Chapter 1 General Introduction and Review of the Literature

1.1 The Immune System

Living animals are able to fight off infections and tumor growth because of their immune systems. On the other hand, the presence of an immune system can leave an animal suffering from allergies or autoimmune diseases. Immunologists attempt to study how the immune system functions and to characterize and treat malfunctions.

ORGANS OF THE IMMUNE SYSTEM

The immune system is different from most other organ systems in that there is not a distinct set of organs. Instead, the immune system is comprised of a collection of organs, tissues, and cells which interact to produce a complete immune response. The immune system can be divided into primary organs, in which the cells develop, and secondary or peripheral organs, in which the majority of the immune response takes place (Roitt *et al.*, 1996).

The primary organs of the immune system are the bone marrow and the thymus. The bone marrow is the central lymphoid organ; all lymphoid and erythroid cells begin their development in the marrow of long bones. Bone marrow can be divided into two types: red marrow and yellow marrow. The red marrow begets the red blood cells, and the yellow marrow begets other lymphoid cells. All classes of lymphoid cells originate from pluripotent stem cells. As the pluripotent stem cells mature and differentiate, three major groups of cells (granulocytes, monocytes, and lymphocytes) are formed. Some stem cells differentiate in the bone marrow to mature and are known as the B cells, while others migrate to the thymus and differentiate into T cells (Roitt *et al.*, 1996).

The thymus is a lobular organ in which lymphocytes mature into functional T cells. The individual lobules are organized into cortical and medullary regions. The outer region (cortex) contains the majority of immature, rapidly proliferating thymocytes. The inner region (medulla) contains the more mature T cells. In the thymus, T cells undergo a two step selection process to select cells capable of interacting with the rest of the immune system but unable to react against self-antigens (Roitt *et al.*, 1996).

The differentiating cells of the immune system express a variety of cell surface markers, designated as cluster of differentiation (CD) molecules. While mature T cells express either CD4 or CD8, all thymocytes start out as double-positive ($CD4^+$ $CD8^+$). The first step towards maturity is positive selection for those cells that can recognize either Class I or Class II Major Histocompatibility Complex (MHC), proteins expressed on the surfaces of other cells. If a thymocyte is not able to recognize self-MHC, it is programmed to die. Cells that recognize Class II MHC are destined to become $CD4^+$ cells, and cells that recognize Class I MHC subsequently become $CD8^+$ cells. Before these cells can enter the general circulation, however, they should not be able to react against self-antigens. The second step is negative selection for those thymocytes that can recognize self-antigen presented on antigen presenting cells (APCs). Those thymocytes that recognize self-antigen undergo an apoptotic cell death. The T cells can then mature into distinct populations of $CD4^+$ and $CD8^+$ cells (Roitt *et al.*, 1996).

Once the lymphocytes have matured, they may then migrate into the secondary lymphoid organs to encounter foreign antigens. Secondary lymphoid organs include the spleen and lymph nodes. The spleen consists of two distinct regions: the red pulp and the white pulp. The red pulp serves as a filter for the blood, with resident macrophages removing senescent red blood cells and platelets. The white pulp consists of lymphoid tissue, much of which is arranged around a central arteriole. This arrangement is called the periarteriolar lymphoid sheath (PALS). The PALS is organized into T cell and B cell areas. T cells are found around the central arteriole, and B cells are organized into follicles. The follicles may be either primary, “unstimulated” follicles, consisting of

virgin B cells, or secondary, “stimulated” follicles, with germinal centers and memory B cells (Roitt *et al.,* 1996).

The lymph nodes are small, bean-shaped structures located throughout the body and are involved in filtering and trapping foreign antigens from the lymph fluid and in promoting lymphocyte-antigen interaction. Lymph nodes are commonly found near mucous membranes, and are found in close association with the gastrointestinal tract. In addition, organizations of lymph nodes are found in areas such as the neck, axillae, and groin, which drain various regions of the body.

IMMUNE RESPONSES

There are two basic and complementary components of the immune system: the innate immune system and the adaptive immune system. The innate immune system comprises the first line of defense after a foreign agent has passed the mucous membrane barriers of the epithelium. As such, the innate immune system is non-specific; the object is to clear foreign substances as rapidly as possible. Phagocytes, such as macrophages and neutrophils, are the cells involved in the innate immune response. When a pathogen, such as a bacterium, invades the host, it is non-specifically phagocytosed by the macrophages and neutrophils (Roitt *et al.,* 1996).

In contrast to the innate immune system, the adaptive immune system is capable of mounting a specific response against an invading pathogen. In addition, the adaptive immune system is capable of a memory response. In this way, if an antigen is encountered again, it will be cleared much more rapidly than it was during the first encounter. This is the theory working behind vaccinations. If one is immunized against a particular agent, the body will be able to mount a primary response without actually contracting the disease. Then, if the specific agent is encountered naturally, the body will be able to resist infection by mounting a quick, specific response.

The cells that play the largest role in mounting this specific, adaptive response are the lymphocytes. Lymphocytes can be divided into two distinct groups: B cells and T cells. Similarly, the adaptive immune response can be divided into two distinct, yet complementary, immune responses. These are the humoral, or antibody-mediated, immune response and the cell-mediated immune response.

B cells can produce antibodies when properly stimulated by an antigen. Memory B cells recognize antigen directly through the antigen receptor, which is similar to the antibodies secreted by the cell. Humoral immunity requires the presence of both B and T cells. While the mature B cells can differentiate into antibody-secreting plasma cells upon activation, they require costimulatory help from T cells. There are some antigens that can activate B cells without requiring T cell help; these antigens are known as T-independent antigens to distinguish them from T-dependent antigens. Though the T-independent antigens are able to elicit an antibody response, the response is greater when T cell help is available.

Cell-mediated immunity is a response that can be adoptively transferred by cells, but not by serum, as humoral immunity is transferred. The major participants in cell-mediated immunity (CMI) are the T cells, although natural killer (NK) cells and macrophages also play an important role. As mentioned previously, there are two distinct populations of mature T cells: CD8+ and CD4+.

The CD8+ T cells are generally classified as cytotoxic T cells; the cytotoxic T cells recognize antigen in association with Class I MHC. Class I MHC is expressed on all of the body's cells; thus, the CD8+ cells are responsible for recognizing virally infected and malignant cells. T cells are "trained" in the thymus to recognize only foreign peptides in association with self MHC. Therefore, the CD8+ T cells will not be able to recognize the self-peptides that are continuously expressed on the Class I MHC. When a non-self antigen, such as viral proteins and tumor specific antigens, is presented on the Class I MHC, the CD8+ T cells can mount a response to eliminate the

infected or tumor cells. Cytotoxic cells can kill tumor cells through a number of different pathways, including ligation of cell receptors, such as Fas expressed on the target cell, which induces apoptosis of the target cell, and secretion of cytotoxic factors, such as perforin and granzymes, which cause cell lysis (Roitt *et al.*, 1996).

Natural killer (NK) cells are also involved in killing virally-infected and malignant cells. Unlike CD8+ T cells, the NK cells are not MHC-restricted. Instead, it seems that the NK cells recognize those cells without Class I MHC. Since some tumors down-regulate the expression of Class I MHC, the NK cells provide an important defense against tumor growth.

The CD4+ cells are known as helper T cells, and their greatest role is in helping other cells to mount efficient immune responses by providing costimulation. Recall that the CD4+ T cells recognize antigen in association with Class II MHC. Class II MHC is expressed on professional APCs and on mature B cells. Professional APCs are phagocytic cells which process the antigen and present it in association with Class II MHC to the helper T cells.

When the CD4+ T cell recognizes the Class II MHC-antigen complex, it provides help via costimulatory molecules to the APC. This leads to activation of the APC. If the APC is a B cell, antibodies will be produced. When a macrophage is activated, it undergoes an oxidative burst, which will kill intracellular pathogens, and produces cytokines such as interferon-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor-α (TNF-α). These cytokines can kill surrounding cells, such as tumor cells, and activate more immune cells to partake in the response.

In addition to providing costimulation for the APCs, the CD4+ T cells produce cytokines. The cytokines that are produced determine whether the response will be primarily cell-mediated or antibody-mediated. These two different types of CD4+ T cell cytokine productions have been denoted Th1 and Th2 immune responses. The primary cytokines produced by Th1 cells are

interferon-γ (IFN-γ) and interleukin-2 (IL-2). The primary cytokines produced by Th2 cells are interleukins 4 and 10 (IL-4 and IL-10). A Th1 response will inhibit a Th2 response, and the converse is also true (Roitt *et al.,* 1996).

Interestingly, CD8+ cells also have two different types of cytokine production. The cytotoxic cells seem to produce a range of cytokines similar to the Th1 cells. There are other CD8+ cells that produce cytokines reminiscent of the Th2 population. It has been suggested that these cells may play an important regulatory or suppressor role (Roitt *et al.*, 1996).

Lymphocytes express a number of different surface glycoproteins and receptors during the maturation process and as mature cells. These markers are classified as clusters of differentiation (CD) and are used to characterize cells. One can characterize the maturity and activation status of cells by examining the expression of these markers. Cells become activated when the receptors are cross-linked sufficiently to cause an intracellular signal to be sent to the nucleus. Full activation of cells requires cross-linking of several costimulatory molecules in addition to cross-linking of the receptor.

B cells are characterized by their expression of surface immunoglobulins, CD19, CD20, and CD22. Macrophages express Fc receptor, MAC-1, and LFA-1. The Fc receptor allows the constant portion of the antibody molecule to bind; this enhances phagocytosis of specific antigens. NK cells are distinguished as being CD3- and CD16+.

Common T cell markers are the T cell receptor (TCR) in conjunction with the CD3 marker. The TCR and CD3 molecule are retained for the life of the T cell. Recall that T cells cannot recognize antigen directly, but can only recognize antigen in association with a Major Histocompatibility Complex (MHC) molecule. Thus the activation of a T cell requires ligation of the T cell receptor in conjunction with either CD4 or CD8. In addition to these basic T cell markers, there are also several important costimulatory molecules. Without the additional

stimulation through ligation of other surface molecules, the T cell will not become fully activated, and may become anergic or die via apoptosis. Some of the important costimulatory molecules that have been characterized are CTLA and CD28, which bind to the B7 molecules on antigen presenting cells.

In addition to the receptor and costimulatory molecules on T cells, there are also a number of activation and adhesion markers. An analysis of the expression of cell surface markers aids in characterizing the activation status of the cells. CD44 and CD45R are molecules which are upregulated in activated T cells. CD44 mediates adhesion of T cells to hyaluronic acid, commonly found in epithelial cell linings. This allows the activated T cells to reach the sites of antigen invasion. CD45R is a tyrosine phosphatase which augments signalling through the T cell receptor. The CD45R molecule allows a T cell to become more fully activated when it is ligated in conjunction with ligation of the T cell receptor.

When the cells become activated through ligation of their receptors, they undergo a period of clonal expansion, during which they divide rapidly to increase the number of cells capable of responding to the specific antigen. Most of these cells are destined to become effector cells and participate in the imminent immune response, but some become memory cells. The effectiveness of the adaptive immune response relies on these memory cells to rapidly recognize and eliminate the foreign body upon subsequent encounters.

After the immune cells have been activated and an immune response has been mounted, there must be some way to terminate the response. If the immune response is not down-regulated, it can cause damage to the host tissue. There are two common ways to down-regulate the response. Firstly, the cells can die. This form of “programmed cell death” is known as apoptosis. Apoptotic cell death is characterized in its early stages by fragmented DNA, and in its later stages by the formation of apoptotic bodies, which are rapidly cleared by phagocytic cells *in vivo*. Fas (CD95) and Fas ligand are intimately involved in apoptotic cell death. It has been suggested that

most of the apoptotic cell death in the periphery occurs through Fas-Fas ligand interactions (Roitt *et al.,* 1996).

Secondly, cellular activation can be suppressed by direct interactions with other cells or by indirect actions, such as the secretion of inhibitory factors. Suppressor cells have been shown to play a role in downregulating some immune responses.

Immunotoxicology

The field of immunology is diverse, and has recently branched into several sub-disciplines such as immunopharmacology and immunotoxicology. Immunopharmacology is the study of pharmaceutical agents on the immune response, and immunotoxicology is the study of effects of xenobiotics on the immune system. The study of these two sub-disciplines in immunology must incorporate the basics of both immunology and pharmacology or toxicology.

Paracelsus, the 16th century father of toxicology, remarked that "all things are poisons, for there is nothing without poisonous qualities. It is only the dose which makes a thing a poison." (Gots *et al*., 1993) This statement exemplifies a basic toxicological principle. Because of this principle, toxicologists study the dose-response relationships between exposure to toxicants and the effect on body systems to determine the level of toxicity.

Potential health hazards are identified and characterized through toxicology testing. The identification and characterization of a hazard include isolating the individual chemicals, determining which organ systems are affected by the toxicant, and establishing dose-response relationships. In order to determine what organ systems are affected and in what manner they are affected, a number of specialized toxicity tests are used.

Because immunotoxicology is a specialized branch of toxicology, there are a series of specialized immunological tests to determine whether a given compound can induce toxicity. Immunotoxicological studies involve examining the ability of chemicals to compromise immune function, elicit hypersensitive reactions, such as allergies, and induce autoimmunity. The National

Toxicology Program has developed a two-tier system of immunological tests to reliably detect immune alterations. The first tier is designed to detect gross alterations in the immune system, such as variations in lymphoid cell counts and organ weights, changes in histological appearances of lymphoid organs, and the changes in the activity of lymphoid cells (Luster *et al.*, 1992).

The second tier is designed to elucidate the reason for alterations observed in the first tier, such as quantification of cell markers (CD markers) on lymphoid cells. The second tier also includes *in vivo* tests to determine whether observed alterations correlate with host resistance to tumors, bacteria, viruses, and parasites (Luster *et al*., 1992).

Once toxicologists have gathered information from the specialized toxicity tests, they must determine the risks to the population as a whole. In addition to knowing how the toxicant affects the immune system, one must also know the level of exposure to the agent. With these two measures (information from the toxicity tests and exposure), toxicologists can perform a risk assessment for the toxicant. For instance, if a toxicant is highly toxic, but not readily encountered, it would not be considered as large a risk as a toxicant that is mildly toxic, yet encountered in large amounts.

Several compounds have been studied for their abilities to induce immunotoxicity. As technology has advanced over the past century, man-made chemicals, both finished compounds and waste byproducts, have been released into the environment. The science of toxicology was not as advanced during the early years of the industrial revolution. Consequently, a number of generally toxic and immunotoxic compounds were released into the environment.

Heavy metals, such as lead, cadmium, and arsenic, have been linked to decreased leukocyte counts, as well as B and T cell dysfunction (reviewed by Luster *et al*., 1992). Man-made compounds such as diethylstilbestrol (DES), dimethylbenz(a)-anthracene, benzo(a)pyrene, and nitrobenzene have all been shown to have immunotoxic effects on both B and T cells (reviewed by Luster *et al*., 1992). There are many man-made environmental pollutants that can induce immunotoxic effects. One of the most toxic and widely studied environmental immunotoxicants is 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD).

1.2 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD)

TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin), generically referred to as "dioxin," is one of the most toxic manufactured substances known. It is the model compound for and most toxic member of the class of molecules, known as halogenated aromatic hydrocarbons (HAH), that exert their effects through binding to the cytosolic Ah (aryl hydrocarbon) receptor (Figure 1) (Okey *et al.,* 1994; Kerkvliet and Burleson, 1994; Poland, 1991; Denison *et al.,* 1991; Gasiewicz and Henry, 1991; Silkworth *et al.,* 1984; Luster *et al.,* 1980). Of all the halogenated aromatic hydrocarbons, TCDD binds with the highest affinity to the Ah receptor, and so exhibits the most biologically potent effects.

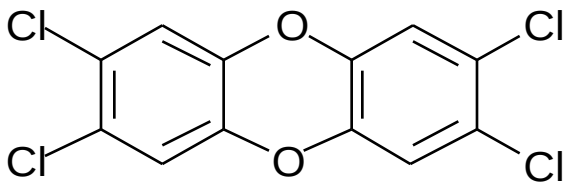

Figure 1. ***Structure of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin)***

TCDD is produced as an unwanted byproduct during the synthesis of chlorinated materials; TCDD residues have been found in herbicides such as Agent Orange, which was used extensively as a defoliant during the Vietnam War, as well as in the bacteriostat hexachlorophene. TCDD is also produced during the combustion of chlorinated materials, such as polychlorinated biphenyls (PCBs), once used extensively in capacitors and transformers as flame retardants. Dioxin is also produced during the chlorine bleaching of pulp (Needham *et al.,* 1991; Rappe, 1991). While TCDD is a ubiquitous environmental contaminant, having been identified in soil samples as old as

150 years, there is some evidence that the background levels have increased between 1940 and 1986 (Schecter, 1991).

High residues of TCDD have been found in the soil and vegetation exposed during environmental disasters, such as the Seveso chemical plant explosion, in Seveso, Italy, and the spraying of dioxin-contaminated waste oil in horse-riding arenas in southern Missouri. In addition, TCDD has been found in low levels in commercial products made of bleached pulp, such as coffee filters, milk cartons, facial tissues, and diapers (Rappe, 1991). The persistence and ubiquitous occurrence of halogenated aromatic hydrocarbons in the environment and the potent toxicity of TCDD in some laboratory animals have raised concerns of potential human health hazards.

The toxicity of TCDD has been well studied and characterized. The most common lesion experienced by humans is chloracne, in which the epidermis undergoes hyperplasia and hyperkeratinization (Knutsen, 1984). In laboratory animals, TCDD has been shown to cause cachexia, teratogenicity, fetotoxicity, dermal lesions, hepatotoxicity and the induction of several xenobiotic-metabolizing enzymes, tumor promotion, thymic atrophy, and generalized immunosuppression (Vos *et al.,* 1974; Kimbrough, 1984; Vos *et al.,* 1991).

The effects of dioxin on the immune system are of particular interest because even at doses that do not produce organ toxicity, TCDD is able to induce a marked immunosuppression. Much work has been done on discovering the target cell in the immune system for TCDD-induced toxicity and on elucidating the molecular pathway through which dioxin acts.

Immunotoxicological studies have shown that TCDD can suppress cell mediated immunity (CMI). TCDD is also able to suppress humoral immunity (HI) involving antibody production by B lymphocytes (Smialowicz *et al.,* 1994). Other immunosuppressive effects of TCDD include suppression of B cell differentiation, a dose-dependent suppression of cytotoxic T cells, cortical

depletion of the thymus (DeHeer *et al.,* 1995), impairment of delayed-type hypersensitivity responses, and increased susceptibility to bacterial infection and tumor challenge (Luster *et al.,* 1980; Dean and Lauer, 1984; Luebke *et al.,* 1994). Despite extensive research, the exact mechanism of TCDD-mediated immunosuppression is not yet known.

TCDD-induced toxicity correlates well with the aryl hydrocarbon receptor (AhR) phenotype. Mice with a high affinity Ah receptor, designated Ah^b, are more susceptible to TCDD than are mice that possess the lower affinity Ah receptor, designated Ah^d. The presence of the higher affinity receptor leads to a greater induction of the xenobiotic-metabolizing enzymes, such as cytochrome P450 1A1 and UDP glucuronyl transferase, that have a dioxin responsive element (DRE) located near the promoter (Vecchi *et al.,* 1983). There are several genes that are known to have DREs (Lusska *et al.,* 1993), and many other genes, such as those controlling the cell cycle and the production of growth factors, that have been postulated to be regulated by a DRE.

A report by Dooley *et al.* (1988) demonstrated that the effects of TCDD on the humoral immune response, specifically on the B lymphocyte, were caused by the activation of a suppressor T cell. Several laboratories have demonstrated specific suppression of IgM secretion by B cells, without any effect on B cell proliferation (Luster *et al.,* 1988; Dooley *et al.,* 1988). This suppression of the antibody response was not limited to IgM; IgG and IgE were also suppressed (Karras *et al.,* 1995). In 1993, Wood *et al.* showed that low density (activated) B cells were a sensitive target for TCDD action while high density (resting) B cells were unaffected by TCDD treatment. TCDD mediated the antibody suppression by inhibiting the formation of fully differentiated B cells. These results suggest that TCDD exerts a selective effect on B cell differentiation.

Because TCDD causes marked thymic atrophy, and because the thymus is the central organ for the maturation of T lymphocytes, the first immunotoxicity studies concentrated on the cell mediated immunity. The effect of TCDD on cell mediated immunity seems to be an age dependent

phenomenon, with younger animals exhibiting greater suppression than older ones.

It appears that TCDD induces the terminal differentiation of the thymic epithelial cells (Kremer *et al.,* 1994), accelerating thymocyte maturation, and leading to apoptosis of thymocytes within the thymus (McConkey *et al.,* 1988; McConkey *et al.,* 1994). There is also some evidence that TCDD drives thymocytes into differentiation faster than the precursor pool can be replenished. Thymic atrophy may be caused, in part, by reduced seeding by prothymocytes (Fine *et al.,* 1990). Prothymocytes in the bone marrow are susceptible to TCDD action, and this action is not secondary to TCDD action in the thymus (Luster *et al.,* 1980; Frazier *et al.,* 1994). TCDD also has effects on the mature thymocyte population.

Clark *et al.*(1983) have suggested that the suppression of the cytotoxic T lymphocyte (CTL) response was due to the induction of suppressor T cells. Nagarkatti *et al.* (1984) showed that TCDD did not affect the frequency of the CTL precursors, indicating that suppressor T cells were induced to mediate the CTL suppression. The development of these suppressor T cells was found to be dependent on the Ah genotype of the host (Nagarkatti *et al.,* 1984). There is more evidence for a functional defect in lymphocyte activation than for the induction of suppressor T cells, however.

While several laboratories have concluded that the helper T cell is not a target for dioxin action (Dooley *et al.,* 1988; Luster *et al.,* 1980), there is at least one report for a defect in helper T cell regulation. Tomar and Kerkvliet (1991) showed that the helper T cell function, in helping B cells to respond to the T-cell-dependent antigen SRBC, is reduced after TCDD exposure. In 1987, Kerkvliet and Brauner showed that T lymphocytes play a central role in mediating humoral immune suppression caused by HpCDD (heptachlorodibenzodioxin). The antibody responses to both the T-dependent (SRBC) and the T-independent (TNP-LPS) antigens were suppressed, but the T-dependent antigen response was suppressed more than the T-independent antigen response. While most of the actions of TCDD have been shown to be immunosuppressive, it can have stimulatory

effects on the immune response as well.

For example, Neumann *et al.* (1993) and Dooley *et al.* (1990) have shown that TCDD actually enhanced the proliferation of activated T cells *in vitro.* However, TCDD is able to inhibit the antigen-specific response. Studies carried out in our lab, as well as studies done elsewhere, suggested that TCDD does not affect naive T cells but inhibits the functions of only those T cells activated *in vivo* through antigen priming (Lundberg *et al.*, 1992; Rhile *et al.*, 1996). In other words, T cell responsiveness of the TCDD-treated ovalbumin primed lymph node cells was suppressed only in response to *in vitro* stimulation with ovalbumin, but not in response to a polyclonal T cell mitogen such as anti-CD3 monoclonal antibodies (Lundberg *et al.*, 1992). These data suggest that TCDD acts on differentiating T cells rather than naive T cells. The exact mechanism by which TCDD inhibits this antigen-specific T cell responsiveness is not clear. However, the fact that Fas-deficient mice are more resistant to TCDD-induced immunotoxicity suggested that apoptosis may play a key role in suppressing the T cell responsiveness (Rhile *et al.*, 1996).

In both the B and the T lymphocyte populations, it appears that TCDD is affecting only the differentiating cells undergoing active proliferation (Dooley *et al.*, 1988). Because B cells are normally more proliferative than T cells, it may appear that the direct effect of TCDD is on the B cells. This view is consistent with the effects of TCDD on non-lymphoid populations, such as adipose tissue (Phillips *et al.*, 1995), in which researchers have found that dioxin affects only those cells undergoing differentiation and rapid proliferation (Luster *et al.*, 1980). This may explain why fetuses and younger animals are at a greater risk for TCDD-induced toxicity than are older animals.

In addition, there is some molecular evidence that suggests that the Ah receptor is not able to effectively bind to DNA that has already undergone cytosine methylation, and so is not able to enhance transcription of the gene (Shen and Whitlock, 1989). Because methylation occurs some

time after replication, this evidence supports the idea that only those cells actively undergoing proliferation will be affected by TCDD.

TCDD also has effects on growth factor and cytokine production. In mice with a TCDD-induced cleft palate, there is overexpression of growth factor by the palatal epithelial cells (Abbott *et al.,* 1994). It has been demonstrated that TCDD can induce expression of interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), transforming growth factor α (TGF-α), and transforming growth factor β (TGF-β) in the human breast cancer cell line MCF-7 (Vogel and Abel, 1995). Expression of these cytokines led to an inhibition of cell growth. In TCDD-treated hairless mice that develop chloracne-like lesions, there was an increase in the dermal expression of TNF-α; this secretion of TNF-α was not mediated by fibroblasts, but more likely, by immune cells at the site of inflammation (Connor *et al.*, 1994).

There is some speculation that TCDD induces much of its toxicity through expression of TNF-α. In this regard, Taylor *et al.*, (1992) neutralized TNF-α through anti-TNFα antibodies and suppressed transcription of the TNF-α gene with dexamethasone, resulting in a 54-92% decrease in TCDD-mediated mortality. These data suggested that TCDD-mediated changes in the TNF pathway may be an important mechanism in TCDD-induced toxicity. Because TNF, TGF, IL-1, and Fas ligand are cytokines involved in the induction of apoptosis (Hernandez-Caselles and Stutman, 1993; Rotello *et al.*, 1991), it is possible that TCDD-induced upregulation in such cytokine production may trigger apoptosis in lymphocytes and may account for TCDD-mediated immunotoxicity.

1.3 Basis for and Goals of Research

Although the toxicity of TCDD has been widely studied, the exact mechanisms through which it exerts its immunosuppressive effects are still not clear. While most studies have shown that TCDD is immunosuppressive, a few studies indicated that TCDD may enhance the lymphocyte activation (Sharma and Gehreing, 1979; Kramer *et al.,* 1987; Tognoni and Bonaccorsi, 1982). In this study, we tested the hypothesis that TCDD would affect resting and activated cell populations differently within the same animal to try to explain this discrepancy.

Within the activated population of cells, Neumann *et al.* (1993) showed that TCDD treatment led to increased proliferation upon culture with growth factors. Upon restimulation, Lundberg *et al.* (1992) and Rhile *et al.* (1996) showed that the activated cells are subject to an antigen-specific proliferative decrease. In this study, we also tested the hypotheses that the antigen-specific immunosuppressive effect of TCDD is mediated through induction of apoptosis and dysregulation of cell surface markers in activated T cells.

When a vertebrate host encounters a foreign antigen, only the antigen-specific B and T lymphocytes are activated. Thus, our studies, focused on addressing the effect of TCDD on resting and activated T cells within the same animal, provide new and useful information on how the TCDD-exposed host would respond to infections, tumorigenesis, allergies, and autoimmunity.

1.4 Specific Objectives

Because dioxin exposure is not limited to just the activated T cells within an animal, it would be beneficial to determine how dioxin differentially affects naive and activated populations of T cells within the same organism. To this date, however, no work has been done comparing the effects of dioxin on naive and activated T cells within the same animal. Therefore, the specific goals of this study were:

1. To determine the immunomodulatory effect of TCDD on resting and activated T cell populations within the same animal.
2. To investigate the mechanisms of the antigen-specific proliferative decrease in activated T cells. Several possible mechanisms were investigated:
    a. Apoptosis: TCDD may trigger apoptosis in activated, but not naive, resting T cells.
    b. Cell surface marker expression:
        i. TCDD may decrease the expression of growth factor receptors, such as the IL-2 receptor, which would lead to decreased responsiveness to growth factors.
        ii. TCDD may alter the expression of cluster of differentiation (CD) molecules on naive or activated T cells.
    c. Altered kinetics of the immune response: TCDD may speed up the differentiation process, allowing less time for clonal expansion, which would decrease the effector cell population.

Chapter 2 Methods and Materials

2.1 Mice

Adult female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Charles River (Boston, MA). The mice were housed in polyethylene cages (5-6 mice/cage) containing wood shavings, and were given rodent chow and tap water *ad libitum*. Mice were housed in rooms maintaining a temperature of 76±2°F and a twelve hour light/dark cycle.

2.2 TCDD exposure

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was generously donated by Dr. K. Chae of NIEHS, Research Triangle Park, NC, and was stored at -20°C. TCDD was dissolved in acetone and diluted in corn oil. The solution was gently heated with stirring to evaporate the acetone. Serial dilutions were done to reach the desired concentrations. Mice were administered 50 µg/kg body weight of TCDD or the vehicle control via an intraperitoneal injection.

2.3 Immunizations

Mice were injected in both rear footpads, with anti-CD3 antibodies in a 1:1 ratio with Complete Freund's Adjuvant (Sigma), to activate T cells in the popliteal lymph nodes. Immunizations were done either two days after TCDD/vehicle treatment, as done in previous studies (Neumann *et al.,* 1993) or at the same time as TCDD/vehicle treatment, to determine if

TCDD could exert immunomodulatory effects when given concurrently with an antigenic challenge.

2.4 Cell preparation

Mice were euthanized at several timepoints (3, 5, 7, 14, and 21 days) after TCDD or vehicle treatment. Axillary and popliteal lymph nodes were removed by dissection. Organs were placed in RPMI-1640 medium (GIBCO Lab., Grand Island, NY) supplemented with 10% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO), 10 mM HEPES, 1 mM glutamine, 40 µM/ml gentamicin sulfate, and 50 µM 2-mercaptoethanol. Single cell suspensions were made with a laboratory homogenizer (Stomacher, Tekmar Co., Cincinnati, OH) and kept on ice. Cells were pelleted by centrifugation and washed twice in medium.

2.5 Cell proliferation to mitogen stimulation

Naive and activated lymph node cells were tested for their abilities to respond to T cell mitogens. Axillary lymph nodes were used as a source of naive, unactivated T cells and popliteal lymph nodes were used as a source of activated T cells.

T cells were cultured with IL-2 (50 U/ml), anti-CD3 antibodies (1:100 final dilution of hybridoma supernatant), and Concanavalin A (2.5 µg/ml). The anti-CD3 antibodies were purified from hybridoma culture supernatants as described (Kakkanaiah *et al.*, 1990; Dean *et al.*, 1990). In short, hybridoma cultures were grown in 10% RPMI. Culture supernatants were collected and concentrated using a dialysis membrane. Concentrated supernatants were tested for activity in cell culture. Axillary and popliteal lymph node cell suspensions were cultured in flat-bottom, 96-well plates at concentrations of $4x10^5$ cells/well in 0.2 ml medium for 18, 24, or 48 hours in an

incubator maintained at 37°C and 5% CO_2. Plates cultured for 18 and 24 hours were pulsed immediately with 2 µCi ^{3}H-thymidine; the plates cultured for 48 hours were pulsed with 2 µCi ^{3}H-thymidine during the last 8 hours of incubation. Cells were harvested using an automated cell harvester (Skatron, Sterling, VA). The amount of radioactivity was determined using a scintillation counter (TM Analytic 6895) and the mean cpm ± SEM of triplicate cultures was calculated.

2.6 Flow Cytometric Staining for Apoptotic Cells

In the current study, we used a kit, based on labeling of DNA strand breaks using terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP, commonly referred to as TdT-mediated nick end labeling or TUNEL technique, (Boehringer Mannheim, Indianapolis, IN) to detect and quantify apoptotic events on a single cell level. Axillary and popliteal lymph node cells were stained directly after removal (fresh) or after 24 hours culture in RPMI or with anti-CD3 antibodies (1:100 final concentration). The cells were washed twice with medium containing phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 minutes at room temperature. The cells were next washed with PBS, permeabilized on ice for 2 minutes, and incubated with FITC-dUTP for one hour at 37°C. Fluorescence of the cell populations was measured by flow cytometric analysis as described (Kamath *et al.,* 1997). The percentage of cells staining positive for each sample was used for analysis (see Appendix I). The analysis was performed on a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

2.7 Flow Cytometric Staining for Activation Markers

Axillary and popliteal lymph node cells were harvested, adjusted to $1x10^6$ cells/ml, and washed twice with phosphate buffered saline containing 0.1% sodium azide. The cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal

antibodies against: CD3 (Armenian hamster IgG), CD4 (rat IgG_{2a}, κ), αβTCR (Armenian hamster IgG), CD122 (rat IgG_{2b}, κ), CD45R (rat IgG_{2a}, κ), CD44 (rat IgG_{2b}, κ), (Pharmingen, San Diego, CA), or CD8 (rat IgG_{2a}) (Sigma Immunochemicals) for 30 minutes at 4°C. Staining for Fas was performed by a primary incubation with purified mouse anti-Fas antibody (Pharmingen), followed by a secondary incubation with FITC-conjugated antibody (goat anti-hamster $F(ab)_2$ IgG) (Jackson Immunochemicals). Fluorescence of the cell populations was measured by flow cytometric analysis as described (Kamath *et al.*, 1997). The parameters used for analysis were percentages staining positive and the mean fluorescent intensity (see Appendix I). The analysis was performed on a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

2.8 Phenotypic Assessment of Apoptotic Cells

Axillary and popliteal lymph node cells were stained directly after removal (fresh) or after 24 hours culture in RPMI or with anti-CD3 antibodies (1:100 final concentration). The cell suspensions were adjusted to $1x10^6$ cells/ml, and washed twice with phosphate buffered saline containing 0.1% sodium azide. The cells were incubated with phycoerythrin (PE)-conjugated monoclonal antibodies against CD3 (Armenian hamster IgG) for 30 minutes at 4°C. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde for 30 minutes at room temperature. Following this step, the cells were stained according to the TUNEL procedure outlined in Section 2.0.6. Fluorescence of the cell populations was measured by flow cytometric analysis. The percentage of cells staining positive for each cell marker and the mean fluorescent intensity of each sample were used for analysis (see Appendix II). The analysis was performed on a Coulter Epics V flow cytometer. Five thousand cells were analyzed per sample.

2.9 Statistical Analysis

In this study, groups of five mice were used for each of the control and TCDD-treated groups. The experimental groups were compared to the control groups, using the Student's t-test. The animals were segregated into two different treatment groups (corn oil and TCDD). In addition, the two different lymph node populations (axillary and popliteal) studied were considered additional groups. Therefore, axillary lymph nodes from TCDD-treated animals were compared only to axillary lymph nodes from oil-treated animals. Similarly, the popliteal lymph nodes from TCDD-treated animals were only compared with other popliteal lymph nodes. This distinction was made because the lymph node populations consist of different cells. The axillary lymph nodes contained resting and naive T cells, whereas the popliteal lymph nodes contained primarily activated T cells due to rear footpad immunizations with anti-CD3 mAbs. Because of the inherent differences in responsiveness to growth factors and upon rechallenge with antigen, as well as the expression of activation molecules, a direct comparison cannot be made between the resting and activated T cells. Differences with p values less than 0.05 were considered to be statistically significant. All assays were repeated at least three times with consistent results.

Chapter 3 Results

3.1 Proliferation Assays

To this date, no work has been done to compare the effects of TCDD on resting and activated cells within the same animal. Therefore, in the first set of experiments, we tested the responsiveness of activated and naive cells from TCDD-treated animals to *in vitro* culture with IL-2, as well as the recall responsiveness of the cells to the specific antigen. To study the activated T cells, mice were immunized with anti-CD3 mAbs into the rear footpads and the draining popliteal lymph nodes were used as the source of activated T cells. Anti-CD3 mAbs were chosen because they would polyclonally activate T cells; this allowed us to study a stronger proliferative/apoptotic response. Because the T cell response to anti-CD3 mAbs was restricted to the popliteal lymph nodes, the distally-located axillary lymph nodes were used as a source of naive, resting T cells.

To this end, mice were injected with vehicle or TCDD on day 0. Both rear footpads were injected with anti-CD3 antibodies on day 2. Axillary and popliteal lymph nodes were harvested on day 3 and were cultured for 48 hours with either RPMI, IL-2, or CD3. Resting T cells (from the axillary lymph nodes) from TCDD-treated mice showed no differences from the oil-treated controls in responsiveness to IL-2 (Figure 2A), while the activated cells (from the popliteal lymph nodes) from TCDD-treated mice showed a slight proliferative increase when cultured with IL-2 ($p=0.005$) (Figure 2B). These data corroborated an earlier study (Neumann *et al.*, 1993), and demonstrated that activated T cells from TCDD-treated mice exhibit increased responsiveness to IL-2.

The next step was to determine if the activated T cells would exhibit an altered response to the recall antigen. To this end, the axillary and popliteal LN cells were cultured *in vitro* with anti-CD3 mAbs. The results indicated that resting T cells from TCDD-treated mice did not show any

significant differences from the oil-treated controls (Figure 3A). The activated cells from TCDD-treated mice, however, showed a significant proliferative decrease upon restimulation ($p=0.01$) (Figure 3B). Responsiveness to another T cell mitogen, Concanavalin A (Con A), was also tested. There was no proliferative difference between either the resting or the activated cells from dioxin-exposed animals in response to stimulation with Con A (Figures 4A and B). Since the activated, TCDD-treated cells were able to respond normally to Con A, but not to anti-CD3 mAbs, this suggests that the immunosuppressive effect of TCDD is antigen-specific.

These studies demonstrated that TCDD affects activated and naive T cell populations differently. *In vivo* TCDD exposure did not decrease the ability of naive (axillary LN) T cells to respond to growth factors and mitogens, but was able to exert an immunosuppressive effect on activated (popliteal LN) T cells undergoing antigenic restimulation.

3.2 Kinetics of TCDD-Induced Immunomodulation

The results provided in Section 3.1 indicated that an antigen-specific decrease could be observed in cells cultured three days after TCDD exposure. These experiments, however, did not address if this decrease would still be present at further timepoints. Therefore, we investigated longer-term effects of TCDD on the resting and activated T cell populations. Mice were injected with either the vehicle or TCDD on day 0. At the same time, both rear footpads were immunized with anti-CD3 antibodies in a 1:1 ratio with Complete Freund's Adjuvant. One, two, and three weeks later, axillary and popliteal lymph node cells were tested for their abilities to respond to *in vitro* culture with IL-2 and antigen restimulation with anti-CD3 antibodies.

One week after treatment, the axillary lymph node cells from TCDD-treated animals showed a significant proliferative increase in response to culture with IL-2 ($p=0.0005$) (Figure 5A). In contrast, the popliteal lymph node cells showed a significant proliferative decrease in response to IL-2 ($p=0.005$) (Figure 5B). It should be noted that the background counts per minute

(cpm) in experiments that used the popliteal LN cells were much higher than those seen for the axillary LN cells, possibly because these cells had been activated *in vivo* with anti-CD3 mAbs. However, even with subtraction of these background cpm, the responsiveness of popliteal LN cells from TCDD-treated mice to IL-2 was significantly less than the vehicle controls.

This is different than the response seen at 3 days (Figure 2B), where the activated cells from TCDD-treated mice exhibited an increased proliferative rate when cultured with IL-2. These data suggested that activated T cells from TCDD-treated mice may initially respond strongly to the growth factors, which is followed by a decline in the responsiveness.

In contrast to the activated T cells, the resting axillary LN cells from TCDD-treated mice responded strongly to stimulation with IL-2. These data suggested that TCDD may activate the resting T cells in such a way that they mount a stronger proliferative response to growth factors.

The results presented above (Section 3.1) demonstrated that upon restimulation, decreased proliferative responses were observed in the activated, *in vivo* TCDD-exposed cells at the 3 day timepoint. We wanted to determine if the antigen-specific decrease observed at the earlier timepoint would still be present at one week. In addition, since the resting cells from dioxin-treated animals showed an increased proliferative ability upon culture with IL-2 at one week (Figure 5A), we wished to determine if there would be a proliferative difference upon *in vitro* stimulation. The results, however, demonstrated that there was no difference in proliferative ability when axillary (resting) lymph node cells from TCDD-treated animals were cultured with anti-CD3 antibodies (Figure 6A). In direct contrast, activated (popliteal) lymph node cells from TCDD-treated mice displayed a greater than 50% decrease in proliferative rate, as compared to the oil-treated control ($p=0.0025$) (Figure 6B).

Two weeks after TCDD-treatment, both the resting and activated lymph node resumed normal responsiveness to *in vitro* culture with IL-2 (Figure 7 A and B). It should be noted that at two weeks the popliteal lymph nodes consisted primarily of resting memory cells. These resting memory cells are different from the resting cells in the axillary lymph nodes, because they are able to mount a more rapid and efficient immune response, a memory response, against the specific

antigen. This was demonstrated by the differences in the magnitude of the immune response (Figure 7), with the popliteal lymph node cells showing a much higher proliferative response than the axillary lymph node cells.

Both populations of cells from TCDD-treated mice were also able to respond normally to *in vitro* antigenic challenge with anti-CD3 antibodies (Figure 8 A and B). Furthermore, the responsiveness of axillary and popliteal LN cells from TCDD-treated groups to IL-2 (Figure 9) and anti-CD3 antibodies (Figure 10) remained normal three weeks post-treatment. This return to normal responsiveness indicates that TCDD does not exert a permanent effect on the immune response.

The possibility that the axillary and popliteal lymph node cell populations were inherently different was investigated. It was deduced from the preceding results that TCDD affects activated and naive cell populations differently. However, these data may have been due to the popliteal lymph nodes having an inherently different responsiveness to *in vitro* stimulation. Therefore, we tested the hypothesis that the naive popliteal lymph nodes did not differ from naive axillary lymph nodes in responsiveness to *in vitro* stimulation. Mice were injected with either the corn oil vehicle or 50 µg/kg TCDD i.p. on day 0. As a proper control for the previous experiments in which the rear footpads had been injected, both rear footpads of the mice were injected with Complete Freund's Adjuvant. On day 7, the axillary and popliteal lymph nodes were harvested, and single cell suspensions were made. The cells were cultured with culture medium (RPMI), IL-2, or anti-CD3 mAbs. In this experiment, both the axillary and popliteal lymph nodes consisted of resting T cells. The T cells in the popliteal lymph nodes were not activated because the rear footpads were not immunized with anti-CD3 antibodies in this experiment. The data demonstrated that the popliteal lymph node cells did not differ from the axillary lymph node cells in their responsiveness to either IL-2 (Figure 11 A and B) or anti-CD3 mAbs (Figure 12 A and B). This indicates that the differential responses observed in the previous studies were due to the different activation states of the cells, and not to inherent population differences.

The restoration of the immune response indicated to us that TCDD exerted only a temporary effect on the studied lymph node cell populations. The transient effect of TCDD on this system was interesting, so we set about determining the mechanism of the transitory immunomodulations.

The first hypothesis tested was that TCDD was altering the terminal differentiation of the cells. Accelerated terminal differentiation would leave less time for clonal expansion, effectively decreasing the effector cell population. In the studies carried out thusfar, the proliferative responsiveness *in vitro* was studied only at one timepoint. Thus, it was possible that TCDD could have had varying effects at other timepoints. To address the above, a kinetic analysis of the immune response was performed. Cells were cultured for differing amounts of time (18, 24, or 48 hours) to determine if the immune response was either accelerated or delayed following TCDD treatment. If the immune response were delayed, the response time would be greater, and the resulting curve would be shifted down and to the right of the control (Figure 13). In contrast, if the immune response were accelerated, the response time would be faster, and the kinetics curve would be shifted up and to the left of the control (Figure 13). The results for cultures with IL-2 and anti-CD3 mAbs at one week and two weeks following TCDD administration are shown in Figures 14 and 15 respectively.

Although not as notably evident at one week after anti-CD3 immunization (Figures 14A and 15A), at the two week timepoint, clear memory responses were detectable in the popliteal lymph node cells. This was demonstrated by a higher basal proliferative rate and indicated that the popliteal lymph node cells were effectively activated *in vivo* (Figures 14B and 15B). In contrast, the axillary lymph node cells had a much lower basal proliferative rate, at both the one- and two-week timepoints, indicating that they were either resting or naive cells (Figures 14A and 15A). At one week, the resting (axillary LN) cells from TCDD-treated animals demonstrated an accelerated proliferative response when cultured with IL-2 at all timepoints tested, as indicated by a higher proliferative response after 48 hours in culture (Figure 14A). However, this was not seen when the resting cells were cultured with anti-CD3 antibodies (Figure 15A).

In contrast, activated (popliteal LN) cells from TCDD-treated animals showed a delayed and decreased proliferative response to the recall antigen (anti-CD3 mAbs) (Figure 15A), but not when cultured with IL-2 (Figure 14A). The delayed responsiveness of activated cells from TCDD-treated mice, in response to antigenic restimulation, is shown as a curve shift downward and to the right of the curve for activated cells from oil-treated mice (Figure 15A). The altered immune responses of both resting and activated cells from TCDD-treated animals disappeared by two weeks (Figures 14B and 15B), as shown by the similarities between the curves for the control and experimental groups (Figures 14B and 15B). These data confirmed our earlier observation that TCDD exerts differential effects on axillary and popliteal LN cells.

3.3 Apoptosis as a Mechanism of TCDD-Induced Toxicity in Activated T Cells

The kinetics assays suggested that TCDD was accelerating the immune responses of resting cells and delaying the responses of activated cells, but the kinetics experiments did not indicate the cause of these altered immune responses. The cause of the delay could be due to changes in the apoptotic rate or dysregulations of cell surface activation molecules. We first tested the hypothesis that the temporary antigen-specific proliferative decrease was due to an increased death rate in the effector cell population. Most cell death occurs through apoptosis, not necrosis, so we analyzed the resting and activated cell populations for apoptotic events. The amount of apoptosis was measured on days 3, 5, 7, and 14 after vehicle/TCDD treatment and immunization with anti-CD3 mAbs.

The timepoints for study of apoptosis were chosen to coincide with data from the proliferation assays. Activated cells from TCDD-treated mice showed the largest proliferative decrease at one week (Section 3.1; Figure 6B). However, it is known that thymocytes undergo apoptosis very shortly after TCDD injection (Kamath *et al.*, 1997). Therefore, shorter time periods

(3 and 5 days post-treatment) were chosen to determine the amount of early apoptosis. The proliferative decrease in response to antigen-restimulation disappeared after two weeks, so this timepoint was chosen to determine the amount of apoptosis occurring without a corresponding proliferative decrease.

In addition to staining the cells freshly recovered from the animal, the cells were cultured *in vitro* for 24 hours with either medium or anti-CD3 antibodies. Kamath *et al.* (1997) was able to show that TCDD induces apoptosis of thymocytes *in vivo*. They found that they could detect apoptosis *in vivo* up to 12 hours after injection of TCDD. After that time period, apoptosis was not detectable because of rapid phagocytosis of the apoptotic bodies *in vivo*. Additionally, Kamath *et al.*(1997) showed that thymocytes from TCDD-treated mice undergo increased apoptosis upon subsequent culture. This method has been shown to be a reliable method to detect low levels of *in vivo* apoptosis with an *in vitro* technique.

We used the TdT-mediated FITC-dUTP nick end labeling (TUNEL) method and flow cytometric analysis to detect apoptotic cells. The TUNEL technique detects DNA strand breaks using TdT (terminal deoxynucleotidyl transferase) and the FITC-labeled nucleotide, dUTP. Because DNA fragmentation is an early sign of apoptotic cells, this labeling method is a recognized method for detecting cells in the early stages of apoptosis. Kamath *et al.*(1997) have shown that the amount of apoptotic cells detected with the TUNEL technique correlates well with other measures of DNA fragmentation and apoptosis, including gel electrophoretic assays and the JAM test. The JAM test was developed by Matzinger (1991) to detect fragmented DNA. In this test, the amount of fragmented DNA is assessed from the amounts of ^{3}H-thymidine incorporated into the DNA of both the control and experimental groups. The JAM test is similar to a proliferation assay in measuring the amount of ^{3}H-thymidine incorporation, but in the JAM test, the incubation time with the radioactive nucleotide is often much shorter than in a proliferation assay. When the cells are harvested, the fragmented DNA passes through the filter; increases in apoptosis are detected as decreased counts per minute. Because of the good correlation and reliability between these

different measures of DNA fragmentation, only the TUNEL technique was employed to detect increases in DNA fragmentation in this study.

We were interested in knowing how lymph node cells from TCDD-treated animals differed from lymph node cells from oil-treated animals. We knew that cells in culture undergo some amount of apoptosis. To detect the differences between the oil- and TCDD-treated animals, therefore, we overlayed results from the experimental groups over the control responses. In Figures 16-19, the control group (cells from oil-treated animals) responses are shown as solid lines, while the experimental group (cells from TCDD-treated animals) results are shown as dotted lines. Increases in apoptosis are indicated by positive shifts in fluorescence (rightward curve shifts). The percentage of increased apoptosis, over the control values for each experiment, is depicted in the top right corner of each histogram. The mean ± SD of four experiments is shown in parentheses below the percentage for the representative experiment.

Previous studies demonstrated that TCDD induces apoptosis in thymocytes (Kamath *et al.*, 1997). In the current study, therefore, we tested the hypothesis that the decreased proliferative response shown by activated T cells from TCDD-treated mice may result from induction of apoptosis in such cells. Furthermore, we also tested whether the naive, resting T cells are resistant to TCDD-induced apoptosis. To study the apoptosis, we used the TUNEL assay and analyzed the cells flow cytometrically.

On day 3 following TCDD administration, no apoptosis was detectable in the fresh samples of either the axillary (Figure 16A) or popliteal lymph (Figure 16D) node cells from vehicle and TCDD-treated groups, indicated by low fluorescence levels (units from 0.1 to 1.0). However, after 24 hours culture with RPMI, the increased fluorescence of the cells (shown as curve shifts) indicated that significant apoptosis was detectable in both resting axillary (Figure 16B) and activated popliteal (Figure 16E) lymph node cells from the control and TCDD-treated groups. This was an expected result, as most cells subjected to *in vitro* culture in the absence of stimulation

undergo apoptosis. Resting axillary LN cells from TCDD-treated animals showed a significantly decreased amount of apoptosis as compared to the oil-treated control, indicated by the decreased fluorescence of the experimental group (Figure 16B). This suggested that TCDD was preventing the resting cells from an apoptotic death in culture. In contrast, the activated cells (popliteal LN cells) from TCDD-treated mice cultured with RPMI showed a significant (30.5%) increase over the control in the amount of apoptotic cells (Figure 16E). These data suggested that TCDD-exposure caused the activated (popliteal LN) cells to undergo increased apoptosis in culture, while preventing the apoptotic death of resting (axillary LN) cells.

When cultured with anti-CD3 antibodies for 24 hours, the resting cells from dioxin-treated mice were again "saved" from apoptosis, as demonstrated by the leftward shift of the dotted-line curve compared to the curve of the control (Figure 16C). However, the activated cells from dioxin-exposed mice, when cultured with anti-CD3 mAbs, did not show an increase in apoptosis when compared to the vehicle controls (Figure 16F).

On day 5 following TCDD treatment, little apoptosis was detectable in the fresh axillary (Figure 17A) and popliteal (Figure 17D) lymph node cells. After 24 hours culture with RPMI, significant levels of apoptosis were seen in the axillary lymph node samples from vehicle-treated mice (Figure 17B). However, no increases in apoptosis, over the amount detected in the control, were detected in resting cells from TCDD-treated animals (Figure 17B). In contrast, the axillary lymph node cells from dioxin-exposed mice again showed a significantly decreased amount of apoptosis as compared to the oil-treated control (Figure 17B). Furthermore, activated T cells from TCDD-exposed mice exhibited a significant increase (31.3%) in apoptotic cells after 24 hours culture with medium when compared to the vehicle controls (Figure 17E).

At the 5 day timepoint, the differences in activation state between axillary and popliteal LN cells are clearly evident by the basal levels of apoptosis. The popliteal lymph node (activated) cells showed less apoptosis after 24 hours culture than the axillary lymph node (resting) cells (Figures

17 B and E). This can be explained by the fact that activated cells retain viability in culture for longer periods than resting cells because unlike the resting cells, activated T cells can produce autocrine growth factors.

The change is activation state is also clear upon comparison of results obtained from experiments performed 3 and 5 days after vehicle or TCDD-treatment (Figure 16E). For example, at 3 days post-treatment with the vehicle, the popliteal lymph node cells cultured in medium alone for 24 hours demonstrated greater levels of apoptosis (Figures 16 E and F) when compared to similar cells obtained 5 days after treatment (Figures 17 E and F). Furthermore, similar results were obtained with TCDD-exposed popliteal LN cells (Figure 16E *vs*. 17E, dotted histograms), except that the degree of apoptosis was enhanced following TCDD-treatment.

When cultured with anti-CD3 mAbs, the axillary lymph node cells collected 5 days after TCDD-treatment did not show any apoptosis, in direct contrast to the axillary lymph node cells from oil-treated animals, which underwent normal levels of apoptosis for cells in culture (Figure 17C). In contrast, popliteal lymph node (activated) cells from TCDD-treated animals, similarly cultured, displayed significant (30%) increases in the percentage of apoptotic cells when compared to the vehicle control (Figure 17F). These results indicated that the axillary lymph node cells from TCDD-treated mice were less susceptible, while the activated cells were more susceptible, to apoptosis than the control cells when restimulated by the specific antigen (anti-CD3 mAbs) *in vitro*.

On day 7, no significant apoptosis was detected in the fresh axillary lymph node cells from oil- or TCDD-treated mice (Figure 18A). In contrast, a small, but significantly increased percentage of apoptotic cells (11.5%) was detected in the popliteal lymph node cells from TCDD-exposed mice (Figure 18D), although the intensity of fluorescence of this population was very low.

Also on day 7, TCDD-exposed axillary LN cells exhibited a decrease in apoptosis when

compared to the controls, when cultured for 24 hours with medium (Figure 18B). Also as seen in the previous timepoints studied, on day 7, a significantly increased amount (23.5%) of apoptosis was detected in popliteal lymph node cells from TCDD-treated mice when compared to the cells from the vehicle controls (Figure 18E).

When cultured with anti-CD3 antibodies, axillary lymph node cells from dioxin-exposed mice collected on day 7 showed no significant change in the amount of apoptosis (Figure 18C). In direct contrast, on day 7, popliteal (activated) lymph node cells from TCDD-treated mice displayed significantly more apoptotic cells (32%) than the control upon *in vitro* restimulation with anti-CD3 mAbs (Figure 18F). The increased amount of apoptotic cells in the popliteal lymph node cells from TCDD-treated animals corresponds directly to the decreased proliferative ability upon restimulation with anti-CD3 mAbs (Figure 6B), and strongly suggests that the proliferative decrease seen in TCDD-treated popliteal LN cells is due to increased apoptosis.

Insignificant amounts of apoptosis were detected in fresh axillary (Figure 19A) and popliteal (Figure 19D) lymph node cells two weeks after vehicle/TCDD treatment. After 24 hours culture in RPMI, the axillary (resting) lymph node cells from TCDD-treated animals showed a slight (8.2%), but insignificant, increase in apoptotic cells (Figure 19B). The popliteal lymph node cells from TCDD-exposed mice exhibited a significant increase (16.1%) in apoptotic events (Figure 19E). Though there was a significant apoptotic increase in the activated (popliteal LN) cells, the great majority of the cells from TCDD-treated animals were found to be as viable as the oil-treated control (Figure 19E), as indicated by the high number of cells from the experimental group exhibiting low levels of fluorescence.

After 24 hours stimulation with anti-CD3 antibodies in culture, resting cells from dioxin-exposed animals collected two weeks later showed a profound (32.8%) increase in the number of apoptotic events over the oil-treated control (Figure 19C). This result was unexpected, as the axillary lymph node cells from the experimental group showed equivalent proliferative responses

when compared to the oil-treated control at the two week timepoint (Figure 8A). If axillary lymph node cells from dioxin-treated mice are still proliferating at a higher rate than resting cells from the oil-control, then the loss of cells through apoptosis may serve to neutralize the proliferative difference. Alternatively, the apoptosis seen following anti-CD3 stimulation may not correlate with the proliferative responses because it measures the end result after 24 hour culture. For this reason, cells cultured with medium alone may more accurately predict how such cells would respond to further stimulation. In this context, it should be noted that axillary LN cells from TCDD-treated mice cultured with medium alone showed similar levels of apoptosis to the control (Figure 19B).

In contrast, at two weeks, the popliteal lymph node cells from TCDD-treated mice showed no significant increase in apoptosis after 24 hours of *in vitro* restimulation with anti-CD3 mAbs (Figure 19F). These data together corroborate our hypothesis that the antigen-specific proliferative decrease observed in activated cells from dioxin-exposed mice is due to induction of apoptosis. Increases in apoptosis were seen at earlier timepoints (5 and 7 day), corresponding to decreased proliferative abilities observed in the proliferation assays. Also at two weeks, neither proliferative decreases nor significant amounts of apoptosis were detected in activated cells from TCDD-treated mice.

3.4 Phenotypic Analysis of the Apoptotic Cell Populations

The results in Section 3.3 suggested that activated (popliteal LN) cells from TCDD-treated animals underwent significantly more apoptosis *in vitro* than the control. In contrast, resting (axillary LN) cells from dioxin-treated mice were more viable than the control *in vitro*. These data correlated with our findings that activated, but not naive, LN cells from TCDD-treated mice

demonstrated decreased proliferative responses to anti-CD3 restimulation *in vitro*. However, because LN cells consist of a mixture of T and B lymphocytes, as well as macrophages, it was not clear from our apoptosis studies whether the activated cells from TCDD-treated mice were undergoing increased apoptosis were really T cells.

To determine this, we used a double-staining technique. One week after vehicle/TCDD-treatment, axillary and popliteal lymph nodes were harvested. As in Section 3.3, the cells were stained immediately after harvesting and after 24 hours culture with either medium or anti-CD3 antibodies. The cells were first stained with PE-conjugated anti-CD3, followed by FITC-dUTP, using the TUNEL technique. In this way, we could determine if the population of apoptotic cells (staining positive with the FITC) was also $CD3^+$ (staining positive with the PE). Figures 20 and 21 show dual parameter histograms in which quadrant 1 represents $CD3^+$ non-apoptotic cells, quadrant 2, $CD3^+$ apoptotic cells, quadrant 3, $CD3^-$ non-apoptotic cells, and quadrant 4 depicts $CD3^-$ apoptotic cells. The quadrants (1-4) are labeled, and the percentages of cells appearing in each quadrant are indicated either above or below each quadrant.

As seen previously in Section 3.3, very little apoptosis was detected in fresh axillary lymph node samples (Figure 20C). No significant differences between the freshly stained control (4.2%) and TCDD-treated (5.7%) groups were observed in the numbers of CD3+ cells undergoing apoptosis (Figures 20 A and B). After 24 hours culture in RPMI, resting lymph node cells from TCDD-treated animals showed a significant decrease in the amount of apoptosis, as indicated by a curve shift to the left (Figure 20F). This was seen previously in Figure 18B. There was a significant decrease in the number of CD3+ cells undergoing apoptosis in the TCDD-exposed group (55.1% *vs*. 66.4%) (Figures 20 D *vs*. E).

After 24 hours culture with anti-CD3 antibodies, axillary lymph node cells from dioxin-treated animals showed a slight, but insignificant, increase (4.9%), over the control, in the amount of apoptosis (Figure 20I). The number of CD3+ cells undergoing apoptosis in resting cells from TCDD-treated animals remained similar (55.1% for the control group *vs*. 54.1% in the experimental group) (Figures 20 G and H). There was also an insignificant increase in the number

of $CD3^-$ cells undergoing apoptosis in the experimental group (0.5% *vs*. 1.4%) (Figures 20 G and H).

Freshly stained popliteal lymph node cells from TCDD-treated mice showed a slight (6.9%) increase, over the control, in the amount of apoptosis (Figure 21C). There was a slight, but insignificant increase in the number of $CD3^+$ cells undergoing apoptosis in the freshly stained, experimental group samples (3.9% *vs*. 1.9%) (Figure 21 B and A). After 24 hours culture with RPMI, there was, as seen previously, a significant increase in the amount of apoptosis in popliteal lymph node cells from TCDD-treated animals (Figure 21F). There was also a significant increase in the number of CD3+ cells undergoing apoptosis in the popliteal lymph node samples from TCDD-treated mice (28.9% for the control *vs*. 34.6% for the TCDD-treated group) (Figures 21 D and E).

After 24 hours restimulation with anti-CD3 antibodies, there was a significantly increased amount, over the control, of apoptosis (22.9%) in the popliteal lymph node cells of TCDD-treated animals (Figure 21I). This corresponded to a significant increase in the number of CD3+ cells undergoing apoptosis in activated cells from TCDD-treated animals (11.6% in the control *vs*. 28.4% for the experimental group) (Figures 21 G and H). These results indicated that TCDD induces apoptosis preferentially upon the $CD3^+$ lymph node cells. Since the popliteal lymph node cells were activated *in vivo* with anti-CD3 antibodies, the $CD3^+$ cells were activated. Therefore, these results suggested that TCDD induces apoptosis in only the activated cells at one week. These results also help to explain the proliferative decrease seen in Figure 6B.

3.5 Flow Cytometric Analysis of Cell Surface Markers

The results of proliferation assays with resting cells from oil- and TCDD-treated animals suggested that TCDD was exerting its effects through an activation mechanism. Because of this,

we tested the hypothesis that TCDD affects the expression of cell surface receptors and activation markers. Current studies in our lab (Kamath *et al.,* unpublished communication) have shown that TCDD affects the expression of surface activation markers on thymocytes. Because Fas plays an integral role in triggering apoptosis, and because the results from Section 3.3 indicated that activated cells from dioxin-exposed mice are more susceptible to apoptosis, we studied the expression of this molecule on axillary and popliteal lymph nodes. In addition to examining the expression of Fas, we also looked at the expression of CD3, αβ−TCR, IL-2R, CD4, CD8, CD44, and CD45R. The expression of these markers was investigated to determine if TCDD had other immunotoxic effects beyond the increased apoptosis (Section 3.3) and to characterize the phenotypes of resting and activated lymph node cells from TCDD-treated animals.

Flow cytometric analysis of cell surface markers was done at days 3, 5, and 7 following TCDD-treatment and antigen immunization. These timepoints were chosen to coincide with data from the proliferation assays. Both the percentage of cells staining positive and mean fluorescent intensity were analyzed for each group. The percentage of cells staining positive gives an indication of the proportion of cells expressing a specific marker within the population, while the mean fluorescent intensity (MFI) is a descriptor of the density of cell marker expression on each cell. The mean fluorescent intensity in TCDD-treated groups was compared to the vehicle-treated groups by considering the control expression being taken as 100%. Thus, values less than 100% represent decreased density of cell marker expression on TCDD-treated cells when compared to the controls, while values greater than 100% represent increased density of expression.

On day 3 post-treatment, a comparison of axillary lymph node cells from oil- and TCDD-treated mice showed a significant increase ($p=0.02$) in the percentage of cells expressing Fas after TCDD treatment (Table 1). The expression of other cell markers remained similar to the control. No significant changes were seen in the density of expression of any of the markers studied (Table 1).

Also on day 3, there was a significant increase in the proportion of cells expressing CD45R

in popliteal lymph node cells from dioxin-exposed mice when compared to cells from oil-treated mice ($p=0.05$) (Table 2), but no significant differences were observed in the expression of any other molecules. While there were no differences in the percentages of cells expressing CD3 and the $\alpha\beta$−TCR, the density of these markers was decreased on activated popliteal LN cells from TCDD-treated animals ($p=0.025$) (Table 2).

These data suggest that the axillary lymph node cells from TCDD-exposed mice are more activated than the corresponding resting cells from the oil-treated control. This was demonstrated by the increased percentage of axillary lymph node cells from TCDD-dosed mice expressing CD45R, a tyrosine kinase costimulatory molecule. Also, Fas is upregulated following T cell activation (Roitt *et al.,* 1996). Thus, the increased expression of Fas on axillary LN cells from TCDD-treated mice suggested that these cells were activated *in vivo*. Similarly, the popliteal lymph node cells from the TCDD-exposed group were more activated than the corresponding control as seen from the increased expression of CD45R (Table 2). The decreased density of CD3 and the T cell receptor on activated cells from TCDD-treated mice (Table 2) may constitute one possible reason why the popliteal LN cells respond poorly to *in vitro* stimulation with anti-CD3 mAbs, as seen in Figure 3.

Table 1. ***Percentage of axillary lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 3 after vehicle/TCDD treatment and rear footpad immunization.***

Cell Marker	Oil Axillary LN[1]	TCDD Axillary LN[1]	Percent change in Mean Fluorescent Intensity for TCDD axillary LN[2]
CD3	73.7±12.5	81±13.9	112±4.8
αβ–TCR	75.3±17.4	80.6±13.3	89.7±15.9
CD4	34.8±19	30.7±9	105±9.3
CD8	36.5±7	41.3±5.9	114.9±34.4
IL-2R	19.55±2.5	21.85±5.5	103.5±12.5
CD44	75.9±5.7	75.3±7.9	99.9±15.1
CD45R	39.7±12.7	44.45±18.4	91±2.4
Fas	32.9±11.9	51.3±10.9*	95.8±1.6

[1] Three days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, axillary lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for axillary LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Significant differences are denoted with an asterisk. (n=3, * p=0.02)

Table 2. ***Percentage of popliteal lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 3 after vehicle/TCDD treatment and footpad immunization.***

Cell Marker	Oil Popliteal LN[1]	TCDD Popliteal LN[1]	Percent change in Mean Fluorescent Intensity for TCDD popliteal LN[2]
CD3	77.8±13.6	74.3±17.7	78.5±8.5**
αβ–TCR	78.9±9.7	72.2±11.7	86.9±4.9**
CD4	29.1±14.5	30.2±15.9	90±7.1
CD8	38.1±7.3	35.8±8.7	83.9±21.2
IL-2R	52.6±22.5	58.4±18.5	87.8±5.8
CD44	86.2±6.3	84.2±7.6	112.6±32.3
CD45R	48.8±7.4	78.9±17.2*	105.3±19.4
Fas	57.8±6.9	52.6±0.05	99.9±15.2

[1] Three days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, popliteal lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for popliteal LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Significant differences are denoted with an asterisk. (n=3, * p=0.05, ** p=0.025)

At day 5, there was a significant increase in the percentage of resting axillary LN cells from dioxin-dosed mice expressing CD45R when compared to axillary lymph node cells from oil-treated mice ($p=0.01$) (Table 3). However, the density of CD45R expression was decreased ($p=0.0005$). In contrast, the density of expression of Fas was increased ($p=0.0005$) over the control. The expression and density of all other cell markers studied remained similar to control levels (Table 3).

Popliteal lymph node cells from TCDD-treated animals, when compared to activated cells from oil-treated animals, showed a significant decrease in the expression of the T cell receptor at the 5 day timepoint ($p=0.02$), as well as decreased expression of CD3 ($p=0.025$) (Table 4). Additionally, the density of CD3 was decreased on the activated cells ($p=0.0025$). The expression and density of all other cell markers remained similar to the oil-treated control levels (Table 4).

The markers expressed on the cell surface change during the time of activation. The times of expression of various cell markers have not been well characterized, but it is known that some markers are preferentially expressed on activated cells. Thus, it is not appropriate to compare the expression of cell markers at different timepoints during the activation process, as during different stages of activation, the expression of markers changes. We can use these data, however, to determine whether the cells from TCDD-treated animals are differing in activation state from the oil-treated control at various timepoints.

These data are in good agreement with the hypothesis that TCDD acts as an activation signal. The axillary lymph node cells from dioxin-treated mice appear to be more activated than the control, with increased numbers of cells expressing CD45R. Interestingly, however, the density of CD45R expression is decreased in axillary lymph node cells from TCDD-exposed mice. This may indicate that resting cells from TCDD-treated animals are more activated and that they apparently require less costimulatory help to be able to respond to an antigen. The decreased density of CD45R would decrease the potential amount of costimulation through that receptor. Despite the decreased density of CD45R expression, axillary lymph node cells from TCDD-treated

animals are able to respond similarly, or at increased rates, to *in vitro* stimulants. The density of Fas expression is increased in the experimental group, indicating a higher activation state.

The number of cells expressing CD3 and TCR are decreased in activated (popliteal LN) cells from TCDD-treated mice, indicating either toxicity to the T cells or that the immune response is being down-regulated. A down-regulated immune response is supported by the decreased density of CD3 expression on popliteal LN cells from TCDD-treated mice. The possibility of TCDD-induced T cell toxicity in the activated cell population was addressed at the one-week timepoint because the largest amount of apoptosis was detected at this timepoint (Figure 18 D, E and F).

Table 3. ***Percentage of axillary lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 5 after vehicle/TCDD treatment and footpad immunization.***

Cell Marker	Oil Axillary LN[1]	TCDD Axillary LN[1]	Percent change in Mean Fluorescent Intensity for TCDD axillary LN[2]
CD3	90.4±0.4	90.3±1.6	80.9±11.4
αβ–TCR	85.8±1.2	89.8±2.9	104.3±8.3
CD4	41.3±2.2	35.6±5	97.3±1.4
CD8	46.8±2.1	50.8±1.4	83±14
IL-2R	12.6±7.3	8.6±5.9	88.3±16.9
CD44	82.3±2.9	85.5±0.9	78.9±14.9
CD45R	32.8±3.8	48.2±4.7*	40.8±2.8**
Fas	31.8±20.4	26.4±7	129.3±1.3**

[1] Five days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, axillary lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for axillary LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Significant differences are denoted with an asterisk. (n=3, * p=0.01, ** p=0.0005)

Table 4. ***Percentage of popliteal lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 5 after vehicle/TCDD treatment and footpad immunization.***

Cell Marker	Oil Popliteal LN[1]	TCDD Popliteal LN[1]	Percent change in Mean Fluorescent Intensity for TCDD popliteal LN[2]
CD3	73.7±14	59.1±12.9*	60±4*
αβ–TCR	70.2±1.8	64.2±0.6**	103±10
CD4	20.3±5.4	20.9±4.1	99.9±4.6
CD8	32.9±7.9	34.1±6.1	109.6±24.9
IL-2R	18±5.8	12.45±5.35	110.5±7.5
CD44	87.5±1.6	82.6±5.3	108.1±23.2
CD45R	58.8±11.6	48.7±7.5	110.5±22.5
Fas	41.4±8.1	33.6±13.5	98.3±29.1

[1] Five days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, popliteal lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for popliteal LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Significant differences are denoted with an asterisk. (n=3, * p=0.025, ** p=0.02)

One week after treatment, there were no significant changes in the expression or density of any of the cell markers on resting lymph node cells from TCDD-treated animals (Table 5). This indicated that resting cells from TCDD-treated animals return to a normal phenotype at one week, although they showed an increased proliferative responsiveness to IL-2 at this timepoint (Figure 5A).

When compared to activated cells from oil-treated mice, activated cells from TCDD-treated animals tested at one week showed a significantly increased density of Fas expression ($p=0.02$) (Table 6). The expression and density of all other cell markers studied remained similar to control levels (Table 6). The finding that the density of Fas was increased correlated well with the results obtained in the studies of DNA fragmentation (Figure 18 D, E, and F). Because Fas is intimately involved in triggering apoptosis, an increased density of Fas expression would be expected to result in increased apoptosis.

Table 5. ***Percentage of axillary lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 7 after vehicle/TCDD treatment and rear footpad immunization.***

Cell Marker	Oil Axillary LN[1]	TCDD Axillary LN[1]	Percent change in Mean Fluorescent Intensity of TCDD axillary LN[2]
CD3	73.1±11.3	82.8±10.7	92.6±2.3
αβ–TCR	75.1±17.7	64±6.9	94.2±10.1
CD4	25.3±7	14.4±4.1	125.3±23
CD8	37.5±6.9	28.6±6.6	96.7±8.2
IL-2R	18.3±9.5	12.3±4	91.5±17.4
CD44	57.4±8.1	51.5±14.7	86.8±14.4
CD45R	15.7±2.5	18.8±5.4	101.5±28.3
Fas	31.5±13.7	39.6±13.2	138.5±43.7

[1] Seven days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, axillary lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for axillary LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Table 6. ***Percentage of popliteal lymph node cells staining positive and mean fluorescent intensity for various cell markers on day 7 after vehicle/TCDD treatment and footpad immunization.***

Cell Marker	Oil Popliteal LN[1]	TCDD Popliteal LN[1]	Percent change in Mean Fluorescent Intensity of TCDD popliteal LN[2]
CD3	59.4±5	53.6±7.1	93±31.7
αβ–**TCR**	59.4±12.3	62.8±13.1	88.4±15.9
CD4	23.4±8.4	20.3±6.7	75.4±16.3
CD8	33.1±4.2	35.4±3.4	104.8±10.3
IL-2R	23.1±4.8	27.8±10.9	103.5±4.7
CD44	90.1±1.1	90.1±3.5	98.5±11.5
CD45R	36.5±13.4	37.8±3.6	84.9±17.2
Fas	36.1±20	47.4±13.9	150.8±12.9*

[1] Seven days after oil/TCDD treatment and rear footpad immunization with anti-CD3 mAbs, popliteal lymph nodes were removed. The cells were stained for various cell surface markers and expression was analyzed flow cytometrically. Percentages of cells staining positive ± SD and MFI ± SD are presented.

[2] The percent change in mean fluorescent intensity (MFI) for popliteal LN cells from TCDD-treated mice was calculated by considering the MFI for control cells as 100%.

$$\text{MFI for TCDD-treated cells} = \frac{\text{(Mean channel number for TCDD-exposed cells)}}{\text{(Mean channel number for vehicle-control cells)}} \times 100$$

Significant differences are denoted with an asterisk. (n=3, * p=0.02)

Chapter 4 Discussion of Significance

We have demonstrated in this study that TCDD affects resting and activated T cells differently, even within the same animal. This is the first report that TCDD exerts differential effects on separate lymph node cell populations, based solely upon the activation status of the cells. Early activated cells, taken from the animals three days after TCDD-treatment, exhibit an increased proliferative response to culture with IL-2, as described previously by Neumann *et al.*(1993). At this early timepoint, no effects were seen in the resting cell population.

Upon restimulation, activated cells from TCDD-treated mice exhibited a significantly decreased proliferative response. As described in previous studies, this decreased proliferative response was antigen specific (Lundberg *et al.*, 1992; Rhile *et al.*, 1996). The antigen specificity of this response indicates that TCDD exerts these effects on only the cells undergoing an immune response to the particular antigen. No proliferative effects were detected in the resting cell population at this early timepoint. Later timepoints were examined to determine if TCDD exerted its effects through the memory cells.

One week after TCDD treatment, the activated cells showed a greater than 50% proliferative decrease upon *in vitro* restimulation with the specific antigen. In contrast to the earlier timepoint, however, activated cells from TCDD-treated mice showed a decreased responsiveness to IL-2 as late as one week post-treatment. Furthermore, the resting cells from TCDD-treated animals showed enhanced responsiveness to IL-2 without differing in responsiveness to culture with the antigen. These results clearly showed that TCDD does have effects on resting and naive cells, and furthermore, that TCDD exerts different effects on resting and activated cells, even within the same animal. The immunomodulatory effects of TCDD on the lymph node cells lasted one week after TCDD treatment, but were undetectable two weeks after treatment. The reversibility of this

response is important; it indicated that TCDD does not affect memory cells, and must exert its effects on the effector cells.

These results suggested that there was a change in the activation status of the cells. Resting cells from TCDD-treated animals appeared to be more activated than the control, as demonstrated by the increased responsiveness to IL-2 at one week. At the same time, activated cells from TCDD-treated animals seemed to differ widely in activation state during the timepoints from three days to one week after treatment. From the proliferation assays alone, though, a clear discernment of the activation states could not be made. For a clear characterization of the activation states, it was necessary to examine other parameters of activation, such as the expression of surface markers and rates of apoptosis.

The antigen-specific decrease appears to be mediated through apoptosis, as activated cells from TCDD-treated animals exhibited higher levels of DNA fragmentation than the control. The TUNEL technique was employed as a sensitive and accurate method for early DNA fragmentation, as shown previously by Kamath *et al.*(1997). Cells were stained directly after recovery and after culture to simulate the conditions of the proliferation assays and to eliminate the issue of rapid phagocytic clearance of apoptotic bodies (Kamath *et al.,* 1997).

Increased apoptosis of activated cells from TCDD-treated animals occurred *in vitro,* corresponding directly to the decreased proliferative abilities observed *in vitro* (Figures 16E, 17 E and F, 18 D, E, and F). The increased apoptosis of activated cells from TCDD-treated animals was not limited to *in vitro* studies, however. At one week, DNA fragmentation was detectable in freshly recovered lymph node samples, indicating *in vivo* apoptosis (Figure 18D). The timepoints for the increased number of apoptotic cells correspond to the timepoints of proliferative decrease in the activated cells. This is consistent with previous studies that suggest TCDD induces apoptosis in rapidly dividing cells, such as thymocytes (Kamath *et al.,* 1997; McConkey *et al.,* 1988; McConkey *et al.,* 1994). Additionally, our analysis of the apoptotic cell population in activated cells indicated that the $CD3^+$ cells were more likely to be apoptotic in activated cell samples from TCDD-treated animals. Together, these results suggest that the immunotoxicity of dioxin on

activated T cells may be mediated through its direct ability to induce a specific apoptosis of the activated effector cells.

In contrast, resting cells from dioxin-treated animals exhibited significantly lower levels of DNA fragmentation than the control at early timepoints (Figures 16 A, B, C, 17 A, B, C, and 18 A and B). In fact, it appeared the resting cells from the TCDD-treated group were exhibiting DNA fragmentation levels similar to the activated cells from the control group (Figures 16 A and D, B and E, C and F, 17 A and D, C and F, 18 A and D, and C and F). At the two-week timepoint, however, resting cells from TCDD-treated animals exhibited significantly higher levels of DNA fragmentation in response to *in vitro* stimulation with anti-CD3 antibodies (Figure 19C). Though there was an increase in apoptosis, there was no decrease in proliferative ability. If the resting cells were still proliferating at a higher rate than the control, increased apoptosis could bring the functional responsiveness back to normal levels.

The exact mechanism by which antigen-primed T cells from TCDD-treated mice undergo increased apoptosis *in vitro* is still unclear. It is possible that TCDD may activate naive T cells in such a way that these cells, upon further stimulation through the T cell receptor *in vivo*, as accomplished by using anti-CD3 mAbs, may undergo apoptosis as described in other systems, termed propriocidal regulation (Lenardo, 1991; Critchfield, 1994). In support of this, earlier studies have demonstrated that TCDD increases the activity of tyrosine kinases (Clark *et al*., 1991; Bombick and Matsumura, 1987).

Furthermore, TCDD evokes a sustained increase in cytosolic free Ca^{2+} concentration leading to possible activation of endonucleases and DNA fragmentation (McConkey *et al*., 1988; Canga *et al*., 1988; Al-Bayati *et al*., 1991). Alternatively, TCDD is known to be a potent transcriptional regulator of several genes in a variety of tissues, including cytochrome P450 1A1, transforming growth factor (TGF)-α, nuclear estrogen receptor, interleukin-1β, and plasminogen activator inhibitor-2 genes (Poland and Knutsen, 1982; Choi *et al*., 1991; Sutter *et al*., 1991). Also, TCDD is known to enhance tumor necrosis factor (TNF) production (Clark *et al*., 1991). Inasmuch as several cytokines such as TGF-β and TNF are actively involved in the induction of

apoptosis (Hernandez-Caselles and Stutman, 1993; Rotello *et al*., 1991), it is possible that increased production of such cytokines by activated T cells from TCDD-treated mice could result in their apoptosis.

Lastly, TCDD may upregulate the expression of Fas or Fas ligand (FasL). It should be noted that FasL is a cytokine belonging to the TNF family (Nagata, 1997). In the current study, we observed that the density of expression of Fas on activated T cells increased on day 7 following TCDD-administration (data not shown). Also, earlier studies from our lab demonstrated that Fas-deficient mice were more resistant to TCDD-induced thymic atrophy and peripheral T cell dysfunction (Rhile *et al*., 1996). Thus, TCDD may differentially regulate the expression of Fas or FasL in naive and activated T cells, which may account for altered induction of apoptosis.

Recently it was demonstrated that TCDD suppressed the apoptosis induced in rat hepatocytes by UV light (Worner, 1996). These studies are consistent with our data that TCDD prevented the naive T cells from undergoing apoptosis. The differential effects of TCDD is of critical importance and suggests how in naive cells by suppressing apoptosis, TCDD may facilitate tumor promotion, whereas in activated cells, by enhancing apoptosis, it may cause toxicity.

From this study, it appears that TCDD is able to exert its effects by sending an activation signal to the cell. This hypothesis is further supported by a phenotypic analysis of surface marker expression. At early timepoints (3 and 5 days post-treatment), activated cells from TCDD-treated animals showed increased expression of CD45R, an activation marker, while at the same time down-regulating the expression of CD3 and the TCR. These phenotypic changes are indicative of increased activation and down-regulation of the immune response, changes not normally observed until later stages of cell activation. Similarly, resting lymph node cells from TCDD-treated mice expressed significantly higher levels of some activation markers (CD45R, Fas) at the 3 and 5 day timepoints than the corresponding control.

This activation signal may come through increased expression of growth factors, as proposed by Abbot *et al*.(1994). It is known that the TCDD-Ah receptor complex binds to dioxin responsive elements (DREs) on the DNA (Vecchi *et al.,* 1983). There are several genes that are

known to have DREs (Lusska *et al.*, 1993), and many other genes, such as those controlling the cell cycle and the production of growth factors, that have been postulated to be regulated by a DRE. The increased activation of resting and activated cells may be brought about by TCDD-induced dysregulation of the cell cycle and of cytokine expression.

Resting cells from TCDD-treated mice showed increased responsiveness to antigens while activated cells showed decreased responsiveness *in vitro*. Both the resting and the activated TCDD-treated cells regained normal functionality by two weeks, but DNA fragmentation analysis suggests that TCDD may exert longer-term effects on the resting immune cells.

Since TCDD is a ubiquitous environmental contaminant, the long-term effects deserve consideration. Increased responsiveness to antigens is not always beneficial; TCDD may be found to be an implicating factor in hyperactive immune reactions, such as allergies and autoimmune diseases. Taylor *et al.*(1992) showed that TCDD induces much of its toxicity through TNF-α, and studies by Connor *et al.*(1994) indicated that the increased dermal expression of TNF-α in mice with chloracne is likely due to immune cells in the skin, though this explanation was not fully tested. It is possible that the resting cells in the TCDD-treated animals are being stimulated to produce TNF-α, but demonstration would require further experiments. Since, at any one time, the majority of the cells in the body are resting, increased expression of TNF-α by these cells could be enough to cause the cachexia associated with TCDD toxicity. Although the functionality of resting cells from TCDD-treated animals returns to normal within two weeks in our *in vitro* studies, this may not be true of an *in vivo* response. Additionally, environmental exposure to TCDD is continuous, not a single dose exposure, as in the current study. Thus, it is possible that the enhanced responsiveness of resting cells from dioxin-treated mice to IL-2 is continuous *in vivo*.

Although the activated cells from TCDD-treated animals return to control levels in the functional and phenotypic assays, this may not be indicative of an *in vivo* immune response. It appears that TCDD does not affect memory cells, which means that a successful immune response can be mounted after the initial challenge (Figure 7B). If the antigenic challenge were ongoing,

though, as in the case of chronic infections, there would be a continuous population of actively proliferating cells. If the *in vivo* effect of TCDD is similar to what we have observed in the *in vitro* studies, a chronic infection may be able to overwhelm the individual, resulting, possibly, in death. Therefore, further studies should be done to determine the extent that this *in vitro* data is applicable to the *in vivo* situation, especially with regard to the responsiveness to recall antigens.

Also, the involvement of other cell markers and cytokine expression in the generation of TCDD-induced immunotoxicity should be more fully explored before making the bold statement that TCDD has no long-term effects on the peripheral immune system.

Significance of the Current Study

In the current study, we have made a novel and original finding that TCDD inhibits the proliferative responsiveness of previously activated T cells to rechallenge with the specific antigen through induction of apoptosis. These data suggested that when a TCDD-exposed host is simultaneously exposed to an infection or tumorigenesis, there may be a decreased immune responsiveness and therefore, increased susceptibility to infections and cancer. In addition, our studies also suggested that TCDD does not affect the proliferative responsiveness of naive, resting T cells. This is an important finding which suggests that TCDD does not non-specifically suppress the functions of all T cells. However, it should be noted that TCDD does cause significant phenotypic alterations in naive T cells, and that it possibly activates resting T cells in such a way that they resist undergoing apoptosis. Increased resistance to apoptosis has been previously shown to be associated with the development of malignancies. Thus, TCDD may use such a mechanism to promote tumorigenesis of naive, resting cells.

Chapter 5 References

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Appendix A Flow Cytometry and Analysis of Flow Data

A flow cytometer uses a laser to detect the fluorescence of different cell populations. Flow cytometry can be used to sort cells based on their fluorescent properties and to study the expression of cell surface molecules. The presence of surface molecules can be detected by staining the cells with color-tagged monoclonal antibodies against the marker of interest. Commonly used color tags include fluorescein isothiocyanate (FITC) and phycoerythrin (PE). In order to determine the amount of expression of the particular cell surface marker, however, a control is used to account for the autofluorescence of the cells. The fluorescence obtained from the unstained cells (autofluorescing) is subtracted from the fluorescence obtained with the stained cells to get the percentage of cells that stain positively for the cell marker. In Appendix Figure 1, the autofluorescing cells are shown as the solid line, and the stained cells are shown as the dotted line. From such an overlay, the percentage of cells staining positively can be calculated.

In addition to determining the percentage of cells staining positive, the mean fluorescent intensity of the cell populations can be determined. Mean fluorescent intensity (MFI) is a reflection of the density of surface markers present on any one cell. This parameter gives us an indication of whether the expression of a cell surface marker is up- or down-regulated on the cells.

In the current study, we have been interested in understanding how lymph node cells from TCDD-treated animals are phenotypically different from lymph node cells from oil-treated animals. In order to compare these results, we have overlayed the positive responses (cells stained with fluorescently tagged Abs) of the experimental group over the positive responses from the control group. Differences between lymph node cells from TCDD- and oil-treated animals are detected as shifts in the fluorescence. With this type of overlay, the increase in percentage of cells staining positive over the control can be calculated. In Figures 16, 17, 18, 19, 20 C, F, and I, and 21 C, F, and I, this type of overlay is used. The results are considered significant when a clear shift in the fluorescent peaks is detected.

Appendix B Flow Cytometric Analysis of Double-Stained Samples

Double-staining is done to more fully characterize the phenotypes of cell populations. In a double-staining procedure, two differently color-tagged monoclonal antibodies are used to tag the markers of interest. In this study, we have used phycoerythrin (PE)-conjugated antibodies against CD3 and FITC-conjugated dUTP. PE-tagged cells fluoresce red, and FITC-labeled cells fluoresce green. The flow cytometer is able to detect both colors at once, and to determine which cells possess both color markers. The resulting printout is organized into four quadrants, as shown in Appendix Figure 2.

Double-positive cells appear in the second quadrant. Cells staining positive for only the PE-conjugated marker appear in the first quadrant. Cells staining positively for only the FITC-conjugated marker appear in the fourth quadrant. Cells that do not stain positively for either cell marker appear in the third quadrant.

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Publications

Pryputniewicz, S.J., Nagarkatti, M., and Nagarkatti, P.S. **Differential Induction of Apoptosis in Activated and Naive T Cells by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and its Repercussion on T Cell Responsiveness.** Submitted for publication to*Toxicology and Applied Pharmacology*, November 1997.