IL-2R). The attention on IL-2 is also driven by the observation that mice deficient in IL-2 or components of the IL-2R, namely CD25 (α-chain) or CD122 (β-chain), develop a lymphoproliferative autoimmune disorder (Sadlack *et al.* 1995; Schorle *et al.* 1991; Suzuki *et al.* 1995; Willerford *et al.* 1995). Recent studies have shown that neutralization of IL-2 by anti-IL-2 monoclonal antibody (Ab) selectively reduces the number of peripheral $Foxp3^+CD25^+CD4^+$ cells (Setoguchi *et al.* 2005). It also appears that although mice deficient in IL-2 or CD25 do have $Foxp3^+$ cells in the periphery, their numbers are significantly reduced compared to wildtype mice (D'Cruz and Klein 2005; Fontenot *et al.* 2005). Collectively, these data suggest that IL-2/IL-2R plays an essential role in the expansion and/or survival of Treg cells in the periphery. While these studies have examined the development and function of Foxp3-expressing natural Treg cells, fewer studies have focused on the development and function of adaptive Treg cells. Of these, there is some evidence that IL-2, TGF-β, and IFN-γ, or a combination of these cytokines, may influence the development of adaptive Treg cells in the periphery (Fantini *et al.* 2005; Wood and Sawitzki 2006; Zheng *et al.* 2006)

The mechanism by which TCDD expands the donor $CD4^+CD25^+$ subpopulation is not known, but several lines of evidence suggest that IL-2 may be involved. First, expression levels of CD25 have been shown to be regulated by IL-2 in a dose-dependent manner (Sereti *et al.* 2000) and TCDD not only increases the frequency of $CD25^+$ cells, but also increases the level of CD25 expressed on those cells (Funatake *et al.* 2005). Previous studies have also shown that the promoter for the *IL-2* gene has three functional dioxin response elements (DRE) and when splenocytes were stimulated with anti-CD3 *in vitro* in the presence of TCDD, increased secretion of IL-2 was observed compared to splenocytes stimulated with anti-CD3 alone (Jeon and Esser 2000). Indeed, studies from our own laboratory have revealed an increase in *ex vivo* secretion of IL-2 on day 5 of the response to P815 tumor allograft (Kerkvliet *et al.* 1996). The possibility that excess IL-2 might play a role in TCDD-mediated suppression of the CTL response is also suggested from studies in which exogenous IL-2 administered to mice during the first three days of the response to P815 tumor allograft suppressed CTL activity measured on day 10 (Prell *et al.* 2000). Together, these data suggest that excess IL-2 early in the activation of T cells may be detrimental to the development of the immune response, possibly by promoting the development of adaptive Treg cells.

The current studies were undertaken to test the hypothesis that exposure to TCDD leads to increased secretion of IL-2 from donor $CD4^+$ T cells that drives the expansion of the donor $CD4^+CD25^+$ subpopulation by increasing signaling through the IL-2R. B6D2F1 mice were treated with TCDD or vehicle one day before adoptive

transfer of B6 donor T cells. Donor $CD4^+$ T cells were analyzed at various time points after adoptive transfer for secretion of IL-2, expression of CD25 and other activation markers, and phosphorylation of STAT5. In other studies, F1 mice were treated with IL-2 alone to determine if IL-2 could mimic the effects of TCDD on the phenotype of donor $CD4^+$ T cells as well as the suppression of the CTL response on day 10.

Materials and Methods

Mice and reagents

C57Bl/6J (B6) mice (H-2^b, Thy1.2) and B6D2F1/J (F1) mice (H-$2^{b/d}$, Thy1.2) were purchased from The Jackson Laboratory. B6.PL-Thy1^a/CyJ (Thy1.1) mice and B6.129-AhR^{tm1Bra}/J ($AhR^{-/-}$) mice (originally purchased from The Jackson Laboratory) were bred and maintained in our specific pathogen-free animal facility at Oregon State University. B6 mice purchased from The Jackson Laboratory were used as wildtype ($AhR^{+/+}$) controls for $AhR^{-/-}$ mice. All animal procedures were approved by the Institutional Animal Care and Use Committee. F1 mice were dosed orally with vehicle or 15 μg TCDD/kg body weight one day prior to the injection of donor B6 T cells as previously described (Kerkvliet *et al.* 2002). In some experiments, F1 mice were injected i.p. with recombinant mouse (rm)IL-2 (eBioscience) or diluent (0.1% BSA in DPBS) twice per day on days 0 – 2 or 3 following adoptive transfer of donor T cells. In the first experiment, mice were dosed with 0, 25, 50, or 500 U rmIL-2, based on previous studies which found that those doses suppressed allo-CTL activity on day

10 (Prell *et al.* 2000). However, when these doses of IL-2 produced no effect on the phenotype of donor $CD4^+$ T cells on day 2, a second study was performed in which mice were treated with 0, 1000, or 5000 U rmIL-2. Due to minimal effects on CTL activity on day 10 with these intermediate doses of IL-2, a third experiment was done in which mice were treated with 0, 10,000, or 25,000 U rmIL-2.

Preparation of donor T cells

T cells were purified from pooled B6 spleens by magnetic bead sorting (Pan T isolation kit, Miltenyi Biotec). The purity of the T cells was ≥ 90% and viability was > 95%. F1 host mice were injected i.v. with 2×10^7 donor T cells. In some experiments, donor T cells were further purified into CD4 and CD8 subpopulations. In this case spleen cells pooled separately from either $AhR^{+/+}$ or $AhR^{-/-}$ mice were first labeled with CD4 beads (Miltenyi Biotec) and positively sorted for $CD4^+$ cells. The $CD4^-$ fraction (< 1% $CD4^+$ cells) was then labeled with CD8 beads (Miltenyi Biotec) and positively sorted for $CD8^+$ cells. The $CD4^+$ cells were > 95% pure and the $CD8^+$ cells were > 85% pure, and in both cases the contaminating cells were not T cells. The donor $CD4^+$ and $CD8^+$ cells from $AhR^{+/+}$ or $AhR^{-/-}$ mice were then recombined, in all four possible combinations, at a ratio of 1.5:1 CD4:CD8 T cells (the same as unseparated donor T cells) and 2×10^7 T cells were injected into F1 mice as described above.

Analysis of IL-2 secretion by ELISA

Spleen cells from F1 mice were isolated 2 or 3 days after adoptive transfer of donor T cells and cultured in complete RPMI (RPMI-1640 containing: 10% FBS, 15 mM HEPES, 50 μg/ml gentamicin, 100 μM NEAA, 2 mM l-glutamine, 1 mM Na-pyruvate, 5 x 10^{-5} M 2-ME) for 22-24 hours. Supernatants were analyzed for IL-2 using Ab sandwich ELISA techniques as previously described (Kerkvliet *et al.* 1996).

Flow cytometry

Spleen cells were stained with anti-H-2D^d or anti-Thy1.1 and anti-CD4 or anti-CD8 Ab to identify the donor CD4$^+$ or CD8$^+$ T cells along with Ab to the following markers: CD44, CD62L, CD25, CD28, and CD71 (BD Pharmingen). In some studies, the cells were fixed and permeabilized (Cytofix/Cytoperm Plus Kit™; BD Pharmingen) after surface staining and stained with anti-CTLA-4 (BD Pharmingen). In studies where secretion of IL-2 was examined, spleen cells were isolated from F1 mice and used in an IL-2 secretion assay (mouse IL-2 secretion assay detection kit; Miltenyi Biotec) according to the manufacturer's instructions. Following the secretion assay, the cells were stained for surface proteins as described above with the addition of anti-IL-2 Ab (Miltenyi Biotec). Isotype-matched fluorochrome-conjugated Ab were used as controls for nonspecific fluorescence. After gating on live spleen cells, listmode data on 5000 – 10,000 donor CD4$^+$ T cells were collected using an FC500 flow cytometer (Beckman Coulter). All analyses of listmode data, including software compensation, were performed using WinList software (Verity Software House).

Measurement of phosphorylated STAT5

The measurement of phosphorylated STAT5 was done according to the methods developed by Krutzik *et al.* (Krutzik *et al.* 2005a; Krutzik *et al.* 2005b; Krutzik and Nolan 2003). Briefly, spleen cells were isolated from F1 mice 36 hours after adoptive transfer of donor T cells. The cells were cultured in complete RPMI for 15, 30, or 60 minutes with rmIL-2 at 0 or 10 ng/ml. After the indicated incubation times, formaldehyde was added to a final concentration of 1.5% and the cells were incubated for an additional 15 minutes at room temperature. The cells were then stained with FITC-anti-Thy1.1, APC-Cy7-anti-CD4, then permeablized with methanol, followed by additional staining with PE-anti-CD25 and AlexaFluor-647-anti-phospho-STAT5 (Y694) (all from BD Pharmingen). Listmode data were collected on 5000 – 10,000 Thy1.1^{+}CD4^{+} cells.

CTL assay

The cytolytic activity of spleen cells to P815 (H-2D^{d}) target cells was measured in a standard 4-hr ^{51}Cr-release assay as previously described (Kerkvliet *et al.* 1996; Prell and Kerkvliet 1997). E:T ratios from 200:1 to 12.5:1 were tested in duplicate. The percentage of cytotoxicity at each E:T ratio was calculated as:

$$\% \text{ cytotoxicity} = \frac{ER - NR}{MR - SR} \times 100$$

where ER is the experimental release using spleen cells from F1 mice that had received donor T cells; NR is the nonspecific release using splenocytes from naive mice; MR is the maximum release of ^{51}Cr from cells incubated with sodium dodecyl

sulfate, and SR is the spontaneous release of ^{51}Cr from P815 cells incubated in media alone.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Inc.). All data are given as the mean ± SEM, unless stated otherwise. Comparisons between means were made using the least significance difference multiple comparison *t*-test, with $p < 0.05$ considered statistically significant.

Results

Increased frequency of $CD25^+$ donor T cells is dependent on activation of AhR in donor $CD4^+$ T cells only

Previous studies had shown that expression and activation of AhR in both donor $CD4^+$ and $CD8^+$ T cells was required for full suppression of CTL activity on day 10 (Kerkvliet *et al.* 2002). Subsequent studies showed that activation of AhR in the donor T cells was necessary for the increase in a $CD25^+$ subpopulation on day 2 of the GVH response (Funatake *et al.* 2005), but it was not known if this increase was dependent on AhR in the donor $CD4^+$ cells, the donor $CD8^+$ cells, or both. To determine the precise role of AhR in the increased subpopulation of $CD25^+$ donor T cells, donor T cells from $AhR^{+/+}$ (wt) and $AhR^{-/-}$ (ko) mice were further separated into $CD4^+$ and $CD8^+$ subpopulations (CD4wt, CD8wt, CD4ko, CD8ko), recombined, and adoptively transferred into F1 mice. Two days after adoptive transfer, splenocytes

were analyzed for expression of CD25 on donor $CD4^+$ and $CD8^+$ T cells. As shown in Figure 4-1A, the frequency of $CD25^+$ donor $CD4^+$ T cells was increased in mice treated with TCDD only when the donor $CD4^+$ T cells expressed functional AhR (i.e., the $CD4^+CD25^+$ subpopulation was increased in TCDD-treated F1 mice receiving CD4wt CD8wt and CD4wt CD8ko donor T cell inoculums, but not CD4ko CD8wt or CD4ko CD8ko donor T cell inoculums). Interestingly, the frequency of donor $CD8^+$ T cells expressing CD25 was also increased only when the donor $CD4^+$ T cells expressed AhR, independent of the AhR-status of the donor $CD8^+$ T cells (Fig. 4-1B). These data demonstrate that AhR-dependent signaling in the donor $CD4^+$ T cells alone is required for the increased frequency of $CD25^+$ cells for both $CD4^+$ and $CD8^+$ donor T cells.

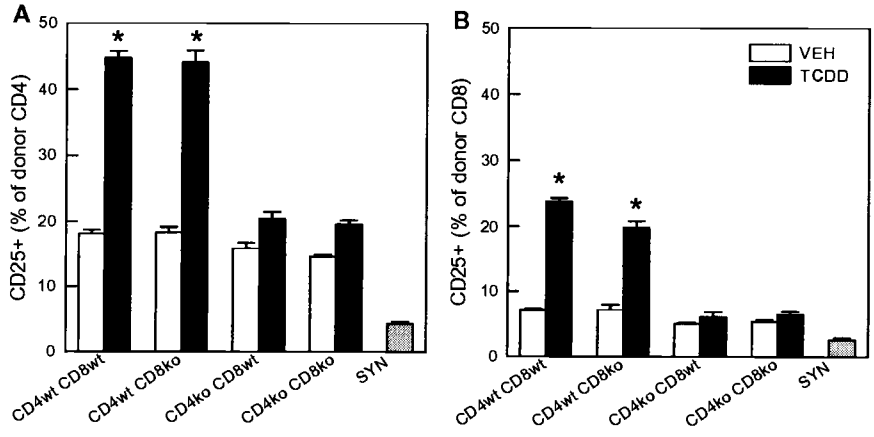

Figure 4-1. Increased expression of CD25 on both donor $CD4^+$ and $CD8^+$ cells is dependent on activation of the AhR in donor $CD4^+$ cells only. *Mice were treated with TCDD or vehicle one day before injection with donor T cells as described in the Materials and Methods. Donor T cells were identified as H-$2D^{d-}$ and $CD4^+$ or $CD8^+$. The expression of CD25 was analyzed on donor $CD4^+$ (A) and donor $CD8^+$ (B) T cells on day 2. Data shown are the mean ± SEM of 3 – 4 mice per group. * $p < 0.05$ compared to vehicle-treated mice with the same donor T cell inoculum.*

Exposure to TCDD increases the frequency of donor CD4$^+$ T cells secreting IL-2

Several lines of evidence point to IL-2 as the CD4-derived factor responsible for expanding the donor CD25$^+$ subpopulation in TCDD-treated mice. First, studies by the Esser laboratory demonstrated that the *IL-2* gene possesses three functional DRE in its upstream promoter and exposure of anti-CD3 stimulated splenocytes to TCDD resulted in increased secretion of IL-2 *in vitro* (Jeon and Esser 2000; Lai *et al.* 1996). In addition, IL-2 is a key cytokine in the development of the acute GVH response and is expressed early and transiently during the initiation of the response (Rus *et al.* 1995; Via 1991). However, other studies have shown that treatment of mice with exogenous IL-2 early can suppress development of GVH disease (Abraham *et al.* 1992; Sykes *et al.* 1993; Sykes *et al.* 1990; Wang *et al.* 1995). When using an ELISA technique to measure secretion of IL-2 from spleen cells isolated from F1 mice on day 2 or 3 after

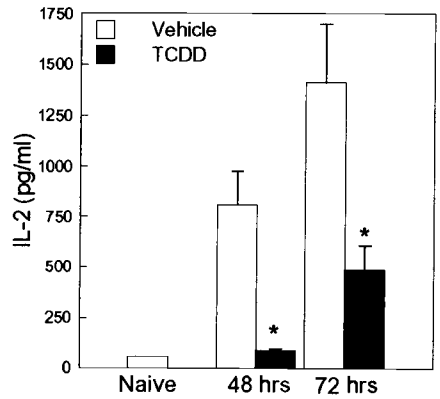

Figure 4-2. Exposure to TCDD decreases secretion of IL-2 from F1 spleen cells as measured by ELISA on days 2 and 3 after injection of donor T cells. *Mice were treated with TCDD or vehicle one day before injection with donor T cells as described in the Materials and Methods. On day 2 or 3 after injection, spleen cells from F1 mice were collected and cultured in complete RPMI for 22-24 hours. The supernatants were collected and analyzed for IL-2 by Ab sandwich ELISA as described in the Materials and Methods. Data represent the mean ± SEM of 3 – 4 mice per group per day. * $p < 0.05$ compared to vehicle-treated mice on the same day.*

adoptive transfer, exposure to TCDD resulted in significantly less IL-2 in the supernatants (Fig. 4-2). Since Treg cells have been shown to suppress T cell proliferation by both competing for and inhibiting the production of IL-2 (Barthlott *et al.* 2005; Thornton and Shevach 1998), these findings support our earlier observation that TCDD expands a subpopulation of donor $CD4^+$ T cells with Treg-like characteristics on day 2 (Funatake *et al.* 2005).

To determine if TCDD increases production of IL-2 specifically from donor $CD4^+$ T cells prior to the expansion of the $CD4^+CD25^+$ subpopulation, spleen cells from F1 mice were assayed directly *ex vivo* for secretion of IL-2 one day after adoptive transfer using a secretion assay designed to assess cell-specific IL-2 secretion (see Materials and Methods). As shown in Figure 4-3A, a small but statistically significant increase in the percentage of donor $CD4^+$ T cells secreting IL-2 was observed in TCDD-treated mice. Because only 5 – 10% of all donor T cells have the potential to respond to allo-antigen, we also looked at the secretion of IL-2 from activated donor $CD4^+$ T cells, which should reflect those cells that are responding to allo-antigen and were identified as $CD25^+CD28^{hi}$ or $CD25^+CD71^{hi}$ in two separate experiments. Interestingly, exposure to TCDD resulted in a small increase in the frequency of activated T cells in both experiments ($CD25^+CD28^{hi}$: vehicle = 21.2 ± 2.4%, TCDD = 28.7 ± 0.6%, $p = 0.04$; $CD25^+CD71^{hi}$: vehicle = 16.3 ± 0.7%, TCDD = 20.7 ± 0.6, $p = 0.01$). This increase seemed to be due primarily to the increase in CD25 single-positive cells (experiment 1: vehicle = 24.2 ± 2.8%, TCDD = 32.4 ± 0.5%, $p = 0.10$; experiment 2: vehicle = 23.4 ± 1.3%, TCDD = 27.4 ± 0.5%, $p = 0.05$),

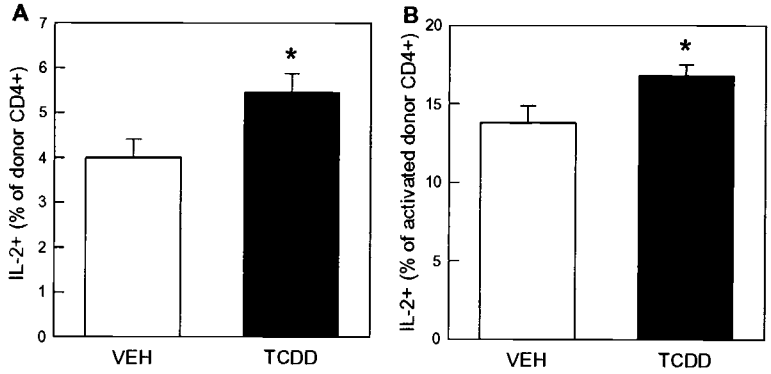

Figure 4-3. Exposure to TCDD increases the frequency of donor CD4$^+$ T cells secreting IL-2. *Mice were treated with vehicle or TCDD one day before adoptive transfer as described in the Materials and Methods. One day after adoptive transfer, spleen cells from B6D2F1 mice were collected and assayed for IL-2 using the Miltenyi IL-2 secretion assay as described in the Materials and Methods. The cells were then stained for Thy1.1, CD4, CD25, and CD28 (experiment 1) or CD71 (experiment 2). The frequency of IL-2$^+$ cells was determined as a percentage of all donor CD4$^+$ cells (A) or as a percentage of donor CD4$^+$ cells expressing an activated phenotype (CD25$^+$CD28^{hi} or CD25$^+$CD71^{hi}). The data shown are pooled from two independent experiments, for a total of 6 mice per group and represent the mean ± SEM. * $p < 0.05$ compared to vehicle-treated mice.*

as there was no difference between vehicle- and TCDD-treated groups when CD28 or CD71 were analyzed independently. Figure 4-3B shows that gating on the activated donor CD4$^+$ T cells enriched the population of IL-2$^+$ cells from about 4% to almost 15% in the vehicle-treated mice and from about 5.5% to about 16% in TCDD-treated mice. Exposure to TCDD increased the frequency of activated donor CD4$^+$ T cells that were secreting IL-2. Host CD4$^+$ T cells and donor CD8$^+$ T cells did not secrete IL-2 (data not shown). When these data were analyzed for the mean channel fluorescence (MCF) of IL-2$^+$ cells, which reflects the average amount of IL-2 produced on a per cell basis, no significant differences were observed. This was likely due to the low numbers of IL-2$^+$ cells analyzed since data collection was based on 5000 – 10,000 donor CD4$^+$ cells. Given that only 5% of the donor CD4$^+$ cells were IL-2$^+$, then only

250 – 500 IL-2^+ cells were collected for analysis. With so few cells upon which to base measurements of MCF, the variation was too high to determine if there was a difference between vehicle- and TCDD-treated mice. In some experiments, expression of CD25 was also increased at 24 hours (indicated above and data not shown). However, this increase was quite small and not consistently observed, suggesting that the effect of TCDD on expression of CD25 occurs near or shortly after increased production of IL-2.

Increased expression of CD25 correlates with increased phosphorylation of STAT5

Although expression of CD25 is often thought of as expression of the high affinity IL-2R, which is composed of three subunits, CD25 (α-chain), CD122 (β-chain), and CD132 (γ_c-chain), it is possible that upregulation of CD25 via the AhR leads to the formation of the low-affinity IL-2R, which is composed of CD25 only and is incapable of signaling due to a short cytoplasmic tail and no known association with intracellular kinases or other signaling factors (He and Malek 1998; Nelson and Willerford 1998). STAT5 is a transcription factor that is recruited to the cytoplasmic tail of the γ_c-chain where it is phosphorylated by Jak3, a kinase that is constitutively associated with the γ_c-chain and is itself activated/phosphorylated upon binding of IL-2 to the receptor (reviewed in Nelson and Willerford 1998). To determine if increased levels of CD25 correlated with increased signaling through the IL-2R, the phosphorylation of STAT5 was measured following a short *ex vivo* stimulation with rmIL-2. Spleen cells from F1 mice were isolated 36 hours after adoptive transfer of

donor T cells and cultured for 15-60 minutes with or without rmIL-2. At 36 hours post-adoptive transfer, the frequency of $CD25^+$ cells as well as the levels of expression of CD25 (as measured by MCF) on donor $CD4^+$ T cells was significantly increased by exposure to TCDD (Fig. 4-4). Minimal amounts of phosphorylated STAT5 were detected on donor $CD4^+$ T cells isolated directly from the animals (data not shown).

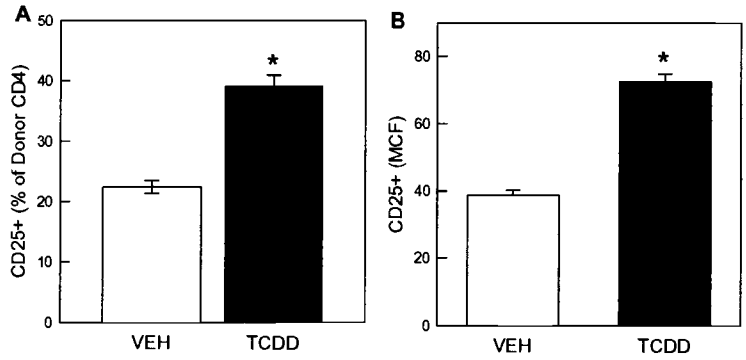

Figure 4-4. The expression of CD25 on donor $CD4^+$ T cells is increased 36 hours after adoptive transfer. *Mice were treated with vehicle or TCDD one day before injection with donor T cells as described in the Materials and Methods. Thirty-six hours after injection, Thy1.1$^+$CD4$^+$ T cells were examined for expression of CD25. Both the percentage of donor CD4$^+$ cells expressing CD25 (A) and the MCF of CD25 (B) were determined. Data shown are the mean ± SEM of 4 mice per group. * $p <$ 0.05 compared to vehicle-treated mice.*

Phosphorylation of STAT5 was dramatically increased following incubation with rmIL-2 for as little as 15 minutes and was maintained through 60 minutes (Fig. 4-5A). During this same time period, the expression of CD25 was stable and remained higher on the donor $CD4^+$ T cells from TCDD-treated mice (vehicle = 10.5 ± 0.5 MCF; TCDD = 17.3 ± 1.0 MCF; $p = 0.0004$). Donor $CD4^+$ T cells from TCDD-treated mice also had significantly higher levels of phosphorylated STAT5 compared to cells from vehicle-treated mice (vehicle = 98.1 ± 2.3 MCF; TCDD = 114.1 ± 2.6 MCF; $p =$

0.002). As shown in Figure 4-5B, the levels of expression of CD25 and phosphorylation of STAT5 were positively correlated ($r^2 = 0.95$). These data indicate that the increase in expression of CD25 correlates with increased signaling through the IL-2R.

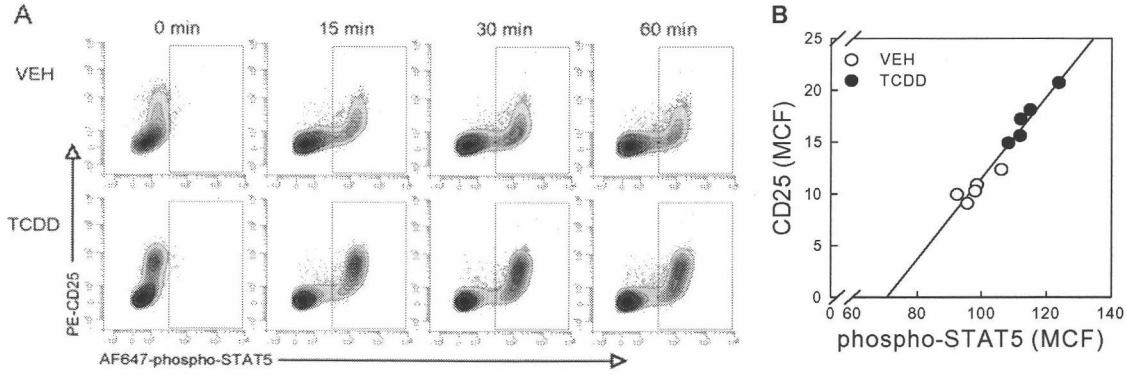

Figure 4-5. Exposure to TCDD increases signaling through the IL-2 receptor as measured by increased phosphorylation of STAT5 following* ex vivo *stimulation with rmIL-2. *Mice were treated with vehicle or TCDD one day before injection with donor T cells as described in the Materials and Methods. Thirty-six hours after injection, the co-expression of CD25 and phospho-STAT5 (A) was analyzed after gating on the donor Thy1.1$^+$CD4$^+$ T cells. The boxes on the histograms represents the region used to determine the MCF of CD25 and phospho-STAT5, which were used in (B) for linear regression analysis. In (B) each point represents the MCF of CD25 and phospho-STAT5 on donor CD4$^+$ T cells from a single animal, with 5 mice per group. The linear regression analysis yielded an r^2-value of 0.95.*

Exogenous IL-2 partially mimics the effects of TCDD

Previous studies in other models of the GVH response have shown that high doses of exogenous IL-2 administered within the first three days of the response inhibits development of GVH disease, suggesting that the allo-immune response is suppressed (Abraham *et al.* 1992; Sykes *et al.* 1993; Sykes *et al.* 1990; Wang *et al.* 1995). In addition, previous studies from our laboratory have demonstrated that much

lower doses of exogenous IL-2 administered during the first three days after injection of allogeneic tumor cells also suppresses the development of allo-CTL activity as measured on day 10 (Prell *et al.* 2000).To determine if excess IL-2 alone was sufficient to induce a Treg cell-like phenotype on donor $CD4^+$ T cells in the GVH response, we administered rmIL-2 to mice on days 0 – 2 following injection of donor T cells. In an initial experiment, mice were given 0, 25, 50, or 500 U rmIL-2 but no changes in the phenotype of donor T cells on day 2 were observed. Thus, in the next experiment mice were given 0, 1000, or 5000 U rmIL-2 and the data from this experiment are shown in Figure 4-6. On day 2, rmIL-2 induced a dose-dependent increase in the frequency of donor $CD4^+$ T cells expressing CD25 as well as the level

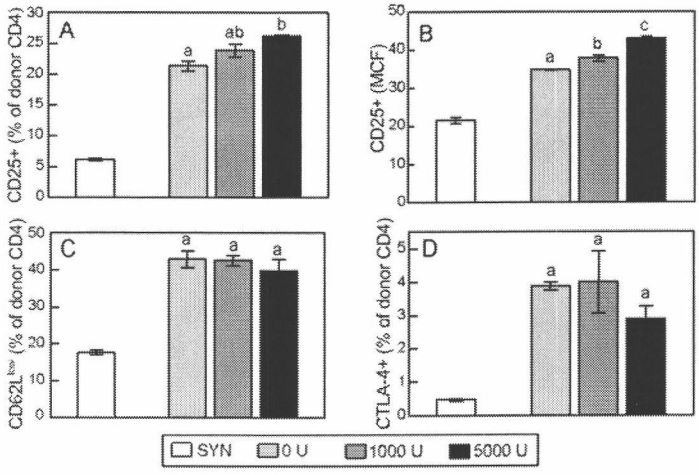

Figure 4-6. Treatment with exogenous IL-2 on days 0-2 increases expression of CD25, but does not alter expression of CD62L or CTLA-4. *Mice were treated with 0, 1000, or 5000 U of rmIL-2 i.p. two times a day on days 0-2, relative to adoptive transfer of donor T cells, for a total of 4 doses. Spleen cells were collected on day 2 and stained for Thy1.1, CD4, CD25, CD62L, and CTLA-4 and analyzed by flow cytometry. The percentage of donor $CD4^+$ cells expressing CD25 (A), CD62L (C), and CTLA-4 (D) was determined after gating on the $Thy1.1^+CD4^+$ donor cells. The MCF of CD25 on $CD25^+$ donor $CD4^+$ T cells was also determined (B). Data represent the mean ± SEM of 4 mice per treatment. Different letters indicate a statistically significant difference ($p < 0.05$) between groups. All groups were statistically different from the syngeneic group.*

of expression of CD25 on those cells (Fig. 4-6A, 4-6B). However, the magnitude of this increase was much less dramatic compared to the 2-fold increased observed in TCDD-treated mice in previous studies. In contrast to treatment with TCDD, treatment with exogenous IL-2 had no effect on the expression of CD62L or CTLA-4 (Fig. 4-6C, 4-6D). Similar findings were observed on day 3, after two additional doses of rmIL-2 (data not shown).

To determine if exogenous IL-2 alone could suppress the allo-CTL response, mice were treated with rmIL-2 on days 0 – 3 following adoptive transfer of donor T cells. On day 10, the spleen cells were assayed for CTL activity against P815 target cells in a standard ^{51}Cr-release assay. In the first experiment, mice were treated with 0, 1000, or 5000 U rmIL-2, but no significant effects on CTL activity were observed. In

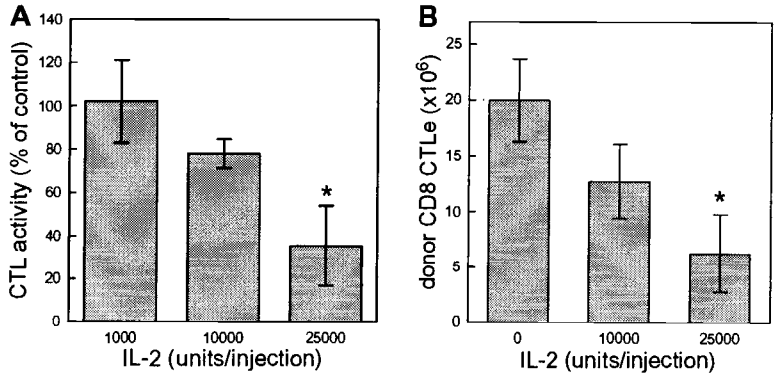

Figure 4-7. Treatment with exogenous IL-2 on days 0-3 dose-dependently suppresses CTL activity and the number of CTLe on day 10. *Mice were injected i.p. two times a day with rmIL-2 on days 0-3 relative to adoptive transfer of donor T cells, for a total of 6 doses. (A) On day 10, splenic CTL activity was measured in a standard ^{51}Cr-release assay as described in the Materials and Methods. Data from two independent experiments were combined and normalized to their respective control groups. Results are presented as the 50:1 E:T ratio and are shown as a percentage of the control (PBS)-treated response. (B) On day 10, Thy1.1^{+}CD8^{+} T cells were analyzed for expression of CD44 and CD62L, to identify CTLe cells, identified as CD44^{hi}CD62L^{low}. Results represent the mean ± SEM of 4 mice per group. * $p < 0.05$ compared to control (PBS)-treated mice.*

a second study mice were treated with 0, 10,000 or 25,000 U rmIL-2. As shown in Figure 4-7A, exogenous rmIL-2 dose-dependently suppressed CTL activity on day 10. Similarly, the number of donor $CD8^+$ T cells expressing a CTLe phenotype ($CD44^{hi}$ $CD62L^{low}$) was dose-dependently decreased in mice treated with high doses of rmIL-2 (Fig. 4-7B). Taken together, the data here suggest that high doses of IL-2 early in the development of a CTL response are capable of suppressing the development of CTLe cells, but the degree of suppression is much more modest than seen following exposure to TCDD.

Discussion

Previous studies from our laboratory have shown that activation of the AhR by TCDD leads to a 2-fold increase in the frequency of $CD25^+$ donor $CD4^+$ T cells (Funatake *et al.* 2005). Not only are there twice as many $CD25^+$ donor $CD4^+$ T cells in TCDD-treated mice, but these cells also express much higher levels of CD25 on their surface and share *in vitro* functional characteristics with Treg cells. The increase in $CD25^+$ donor $CD4^+$ T cells does not depend on the presence of naturally occurring $CD4^+CD25^+$ T cells in the donor T cell inoculum because depletion of these cells before injection into F1 mice does not prevent the increase in the $CD25^+$ subpopulation of donor $CD4^+$ T cells. These results suggest that activation of AhR may expand and/or induce a subpopulation of adaptive Treg cells early in the response to antigen.

While these earlier studies had shown that AhR was required for the upregulation of CD25 in TCDD-treated mice (Funatake *et al.* 2005), studies looking at CTL activity on day 10 had shown that AhR was required in both the $CD4^+$ and $CD8^+$ T cells for full suppression by TCDD(Kerkvliet *et al.* 2002). If only one subpopulation expressed functional AhR, the response was only partially suppressed (Kerkvliet *et al.* 2002). In the present studies, the relative contribution of AhR expressed in either the $CD4^+$ or $CD8^+$ T cells on the expression of CD25 was determined. Interestingly, the upregulation of CD25 on both donor $CD4^+$ and $CD8^+$ T cells was dependent on the expression of functional AhR only in the donor $CD4^+$ T cells. These data indicate that AhR-dependent changes in the $CD4^+$ T cells influence the expression of CD25 on the donor $CD4^+$ T cells themselves as well as the donor $CD8^+$ T cells.

Given that expression of CD25 can be dose-dependently increased by IL-2 (Sereti *et al.* 2000) and because the *IL-2* gene contains three DRE in its promoter (Lai *et al.* 1996), we determined if IL-2 production from donor $CD4^+$ T cells was also increased in TCDD-treated mice. Exposure to TCDD led to an increase in the frequency of donor $CD4^+$ T cells that were secreting IL-2 one day after adoptive transfer into F1 mice. Although the increase in $IL\text{-}2^+$ cells was small, it is important to remember that only 5 – 10% of the donor T cell inoculum is likely to respond to non-self MHC, and in the microenvironment of the spleen where T cell activation is occurring, the concentration of IL-2 could be much higher than can be measured once the spleen cells are prepared for analysis. It is possible that the microenvironment where IL-2 is secreted is even smaller, given the recent findings that IL-2 secretion is

directed primarily toward the immunological synapse (Huse *et al.* 2006). Once this interaction is disturbed, it may be difficult to reactivate the machinery to secrete IL-2 in the absence of cell-cell contact (the cells are kept in suspension during the secretion assay) and this could be another reason we can only detect a small frequency of IL-2^+ donor $CD4^+$ T cells. Nonetheless, a consistent increase in IL-2^+ donor $CD4^+$ T cells was seen in TCDD-treated mice that could drive the expansion of a $CD25^+$ subpopulation of donor $CD4^+$ T cells. The paracrine activity of IL-2 could also explain the upregulation of CD25 on donor $CD8^+$ T cells, even when the $CD8^+$ T cells did not express functional AhR.

The IL-2R is a multimeric complex that can be expressed as a high-, intermediate-, or low-affinity receptor depending on which protein chains are in the complex. Only the high-affinity receptor, which is composed of CD25 (α-chain), CD122 (β-chain), and CD132 (γ_c-chain), and the intermediate-affinity receptor, which is composed of the β- and γ_c-chains, are able to initiate a signaling cascade (Nelson and Willerford 1998). The low-affinity receptor, which is composed of CD25 alone, does not trigger intracellular signaling. Thus, to determine if AhR-dependent up-regulation of CD25 led to the formation of functional, high-affinity IL-2R complexes, the phosphorylation of STAT5 was measured. STAT5 is a transcription factor involved in one of the signal transduction pathways of the IL-2R (Nelson and Willerford 1998). We found that the higher levels of CD25 on donor $CD4^+$ T cells in TCDD-treated mice correlated with increased signaling through the IL-2R, as measured by increased levels of phosphorylated STAT5. These results indicate that

up-regulation of CD25 expression is functional and increases IL-2R-mediated signaling in TCDD-treated mice. IL-2R-mediated signaling has been shown to be essential for the peripheral maintenance of Treg cells (Setoguchi *et al.* 2005), however, the effects of TCDD on responding donor $CD4^+$ T cells occurred even when the natural Treg cells were depleted from the donor T cell inoculum (Funatake *et al.* 2005), suggesting that TCDD does not simply expand the existing population of natural Treg cells. Since exogenous IL-2 alone only partially induced changes in the phenotype of the donor $CD4^+$ T cells and did not suppress the CTL response to the same magnitude as TCDD, these findings suggest that interaction between the signaling pathways for AhR and IL-2R may be responsible for the full phenotypic and functional changes that occur in donor $CD4^+$ T cells.

Our findings that exogenous IL-2 increased expression of CD25 and partially suppressed CTL activity on day 10 (albeit at much higher doses) suggest that IL-2 may play some role in the suppressive effects of TCDD on the GVH response. Similar findings were observed using the P815 tumor allograft model (Prell *et al.* 2000). In the P815 studies, much lower doses of rmIL-2 were required to suppress CTL activity than were used in the current studies. One possible explanation for this discrepancy could be that in the P815 model, the site of antigen is the peritoneal cavity and when rmIL-2 is injected at the site of antigen it may be more effective. Also, there is considerable inflammation in response to P815 tumor cells and the effects of IL-2 may be accentuated by the inflammation. Although, in another model of the GVH response in which host mice were lethally irradiated before receiving bone marrow

transplantation, a condition which also leads to increased inflammation, similarly high doses of IL-2 were required to suppress development of disease (Abraham *et al.* 1992; Sykes *et al.* 1990). These data suggest that perhaps the GVH response is simply not as sensitive as the P815 tumor allograft model to the suppressive effects of IL-2.

Although exogenous IL-2 alone does not appear to be sufficient to reproduce the effects of TCDD, it remains to be determined if IL-2 is required for the immunosuppressive effects of TCDD. Although both high doses of IL-2 and TCDD increased expression of CD25 on the donor T cells and both were able to suppress CTL activity on day 10, TCDD was far more effective in both cases. The mechanism by which high doses of IL-2 suppress the GVH response has not been well-studied but appears to involve inhibition of $CD4^+$ T cells and altered production of IL-2 and IFN-γ (Sykes *et al.* 1993; Wang *et al.* 1995). Recent studies have suggested that TCDD induces the development of potent Treg cell-like suppressive activity in donor $CD4^+CD25^+$ T cells (Funatake *et al.* 2005). It is possible that excess IL-2 administered early in the development of an immune response skews the differentiation of T cells to become adaptive Treg cells. Similarly, TCDD may lead to the development of adaptive Treg cells via increased production of IL-2 early in the immune response. As discussed above, IL-2 alone does not suppress the GVH response to the same magnitude as TCDD, thus, it is possible that "maximal" induction of Treg-like functions in donor $CD4^+$ T cells requires the interaction of AhR- and IL2R-mediated signaling.

Chapter 5

CONCLUSIONS

The results of this dissertation identify a novel role for AhR in inducing Treg-like suppressive activity in responding $CD4^+$ T cells and provide new insight into the immunosuppressive mechanisms of TCDD. Based on these findings, AhR may represent a novel target for treating autoimmunity and allergic responses. Because of its notoriety as a carcinogen and as the "most toxic man-made chemical," TCDD is not likely the ideal "drug" to use for the treatment of these diseases. However, many other compounds have been shown to bind to AhR (Denison and Nagy 2003), and these other ligands may also be able to induce a Treg-like population of T cells capable of controlling over-active immune responses. Interestingly, many dietary components have also been shown to bind to AhR (Ciolino *et al.* 1999; Denison and Nagy 2003), thus the role our diet plays in susceptibility to autoimmunity and allergy may be related to genetic polymorphisms of AhR that affect its ability to bind ligand with high affinity, heterodimerize with ARNT, or bind to DNA. Because AhR is a transcription factor, studies into the specific changes in gene expression may reveal certain signaling pathways that could be targeted through other means to skew the differentiation of T cells toward Treg cells, ideally without the other toxic effects associated with activation of the AhR.

In conclusion, based on our discovery of a putative mechanism by which TCDD suppresses adaptive immunity, that is, the induction of adaptive Treg cells, the AhR has been identified as a potential therapeutic target for the treatment of many immunological disorders. It will be interesting to see if other ligands for AhR induce similar immunosuppressive effects as TCDD but without the overt toxic effects.

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