

Keynote Address: TCDD: An Environmental Immunotoxicant Reveals a Novel Pathway of Immunoregulation - A 30-Year Odyssey.

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### **Abstract**

I was honored to be the Keynote Speaker at the 30<sup>th</sup> Annual STP Symposium “Toxicologic Pathology and the Immune System”. I had the opportunity to reminisce about events in the 1970’s that set the stage for the birth and subsequent growth of the field of immunotoxicology, and to summarize my research career that has spanned the past forty years as well. An initial focus on the immunotoxicity of pentachlorophenol (PCP) led my laboratory into the aryl hydrocarbon receptor (AHR) field, and the study of its most potent ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). My research career has been devoted to trying to elucidate the immunological basis of TCDD’s profound immunosuppressive activity that is mediated by activation of AHR. In recent years my laboratory has focused on the role of CD4<sup>+</sup> T cells as targets of TCDD, and we were the first to describe the induction of AHR-dependent regulatory T cells (Tregs). The ability to induce Tregs using an exogenous AHR ligand to activate the AHR-Treg pathway represents a novel approach to the prevention and/or treatment of autoimmune disease. We are currently searching for such ligands.

Key words: immunotoxicology, 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD, aryl hydrocarbon receptor, AHR, CD4<sup>+</sup> T cells, regulatory T cells

## **Historical Perspectives on the Birth of Immunotoxicology**

The 30<sup>th</sup> anniversary of the STP Symposium was an opportunity to reminisce about events in the 1970's that set the stage for the birth and subsequent growth of the field of immunotoxicology. This was a special time for me personally as I began my graduate studies at Oregon State University in the fall of 1970. In 1971, funding for biomedical research achieved a new peak when President Richard Nixon declared his 'War on Cancer'. This 'War' effort benefited many fields of study beyond cancer research and invigorated the basic research community. At the same time, the field of immunology was burgeoning with new discoveries, such as distinct subsets of T and B lymphocytes and their communication via interleukins (aka cytokines). By coincidence, the realization of the adverse effects of cancer chemotherapeutic agents on bone marrow and immune function paved the way for toxicologists to enter the field of immunology as well.

The 1970's also witnessed the emergence of the environmental movement. Growth of 'environmentalism' was fueled by growing public concerns about the potential effects of pollution on human health and by the empowerment of several new government agencies to regulate the use of industrial chemicals and study their toxicity [e.g., the National Institute of Environmental Health Science (NIEHS) -1968; the Environmental Protection Agency (EPA) - 1970; the Toxic Substances Control Act (TSCA)-1976; and the National Toxicology Program (NTP)-1978]. In retrospect, it is not surprising that the field of immunotoxicology emerged during this era, bringing together the fields of immunology and toxicology to address the premise

that environmental pollutants could impact human health by adversely affecting the immune system.

One of the first publications in the nascent field of immunotoxicology was published in 1973 by J.G. Vos and colleagues (Vos et al., 1973) in a new NIEHS-sponsored journal, Environmental Health Perspectives. The paper, entitled “Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin on the Immune System of Laboratory Animals”, described the immunosuppressive effects of 4-8 weekly doses of TCDD on humoral and cell-mediated immunity in guinea pigs, mice and rats. The results of these studies revealed an unprecedented potency of TCDD to suppress the immune response. The cell-mediated immune response in guinea pigs was most sensitive, with suppression of the tuberculin reaction occurring at a total dose of only 0.32 µg/kg. In mice, a graft-versus host response was suppressed after 4 weekly TCDD doses of 5 µg/kg. In contrast, rats were more resistant to the immunosuppressive effects of TCDD but showed increased liver toxicity. The basis for the interspecies differences in immunotoxicity has still not been resolved.

The Vos paper was followed by numerous publications over the next decade describing the immunotoxic effects of TCDD and structurally-related chemicals such as polychlorinated biphenyls in laboratory animals. Several studies showed that exposure to TCDD decreased host resistance to infection, resulting in increased severity of symptoms and/or increased incidence of disease-induced mortality following challenge with a variety of pathogens. At the time, such changes in host resistance represented the most definitive evidence for biologically significant immunotoxicity. Furthermore, the immunosuppressive effects of TCDD were dose-dependent and occurred at doses that were not overtly toxic to the animal.

The overarching goal of many of these studies was to simulate human environmental exposure conditions and determine the lowest dose of TCDD that would cause effects on

different types of immune responses and/or alter resistance to disease. Such studies were aimed at providing data for human health risk assessments and regulatory decisions. However, the mechanism(s) underlying the remarkable potency of TCDD to suppress immune responses remained elusive.

### **Immunotoxicity of Pentachlorophenol**

My laboratory's contributions during the early years of immunotoxicology emanated from studies on the immunotoxicity of pentachlorophenol (PCP). PCP is a general biocide that acts by inhibiting oxidative phosphorylation. It was widely marketed as a wood preservative that was heavily used in Oregon in the wood products industry in the 1970's. PCP was sold as a technical product (T-PCP) of approximately 85% purity with ~15% dimeric impurities that formed as condensation products during production. These dimeric impurities were primarily hexa-, hepta- and octa-chlorinated dioxins, -furans and -diphenyl ethers. In 1982, we published our first papers that described the ability of T-PCP to suppress humoral and cell-mediated immune responses in mice and to increase the tumor burden of mice injected with Moloney sarcoma virus (Kerkvliet et al., 1982a; Kerkvliet et al., 1982b). Analytical grade (A-PCP) (>99% pure) did not produce immunosuppressive effects at comparable doses. Subsequently we showed that the dioxin/furan fraction, but not the diphenyl ether fraction, of T-PCP was responsible for the immunosuppressive activity (Kerkvliet et al., 1985). The rank order of immunosuppressive potency for the individual dioxin and furan congeners in T-PCP was consistent with the emerging theory of dioxin toxicity as a function of activation of the AHR.

## **The AHR and Dioxin Toxicity**

In 1973, Poland and Glover described a structure-activity relationship between different chlorinated dioxin and furan congeners and their ability to induce the activity of a xenobiotic metabolizing enzyme known as aryl hydrocarbon hydroxylase (AHH) (Poland and Glover, 1973). TCDD was the most potent enzyme inducer while the activity of other congeners varied with the position and degree of chlorination. In 1976 Poland's laboratory reported the discovery of a hepatic protein that bound TCDD with very high affinity (Poland and Glover, 1976). Twenty-three other chlorinated dioxin and furan congeners showed varying binding affinities for this hepatic cytosol-binding species that closely correlated with their potencies as inducers of hepatic AHH activity. The binding protein was named the 'aryl hydrocarbon receptor'.

After many years of study, we now know that the AHR is the ligand-binding member of a heterodimeric transcription factor that regulates gene expression. It is present in most cells of the body. Upon binding a fat-soluble, membrane-diffusible ligand such as TCDD, the cytoplasmic AHR translocates to the nucleus where it binds to another protein known as AHR Nuclear Translocator (ARNT; aka HIF1 $\beta$ ). The ligand-activated AHR-ARNT complex is capable of binding to specific sequences of DNA (-TNGCGTG-) known for many years as "dioxin response elements" (DREs), but now often referred to as XREs or AHR-REs. Once bound, the complex recruits appropriate transcriptional machinery to increase or decrease gene transcription. The resulting alterations in the proteome of the cell are thought to underlie the toxicity of TCDD. The broad spectrum of effects that are produced following exposure to TCDD may reflect the relatively common occurrence of DRE sequences throughout the genome.

TCDD is the most potent AhR ligand. Its potency relates to its high binding affinity as

well as its resistance to metabolism which confers a long biological half-life. Resistance to metabolism is a relatively unique feature of TCDD, making it an ideal ligand for studying the functionality of AHR in the absence of potentially confounding effects of active metabolites. Whether or not the biological effects of TCDD reflect the inherent role of the AHR remains to be proven. Likewise endogenous AHR ligands of functional significance remain to be confirmed. The promiscuous nature of the AHR ligand binding site has led to the identification of numerous endogenous compounds that bind and activate AHR transcription in vitro (e.g., prostaglandins, lipoxin A4, bilirubin, tryptophan metabolites) (Denison and Nagy, 2003). However, the AHR-dependent effects of such compounds in the intact animal are just beginning to be described. AHR knockout (KO) mice are relatively healthy as adults and mount normal immune responses to antigenic stimulation (Vorderstrasse et al., 2001). However, several recent studies report that AHR KO mice have hyper-inflammatory tendencies (Furumatsu et al., 2011; Sekine et al., 2009; Thatcher et al., 2007), consistent with the existence of endogenous AHR ligands that function to down-regulate inflammation and immune function.

### **Immunological Basis of TCDD's Immunotoxicity**

AHR KO mice are completely resistant to the immunosuppressive effects of TCDD, confirming the essential role of AHR activation in TCDD's immunotoxicity (Vorderstrasse et al., 2001). However, the cells that express AHR and the functions of those cells that are compromised by its activation during an adaptive immune response have intrigued many investigators for the past 20 years.

My laboratory joined the search for answers in 1990 with a publication demonstrating a role for the AHR in suppression of the cytotoxic T lymphocyte (CTL) response to allogeneic P815 tumor cells in

C57Bl/6 (B6) mice by TCDD and PCBs (Kerkvliet et al., 1990). This P815 tumor model became our model of choice for mechanistic studies because of the robustness of the alloimmune response that develops over a 10-12 day period. Using flow cytometry, an emerging technology at the time, we could track the differentiation of the allospecific CD8<sup>+</sup> CTL over time, and we could measure autocrine production of cytokines *ex vivo*. It was also important that the CTL response was dose-dependently suppressed by TCDD (2-20 µg/kg) given orally one day before P815 tumor injection (Kerkvliet et al., 1996). The flow cytometric approach allowed us to show that suppression of the CTL response by TCDD was due to a dose-dependent reduction in the number of CTL that developed as opposed to a reduction in the lytic activity of the CTL per se. Furthermore, the reduced number of CTL was preceded by a dose-dependent reduction in the number of CD8<sup>+</sup> T cells expressing an activated CTL precursor (CTLp) phenotype on day 7 (Oughton and Kerkvliet, 1999), suggesting that the activation of the CTLp was compromised by TCDD early in the response. An early effect was supported by data showing that TCDD was no longer suppressive if it was given after the first 3 days of the CTL response (Kerkvliet et al., 1996). These results further imply that, once activated, the allospecific CTLp are no longer sensitive to TCDD and are fully competent to terminally differentiate and clonally expand in the presence of TCDD.

Since the CD8<sup>+</sup> CTL response to P815 tumor cells is dependent on CD4<sup>+</sup> T cells (Kerkvliet et al., 1996), it was of interest to know when the CD4<sup>+</sup> T cells were required. Using an anti-CD4 antibody to deplete the cells, we found that the CTL response was suppressed only if the CD4<sup>+</sup> cells were depleted during the first 3 days of the response (Kerkvliet et al., 1996). Thus, the window in which CD4<sup>+</sup> T cells were required to help activate the CTLp was the same as the window of sensitivity to suppression by TCDD (Kerkvliet et al., 1996).

These results led us to hypothesize that TCDD was suppressing the activation of CD4<sup>+</sup> T helper cells to indirectly suppress the development of the CD8<sup>+</sup> CTL. Unfortunately, the population of allospecific CD4<sup>+</sup> T cells was too small to track in the P815 tumor model due to

lack of expression of Class II antigens on the P815 tumor cells. Thus, in order to directly study the response of allospecific CD4<sup>+</sup> T cells to TCDD, we switched model systems to a parent-into-F1 hybrid acute graft-versus-host (GVH) model in which donor T cells from B6 mice (the graft) are injected i.v. into B6 x DBA/2 F1 host mice. The F1 host mice do not recognize the B6 T cells as foreign but the B6 T cells become activated in response to DBA/2 antigens on F1 host cells and generate an anti-host CTL response similar to the CTL response to P815 tumor cells. However, because F1 host cells express both allo-Class I and allo-Class II antigens, a robust allospecific response occurs in both CD4<sup>+</sup> and CD8<sup>+</sup> donor T cell subsets which can be tracked by flow cytometry. This GVH model has the additional advantage of being very versatile via the choice of donor T cells that are injected, allowing different attributes of the donor T cells to be tested.

After validating that the CTL response generated in the GVH model was sensitive to suppression by TCDD, the first question that we wanted to answer was “Are CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells direct AHR-dependent targets for TCDD?” While we knew that TCDD's suppression of the alloCTL response to P815 tumor was dependent on AHR expression (Vorderstrasse et al., 2001), we did not know if T cells were direct targets. Using donor T cells from AHR KO mice and different combinations of donor CD4<sup>+</sup> and CD8<sup>+</sup> T cells from AHR-WT and AHR KO mice, the results of these studies clearly demonstrated the requirement for AHR expression in the donor T cells (Kerkvliet et al., 2002). When donor T cells were obtained from AHR KO mice, TCDD was unable to suppress the CTL response, while the response of WT cells was 90% suppressed. Furthermore, expression of AHR in CD4<sup>+</sup> T cells appeared to be more important than AHR expression in CD8<sup>+</sup> T cells, although AHR responsiveness of both subsets contributed to maximal suppression of the CTL response.

With the knowledge that CD4<sup>+</sup> T cells may represent the most critical target for TCDD in suppression of the CTL response, we set out to describe the changes in the activation of the donor CD4<sup>+</sup> T cells that were induced by TCDD during the early stages of the GVH response. First, we used carboxyfluorescein succinimidyl ester (CFSE)-labeled donor T cells to track donor cell division and found that the number of donor CD4<sup>+</sup> T cells increased in the spleen of the F1 host from day 1 to day 3 and that this expansion was due to cell proliferation. TCDD did not affect the number of donor CD4<sup>+</sup> T cells or their proliferation (Funatake et al., 2005). However, while numbers of donor CD4<sup>+</sup> T cells in the spleen were maintained on day 4 and 5 in vehicle-treated mice, they significantly declined in TCDD-treated mice. This pattern of normal expansion followed by premature decline in CD4<sup>+</sup> T cells in TCDD-treated mice has been reported in other models (Mitchell and Lawrence, 2003; Shepherd et al., 2000), and is reminiscent of the response of antigen-specific CD4<sup>+</sup> T cells to antigen in the absence of sufficient costimulation. These results suggested that TCDD might be compromising the ability of the CD4<sup>+</sup> T cells to respond to costimulatory signals from antigen presenting cells.

However, instead of suppressed activation of CD4<sup>+</sup> T cells, we were quite surprised to find that CD4<sup>+</sup> T cells in TCDD-treated mice express a super-activated phenotype. On day 2 in particular, a subpopulation of the dividing allospecific donor CD4<sup>+</sup> T cells in TCDD-treated mice expressed very high levels of CD25 and significantly greater down regulation of CD62L as compared to dividing allospecific cells from vehicle-treated mice (Funatake et al., 2005). The high level of expression of CD25 appeared to represent a functional high affinity IL-2 receptor, as incubation of the cells *ex vivo* with IL-2 resulted in a highly correlated increase in phosphorylated STAT5 expression (Funatake, 2006). This TCDD-induced change in activation phenotype was dependent on the AHR and was not induced by TCDD in AHR KO donor CD4<sup>+</sup>

cells (Funatake et al., 2005).

Fortuitously, these studies were ongoing at the same time that the discovery of CD4<sup>+</sup> T Tregs was beginning to dominate the immunology literature. CD4<sup>+</sup> Tregs were described as uniquely immunosuppressive cells that expressed high levels of CD25, along with other markers such as increased GITR and CTLA-4 (Sakaguchi et al., 2009). Later, the transcription factor, Foxp3, was identified as a lineage specific marker for some types of Tregs. When we examined markers of Tregs on donor CD4<sup>+</sup> T cells, we found that 60% of the CD25<sup>+</sup> cells in TCDD-treated mice also expressed GITR and CTLA-4 compared to only 30% of the CD25<sup>+</sup> cells in the vehicle-treated mice. The CD4<sup>+</sup> CD25<sup>+</sup> T cells from TCDD-treated mice also potently inhibited the proliferation of naïve, anti-CD3 activated T cells *in vitro*, a generally accepted assay of Treg function. Additional studies showed that these putative TCDD-induced Tregs did not express Foxp3 but produced elevated amounts of IL-10, an immunosuppressive cytokine associated with type 1 T regulatory (TR1) cells (Grazia Roncarolo et al., 2006). Interestingly, a recent study has shown that mouse and human T cells respond to TCDD *in vitro* by differentiation into TR1 cells as well (Apetoh et al., 2010; Gandhi et al., 2010).

### **TCDD Suppresses Development of Type-1 Diabetes**

We were intrigued by the concept that the uniquely potent, non-cytotoxic immunosuppressive effect of TCDD might be explained by the induction of Tregs, opening up the possibility that TCDD might prevent the development of autoimmune diseases known to be influenced by Tregs. In order to test this hypothesis, we chose to study the effects of TCDD in non-obese diabetic (NOD) mice that spontaneously develop Type-1 diabetes. The onset of diabetes has been linked to disruption of the balance between Tregs and pathogenic anti-beta

cell-specific CTL, with a decline in the number and function of Tregs preceding the onset of overt diabetes (Gregori et al., 2003; Pop et al., 2005).

The influence of TCDD on the development of diabetes was profound. At 22 weeks of age, when 72% of the control vehicle-treated NOD mice had developed diabetes, none of the mice treated with TCDD showed elevated blood sugar (Kerkvliet et al., 2009). Therefore, to maximize the study's value, we decided to stop treating half of the mice in the TCDD-treatment group. Within 8 weeks, 50% of the mice in this group developed diabetes while no diabetes was seen in the mice that continued to be treated with TCDD. Chronic TCDD treatment did not produce any overt toxicity and the mice appeared healthy at 31 weeks of age at study termination. Pancreatic histology revealed greatly attenuated inflammatory infiltrates in the TCDD-treated mice. At the same time, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in the pancreatic lymph nodes of TCDD-treated mice were significantly increased compared to surviving vehicle-treated mice, or mice that had been taken off of TCDD treatment. Interestingly, all of the CD25<sup>+</sup> T cells in the pancreatic lymph nodes also expressed Foxp3. Increased numbers of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells have also been found following TCDD treatment in mouse models of multiple sclerosis (EAE) (Quintana et al., 2008), autoimmune uveitis (EAU) (Zhang et al., 2010) and autoimmune colitis (Furumatsu et al., 2011; Takamura et al., 2010). These results contrast with the absence of Foxp3 expression in donor CD4<sup>+</sup> T cell-derived Tregs induced by TCDD during the GVH response. A Treg pathway that relies on activated AHR rather than Foxp3 is intriguing and deserving of further investigation.

### **Gene Expression in AHR-Tregs: Revelation of Mechanisms and Screening Fingerprint**

Current research efforts in my laboratory are focused on discovering the genes that are directly regulated by AHR activation in CD4<sup>+</sup> T cells that lead to the induction and function of Tregs. Understanding the pattern of gene expression and the signaling pathways that are altered by AHR activation are critical for understanding the mechanisms of TCDD-induced Treg development and function. Changes in gene expression induced by TCDD are also being used for screening alternative AHR ligands that have the potential to induce AHR-dependent Tregs. A gene fingerprint for AHR-dependent Tregs could also be useful for identifying endogenous AHR ligands as well as exogenous dietary chemicals that influence immune function. Our current NIEHS-funded grant made possible by the American Recovery and Reinvestment Act (ARRA), has afforded us the opportunity to demonstrate proof-of-principal of our screening approach. We have validated and extended the number of genes previously reported to be upregulated by AHR signaling in T cells (Marshall et al., 2008), including *Il12rb2*, *Gzmb*, *Il10* and *Tgfb3*, and we have documented higher expression levels in sort-purified, alloantigen-specific CD4<sup>+</sup> donor T cells. We are also very excited about our recent discovery of a novel AHR ligand that is immunosuppressive and induces a robust AHR-dependent TCDD-like Treg phenotype on day 2 of the GVH response. We look forward to continuing these studies with our ultimate goal of understanding the role of AHR in T cell biology and the development of new treatments for immune-mediated diseases.

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