An Abstract of the Dissertation of

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Nancy I. Kerkvliet

In experimentally exposed mice, the environmental contaminant 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) produces significant suppression of adaptive immune responses at low doses. However, the underlying biochemical and cellular mechanisms of TCDD-induced immunotoxicity have remained elusive since the identification of these effects nearly 30 years ago. Antigen-presenting cells (APC) constitute various populations of cells essential for the initiation and maintenance of adaptive immune responses, and represent a potential target of TCDD toxicity. Thus, the studies presented here address the ability of TCDD to directly affect APC. The underlying objectives of these studies focus on the investigation of molecular signaling pathways and cellular processes potentially affected by TCDD. In order to eliminate conflicting variables found *in vivo*, we used *ex vivo* and *in vitro* models to address these objectives. Initial studies investigated the status and behavior of the aryl hydrocarbon receptor (AhR), a transcription factor recognized as the principal mediator of TCDD-induced immunotoxic effects, in the two main APC populations, macrophages and dendritic cells (DC). The results demonstrated that both APC populations expressed AhR. However, TCDD induced binding of AhR to dioxin response elements only in macrophages, and not DC. Because TCDD has been shown to alter DC function and survival *in vivo*, the possibility that TCDD altered other signaling pathways was addressed. Specifically, activation of the transcription

factor NF-κB/Rel, integral in DC generation and function, was found to be suppressed by TCDD. This suppression was apparently mediated by a physical association between the AhR and proteins of NF-κB/Rel. Additional studies demonstrated that TCDD enhances the maturation of DC and appears to sensitize DC to apoptosis. These data establish that TCDD directly affects DC on the molecular and cellular levels and support several potential mechanisms of TCDD-induced immunotoxicity

Direct Effects of 2,3,7,8 Tetrachlorodibenzo-*p*-Dioxin on Antigen-Presenting Cells and Molecular Signaling Pathways in Dendritic Cells

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APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Head of Department of Environmental and Molecular Toxicology

Redacted for Privacy

Dean of Graduate School

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Carl E. Ruby, Author

Contribution of Authors

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

Funatake, Castle J.	Critical review of experimental design and data. Technical support.
Kerkvliet, Nancy I.	Critical review of experimental design, data, and manuscripts; financial support.
Lawrence, B. Paige.	Critical review of experimental design and data.
Leid, Mark.	Critical review of experimental design and manuscripts.

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Direct Effects of 2,3,7,8 Tetrachlorodibenzo-*p*-Dioxin on Antigen-Presenting Cells and Molecular Signaling Pathways in Dendritic Cells

Chapter 1

Introduction

"Eliminate all other factors, and the one which remains must be the truth."

'Sherlock Holmes'
A. Conan Doyle

The mechanism(s) of 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD)-induced immune toxicity has remained a mystery despite extensive research over the past 30 years when Vos and colleagues identified the immune system as a sensitive target of TCDD toxicity. Although the aryl hydrocarbon receptor (AhR) has been shown to be the primary mediator of TCDD toxicity, the specific molecular and cellular mechanisms that follow AhR activation have yet to be identified. The studies presented here address a few of the many "factors" potentially involved in the phenomena of TCDD-induced immune toxicity, in hopes of uncovering the truth.

TCDD

Overview

TCDD, the most toxic member of a group of compounds known collectively as halogenated aromatic hydrocarbons, was first identified in 1957 as a contaminant

produced during the synthesis of trichlorophenol (Kimmig and Schulz, 1957). As shown in Figure 1-1 TCDD is a simple molecule comprised of two benzene rings linked by two oxygen bridges and chlorinated at positions 2, 3, 7, and 8. TCDD is thermally stable to temperatures greater than 800°C (Kearney *et al.*, 1973; Stehl *et al.*, 1973) with low solubility in water corresponding to a high octanol-water coefficient (Marple *et al.*, 1986; Jackson *et al.*, 1993). The sum of these features contribute to the long half life of TCDD in the environment, calculated at about 10 years (di Domencio *et al.*, 1980) and fuel concern over potential toxicity of this molecule.

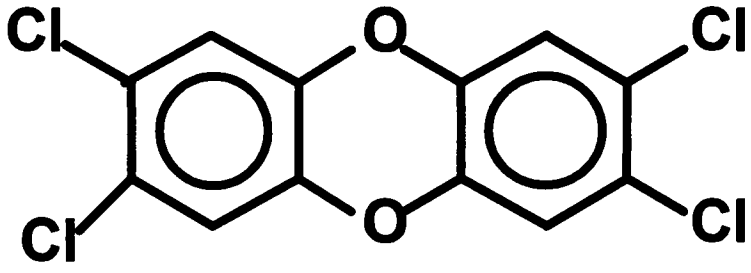
Although TCDD is found widely throughout the environment, it was never intentionally synthesized, simply finding its way into the environment as an unwanted by-product of chemical manufacturing, chlorine paper bleaching, and waste incineration (Zook and Rappe, 1994). The lipophilic nature of TCDD allows it to accumulate in the fat of meat, fish, and milk, constituting the main route of exposure of humans to TCDD (Schecter *et al.*, 1994; Startin, 1994). A less consistent source of TCDD is the rare industrial accident, resulting in the exposure of humans to high concentrations of TCDD and providing insight into the toxicity of TCDD in humans.

Human Toxicity

Following accidental poisonings or industrial accidents, there have been no immediate cases of death due to TCDD exposure. However a commonly observed toxic condition is the skin disease, chloracne, characterized by pigment changes and sores persisting for years after exposure. Other more controversial effects of TCDD in humans are increased risk of diabetes, heart disease, and cancer (Vena *et al.*, 1998; Steenland *et al.*, 1999; Bertazzi *et al.*, 1998). The International Agency for Research on Cancer classifies TCDD as a group I human carcinogen based on epidemiological evidence that has revealed a positive trend for cancer mortality with

Fig. 1-1. The structure of 2,3,7,8 tetrachlorodibenzo-p-dioxin.

Figure 1-1



increasing dose in workers exposed to various levels of TCDD (Steenland *et al.*, 1999). Although epidemiological studies characterize TCDD as a complete carcinogen, TCDD is not genotoxic and is therefor likely to be acting as a promoter.

Alterations in immune parameters have been also examined in human subjects exposed to high concentrations of TCDD. Studies of people exposed to TCDD in industrial accidents or by occupational exposure have shown minor immunological alterations, including a small decrease in numbers of $CD4^+$ T cells and an increase in $CD8^+$ T cells (Lu and Wu, 1985). In contrast, another study showed no significant difference in the distribution and numbers of $CD4^+$ and $CD8^+$ T cells in subjects accidentally exposed to much lower levels of TCDD (Webb *et al.*, 1989). In a study of workers exposed to TCDD, the proliferation of blood mononuclear cells was suppressed following co-culture with IL-2 or allogeneic cells (Tonn *et al.*, 1996). Based on the limited and inconclusive data, the relevance of these findings are difficult to assess. Thus, in order to clarify the potential effects of TCDD on humans, animal models have been employed.

Animal Toxicity

The use of a variety of animal models has allowed for the characterization of a number of TCDD-induced toxic effects, ranging from high dose lethality to low dose carcinogenicity. The dose of TCDD that is lethal depends on the animal model utilized. For example, the LD_{50} for guinea pigs is 2 μg/kg, while 3000 μg/kg is required to produce the same percentage of lethality in hamsters (Pohjanvirta and Tuomisto, 1994). The lethality of TCDD is delayed, often taking weeks following exposure to occur. The exact cause of TCDD-induced lethality remains unknown, but a severe wasting syndrome precedes death (Max and Silbergeld, 1987).

The carcinogenic potential of TCDD, suggestive in human epidemiological studies, has also been addressed through the use of animal models. In several studies, repeated TCDD exposure results in the generation of tumors at a number of

sites in rats and mice (Huff, 1992; Kociba *et al.*, 1978). Carcinogenesis is believed to be a multi-step process involving initiation, a genotoxic event, and promotion. Although TCDD has not been shown to be mutagenic, and in tumor promotion models it is highly potent (Thornton *et al.*, 2001; Dragan *et al.*, 1992). Paradoxically, even though TCDD is unable to initiate genotoxic events, it is widely considered a complete carcinogen (Rao *et al.*, 1988).

Immunotoxicity

Early studies recognized that TCDD-exposed animals were more susceptible to many infectious diseases. Extremely low doses (0.01 μg/kg) of TCDD increased influenza virus-induced mortality (Burleson *et al.*, 1996). Additionally, TCDD enhanced the mortality of mice infected with the herpes simplex type II virus (Clark *et al.*, 1983), *L. monocytogenes* (Hinsdill *et al.*, 1980), and *E. coli* endotoxin (Thomas and Hinsdill, 1979). Based on these and similar studies, TCDD displays a capacity to effectively suppress host resistance to pathogens, suggesting that the immune system is compromised.

Several studies in mice have shown that exposure to TCDD profoundly suppresses adaptive immune responses, including humoral and cell-mediated effector functions (Luster *et al.*, 1979; Clark *et al.*, 1983; Kerkvliet *et al.*, 1990; Kerkvliet *et al.*, 1996). Utilizing *in vivo* animal models, *ex vivo* culturing, and *in vitro* cell lines, individual populations of immune cells have been extensively examined for TCDD toxicity. The effect of TCDD on, T cells, B cells, and antigen presenting cells (macrophages and DC), is summarized below.

The T cell is at the center of nearly every adaptive immune response, orchestrating other cells and factors to vigorously respond to a pathogen. Following presentation of antigen, naive T cells are activated, clonally proliferate into effector cells, and secrete cytokines to generate immune responses. TCDD, at low doses, has been shown to suppress the T cell-dependent humoral response in mice against

sheep red blood cells (SRBC) (Kerkvliet *et al.*, 1990). The other arm of the adaptive immune response, the cell-mediated response, is also suppressed by TCDD in a dose-dependent fashion. TCDD suppression is evident in the cell-mediated response to allogeneic P815 tumor cells, as TCDD effectively suppresses splenic cytotoxic T cell (CTL) activity following injection of tumor, and decreases levels of the cytokines, IL-2 and IFNγ (Kerkvliet *et al.*, 1996).

The ability of TCDD to suppress T cell activity is evident in many *in vivo* models, but studies using *in vitro* or *ex vivo* systems to mechanistically explore these findings have been unsuccessful. TCDD failed to alter the CTL response to P815 tumor cells *in vitro*, and TCDD had no effect on proliferation of T cells in mixed lymphocyte tumor cell cultures (Dekrey and Kerkvliet, 1995). Moreover, TCDD failed to influence *ex vivo* T cell proliferation and cytokine release when T cells were treated with mitogen or antigen (Prell *et al.*, 1995). The lack of *in vitro* effects in these studies makes it difficult to conclude that the T cell is a direct target of TCDD. Recently, an *in vivo* model using antigen-specific transgenic DO11.10 T cells adoptively transferred into Balb/c mice was used to analyze TCDD effects on antigen-specific T cell proliferation and cytokine production. The results demonstrate that TCDD does not significantly alter the proliferation of T cells or their activation, although the immune response is suppressed by TCDD (Shepherd *et al.*, 2000). However, the survival of the antigen-specific T cells was significantly reduced in this model. These data suggest that TCDD might be altering other immune cells that interact with T cells to promote T cell survival.

The B cell functions to produce antibodies against antigens dependent or independent of T cell help. B cells have been shown to be susceptible to TCDD toxicity *in vivo* and *in vitro*. In mice exposed to low doses of TCDD, antibodies produced in response to SRBC are suppressed significantly (Kerkvliet *et al.*, 1990). Additionally, *in vitro* studies have established that TCDD directly alters B cells on the molecular level by increasing Ca^{++} concentrations and increasing protein tyrosine

kinase activity (Kramer *et al.*, 1987; Clark *et al.*, 1991). On the cellular level, TCDD inhibits terminal differentiation of B cells (Luster *et al.*, 1988), and evidence from studies using B cell lines showed LPS-induced IgM production was suppressed by TCDD *in vitro* (Sulentic *et al.*, 1998). Thus, it is evident from numerous studies *in vivo* and *in vitro* that TCDD influences B cells directly, however, these effects cannot fully account for the scope of TCDD-induced immune toxicity.

Antigen-presenting cells (APC) are highly specialized cells that take up antigen and display it in the context of major histocompatibility class II (MHC II) molecules, together with costimulatory molecules to activate naive T cells. Both macrophages and DC function as APC, playing separate, but overlapping roles in T cell activation. Immature DC take up antigen in the tissue and migrate to areas in the draining lymph or spleen, where they mature. Maturation is characterized by an inability to take up antigen and the expression of high levels of MHC II and costimulatory molecules. The mature DC, in an antigen-specific manner, then stimulates the naive T cell to differentiate and proliferate. Alternatively, macrophages residing in the tissue engulf antigen through phagocytosis and present it to T cells at the site of infection. In turn, these activated T cells secrete cytokines that stimulate macrophages to produce cytotoxic levels of superoxide, hydrogen peroxide, and nitric oxide to kill pathogens.

A number of reports have characterized the effects of TCDD on macrophages. Initially, TCDD was shown to increase accumulation of macrophages in various tissues in rodents (Vos *et al.*, 1974; Puhvel and Sakamoto, 1988). Further studies examined the effects of TCDD on antigen presentation by macrophages. Macrophages identified by Mac1 staining in SRBC-treated mice appeared to be relatively unaffected by TCDD and antigen presentation was not altered (Kerkvliet and Oughton, 1993). However, Prell and colleagues (1997) demonstrated TCDD decreased the expression of B7 costimulatory molecules on the surface of Mac1 staining cells in mice treated with P815 tumor cells. Antigen

presentation by the macrophage was further investigated *in vitro*, and in several immune responses adherent macrophages from mice treated with TCDD were not altered (Dooley and Holsapple, 1988). Moreover, macrophages, following *in vivo* treatment with ConA and TCDD, and mixed *ex vivo* with normal T cells, failed to alter T cell proliferation (Rhile *et al.*, 1996). These studies suggest that TCDD has no significant effect on the ability of macrophages to present antigen. However, TCDD appears to be capable of amplifying aspects of nonspecific immune responses carried out by macrophages. It has been shown that *in vivo* exposure to TCDD increased generation of superoxide, hydrogen peroxide and nitric oxide, leading to oxidative stress (Stohs, 1990; Alsharif *et al.*, 1994; Moos and Kerkvliet, unpublished results). Thus, increased oxidative stress mediated by TCDD-exposed macrophages could potentially inflict cellular damage to surrounding tissue and to other immune cells, most notably the T cell.

DC, considered the most potent APC for naive T cells have been shown to be affected by TCDD (Vorderstrasse and Kerkvliet, 2001). Splenic DC numbers were reduced by 40% in TCDD-exposed mice. Interestingly, the remaining DC in the spleen were shown to express significantly more costimulatory molecules, and increase T cell proliferation in *ex vivo* cultures. In a second study, mice exposed to TCDD and challenged with P815 tumor cells also demonstrated a decrease in DC cell numbers in the spleen (Shepherd *et al.*, 2001). Thus certain functions of both DC and macrophages can be influenced by TCDD.

TCDD-induced Immunotoxicity involves a number of different responses and cells, but the underlying primary cellular target remains to be fully identified. The APC represents a unique target whose primary function is to sample the environment for antigen and initiate immune response via T cell activation. Several functions of the APC are potential targets of TCDD and may account for TCDD-induced immune toxicity. These include: (1) antigen presentation, (2) costimulation, and (3) survival. The first function has been addressed and appears to be relatively

unaffected by TCDD. The second function has been shown to be altered by TCDD in several studies. However, the results are conflicting, with one study demonstrating a decrease in costimulatory molecules expression on Mac1 cells (Prell et al., 1995) and another showing an increase on DC (Vorderstrasse and Kerkvliet, 2001). The final factor, survival, has not been fully assessed, but TCDD appears to decrease DC numbers (Vorderstrasse and Kerkvliet, 2001; Shepherd *et al.*, 2001). DC survival is critical for sustained initiation of T cell-dependent immune responses (Josien *et al.*, 2000), possibly mediated by survival factors, including CD40 (Miga *et al.*, 2001).

Ah Receptor

Overview

It has been difficult to identify the ultimate cellular target of TCDD to fully account for the observed immune suppression, but a singular factor has been shown to mediate TCDD immunotoxicity, the aryl hydrocarbon receptor (AhR). The AhR, an orphan receptor, has been linked to TCDD-induced immunotoxicity by the use of congenic strains of mice (Thurmond *et al.*, 1987; Kerkvliet *et al.*, 1990) and through the manipulation of knockout mice. The hallmark of TCDD immunotoxicity, thymic atrophy, was shown to require the presence of the AhR in bone marrow chimeric mice (Staples *et al.*, 1998). Furthermore AhR knockout mice generated normal immune responses to SRBC and P815 tumor cells when exposed to immunosuppressive concentrations of TCDD (Vorderstrasse *et al.*, 2001).

The AhR has been widely studied and numerous reports have reviewed the AhR at the molecular level (Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). The AhR, a ligand activated basic-helix-loop-helix (bHLH) transcription factor, possesses structural regions and motifs integral to the function of the protein.

The basic region of the bHLH structure, found at the N-terminus, is essential for binding to DNA which, in conjunction with a tetratricopeptide repeat half-site located outside of the N-terminus region, is also required for DNA interaction (Levine *et al.*, 2000). Aside from the bHLH structure, the most prominent feature of the AhR protein is the Per-ARNT-Sim (PAS) region positioned near the N-terminus. The PAS region contains the nuclear localization sequence, needed for nuclear translocation, and is the site for ligand binding. The final prominent structural feature of the AhR is the transcription activation domain (TAD), situated near the C-terminus.

The AhR has been identified in organisms as simple as *C. elegans* and as complex as humans, but it is in the mouse that the AhR has been best characterized and most studied. There are four different AhR alleles in mice, categorized by their affinity for TCDD. A low affinity allele, AhR^{d}, corresponding to an AhR protein with a molecular weight of 104 kDa, is found in the mouse strains DBA and 129 (Swanson and Bradfield, 1993). In contrast to the low affinity allele, there are three high affinity alleles, AhR^{b1}, AhR^{b2} and AhR^{b3} coding for AhR proteins ranging from 95 to 105 kDa, as seen in C57Bl/6 and Balb/c mice respectively (Poland *et al.*, 1987; Poland and Glover, 1990). Interestingly, a simple Val to Ala polymorphism in the ligand binding domain defines the primary difference between low affinity and high affinity alleles.

Although an endogenous ligand has not been identified to date, many environmental contaminants and natural compounds are ligands for the AhR. The prototypic ligand of the AhR is TCDD, but compounds known as approximate isosteriomers of TCDD, including polychlorinated dibenzo-dioxins (PCDD), polychlorinated biphenyls (PCB) and polychlorinated dibenzo-furans (PCDF), also function as ligands for the AhR. In addition to the man-made compounds, the amino acid tryptophan has been shown to interact with the AhR, blocking TCDD binding and activating the AhR in hepa1c1c7 cells (Heath-Pagliuso *et al.*, 1998). In

addition, the acid catalyzed form of indole-3-carbinol, indolo[3,2-b]carbazole, has also been characterized as a AhR ligand (Rowlands and Gustafsson, 1997).

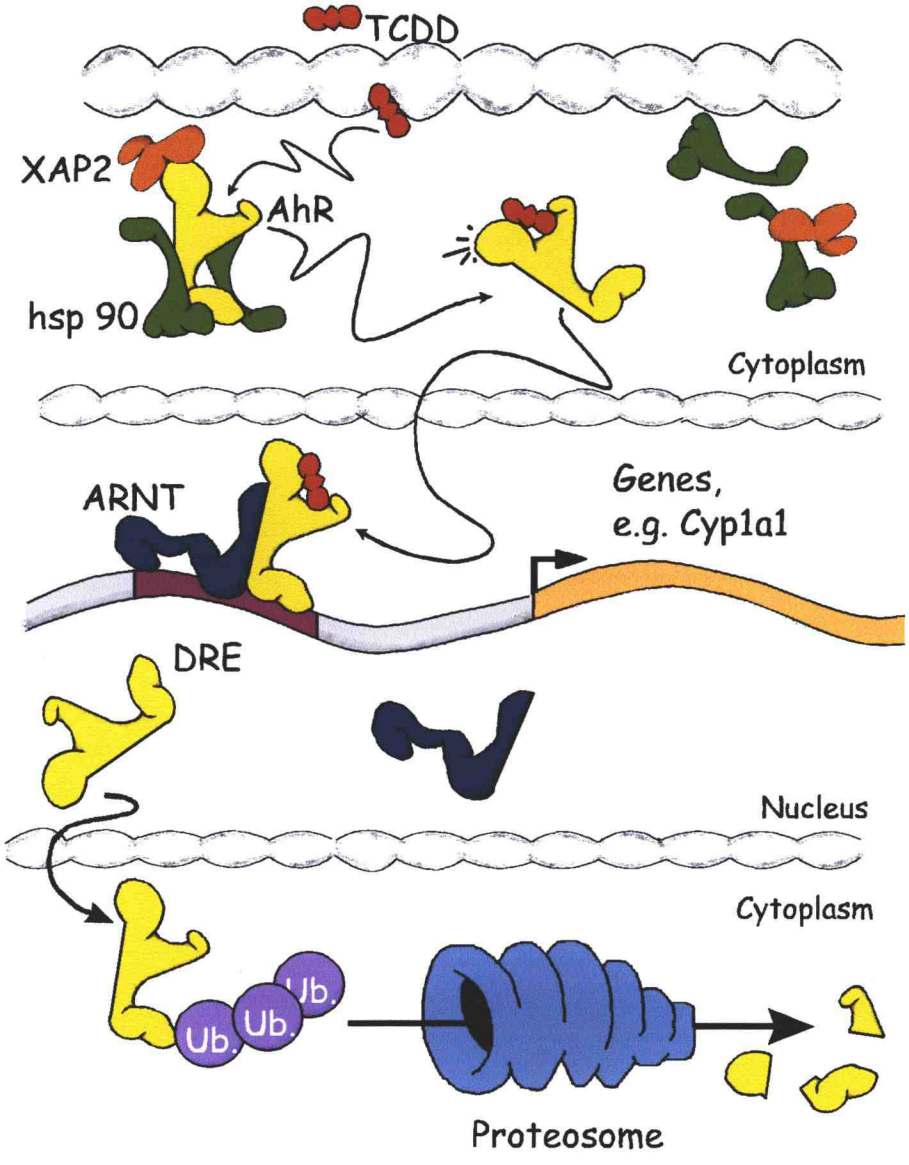
The binding of ligand to the AhR initiates a sequence of events collectively known as activation, culminating in the formation of an AhR-DNA complex (Figure 1-2). Prior to ligand binding, the AhR is found in the cytoplasm in a multi-protein complex. The complex contains two heat shock protein 90s (hsp90) and an immunophilin protein, that bind at the PAS region of the AhR, and aid in receptor stability (Schmidt and Bradfield, 1996). These accessory proteins are shed after a conformational change in the AhR initiated by ligand binding, allowing for rapid translocation of the AhR to the nucleus. The AhR then dimerizes with a second protein closely related to the AhR, Ah receptor nuclear translocator protein (ARNT), at sites in the PAS region (Kronenberg *et al.*, 2000) and the HLH domain (Rowlands and Gustafsson, 1997). The AhR-ARNT heterodimer then binds to DNA at specific sequence motifs called dioxin response elements (DRE). The core sequence of the DRE is an E-box half site consisting of GCGTG (Lusska *et al.*, 1993). The DNA bound AhR-ARNT dimer recruits the coactivators SR-1 and CBP/p300, respectively (Kumar and Perdew, 1999; Kobayashi *et al.*, 1997), and initiates the transcription of various gene products. In the final step, the AhR is down-regulated via a nuclear export signal (NES) located in helix 2 of the bHLH region and degraded by the proteosome following ubiquitination in the cytoplasm (Pollenz and Barbour 2000; Davarinos and Pollenz, 1999; Ma and Baldwin, 2000).

TCDD toxicity mediated by the AhR

Through the use of resistant and susceptible strains of mice carrying the AhR^d and AhR^b alleles, early studies hypothesized that the toxic effects of TCDD were transcriptional events mediated by the AhR. This hypothesis was later verified using congenic (Kerkvliet *et al.*, 1990) and more recently, AhR knockout mice to establish

Fig. 1-2. Activation of the aryl hydrocarbon (AhR) receptor. See text for details. Abbreviations include: heat shock protein 90 (hsp90); AhR nuclear translocator (ARNT); dioxin response element (DRE): ubiquitin (Ub).

Figure 1-2


TCDD
XAP2
AhR
hsp 90
Cytoplasm
ARNT
Genes,
e.g. Cyp1a1
DRE
Nucleus
Cytoplasm
Ub.
Ub.
Ub.
Proteosome

that TCDD toxicity is mediated by the AhR (Revilla *et. al.*, 1997; Schmidt *et. al.*, 1996). Thus, the AhR represents the critical factor in TCDD toxicity.

The AhR mediates TCDD toxicity through several transcriptional events that are dependent on binding to the DRE, its cognate response element. Some of the most widely studied transcriptional events induced by TCDD involve the increased expression of a number of xenobiotic metabolizing gene products. CYP1A1, a common gene studied, contains at least six DREs in its promoter region, resulting in a high sensitivity to TCDD exposure. Other xenobiotic metabolizing genes influenced by the AhR through the DRE are CYP1A2, CYP1B1, and glutathione-s-transferase Ya (Schmidt and Bradfield, 1996). In addition to the xenobiotic metabolizing enzymes, the AhR also influences in the expression of hormone and cytokine genes. The transcription of the hormone ACTH gene has been shown to potentially involve the AhR, as TCDD-induced expression of ACTH was inhibited by alpha napthoflavone, an AhR antagonist (Bestervelt *et al.*, 1998). Levels of the cytokine IL-2, critical in T cell proliferation and survival, were increased in response to TCDD in activated T cells, apparently due to AhR binding to three DREs in the IL-2 upstream promoter region (Jeon and Esser, 2000).

The AhR not only mediates toxicity through transcriptional events directed by the DRE, but also potentially through a number of additional mechanisms recently elucidated. An example of one of the mechanisms not dependent on the DRE is seen when intracellular concentrations of Ca^{++} were significantly increased within two minutes of initial TCDD exposure in Hepa-1 cells and not in AhR-defective cells (Puga *et al.*, 1992). Phosphorylation, another early cellular signaling event, is altered by TCDD. A rapid increase in tyrosine phosphorylation and an increase of phosphorylation mediated by pp60src kinase, were observed following TCDD exposure (Clark *et al.*, 1991; Blankenship and Matsumura, 1997; Enan *et al.*, 1998). These phosphorylation events mediated by src kinase were concluded to play a

significant part in mediating at the least some of the effects by TCDD (Enan *et al.*, 1998).

TCDD, in combination with the AhR, has been shown to alter the activity of a number of other transcription factors. TCDD exposure resulted in the up-regulation of c-jun, a protein involved in the formation of the AP-1 transcription factor, and increased AP-1 binding to DNA (Puga *et al.*, 1992; Puga *et al.*, 2000). Recently a number of studies have demonstrated that the AhR is able to alter transcription factor function through physical interactions, often manifesting in transcription factor inhibition.

The transcription factors Rb, estrogen receptor (ER), COUP-TF and Rel A, have been shown to associate with the AhR, potentially leading to toxicity. Puga et al (2000) demonstrated that the AhR complexed with Rb in a cell-free system and in hepa1c1c7 cells. In a second study, the AhR-Rb interaction was shown to reduce E2A transcription and induce cell cycle arrest, leading the authors to conclude that G1 arrest was an AhR-mediated event (Elferink *et al.*, 2001). The AhR, but not ARNT, appeared to interact with the ER leading to mutual repression (Klinge *et al.*, 2000; Klinge *et al.*, 1999). Interestingly, the transcriptional repressor COUP-TF is capable of binding to DRE motifs, displacing the AhR-ARNT dimer. In one study the COUP-TF protein was shown to physically interact with the AhR in CV-1 cells transfected with both proteins (Klinge *et al.*, 2000). Finally, the AhR was shown to physically interact with Rel A, a protein from the NF-κB/Rel transcription factor family in hepa1c1c7 cells(Tian *et al.*, 1999) and MCF-7 cells (Kim *et al.*, 2000).

The AhR has also been hypothesized to initiate toxicity in other forms of non-DRE mediated events. It has been speculated that activated AhR could lead to coactivator dilution in a scenario similar to that seen following overexpression of steroid hormone receptors resulting in reduced transcriptional activity of other steroid receptors (Meyer *et al.*, 1989). There is also speculation that persistent transcriptional activation of the AhR by TCDD could be a possible mechanism of

toxicity by sequestering ARNT, thereby reducing ARNT dimerization with other proteins (Schmidt and Bradfield, 1996).

In conclusion, the AhR is the principal factor involved in mediating the toxic effects of TCDD. The consequences of the AhR directly altering transcription through DRE motifs or by interacting with other transcription factors are just beginning to be understood. Thus, the AhR is a dynamic factor promoting transcription and potentially influencing many intracellular signaling events.

NF-κB/Rel

Overview

NF-κB/Rel was initially characterized in 1986 by Baltimore and colleagues as a transcription factor important in promoting the transcription of the immunoglobulin kappa light chain in B cells (Sen and Baltimore, 1986). In the fifteen years since its discovery, there has been an explosion of information regarding NF-κB/Rel, making it one of the most widely studied transcription factors. These studies describe an influential transcription factor involved in responses to infection, stress and injury. A substantial amount of information has been published on the details of NF-κB/Rel activation and numerous reviews have provided up-to-date reports on its mode of action (Baldwin, Jr., 1996; Ghosh *et al.*, 1998; Karin and Ben Neriah, 2000).

NF-κB/Rel is the general name given to identify a family of five proteins, differing in behavior, function and structure. The proteins of NF-κB/Rel consist of Rel A, c-rel, RelB, p50, and p52, forming various homo- and heterodimers, with the exception being RelB which only forms heterodimers with p50 and p52. The five proteins of NF-κB/Rel share a structurally similar motif known as the Rel homology domain (RHD). The RHD is a highly conserved region involved in dimerization,

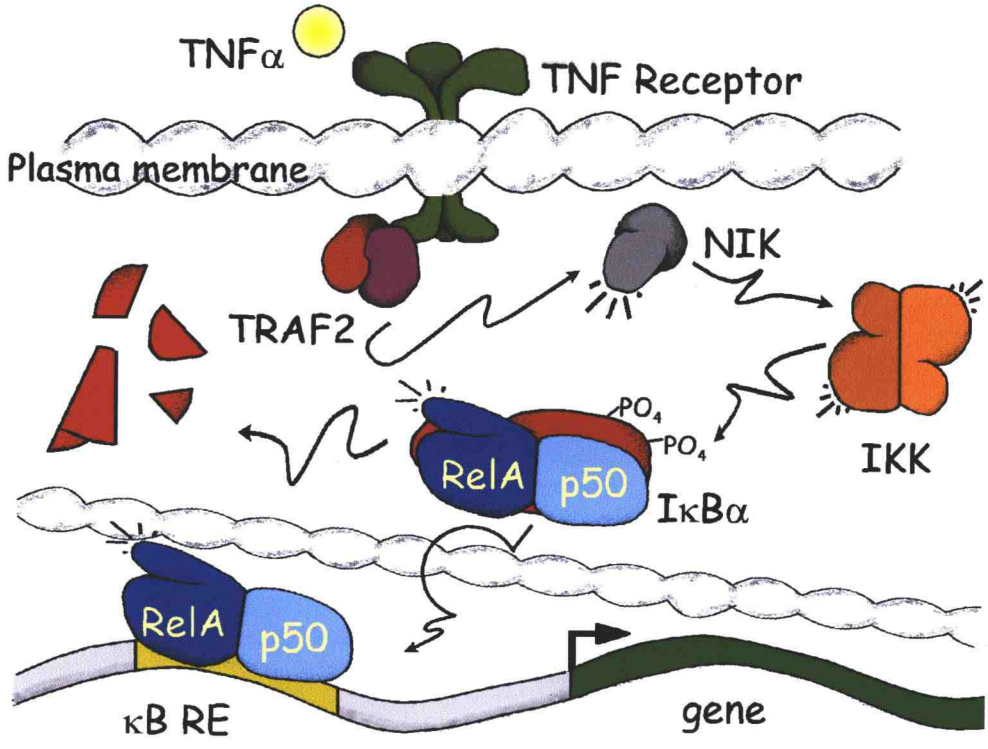
DNA binding, interaction with inhibitor proteins, and translocation. The NF-κB/Rel proteins can further be subdivided into two additional groups, distinguished by the presence of a transcriptional activation domain (TAD). The NF-κB/Rel proteins Rel A, RelB, and c-rel contain a C-terminal domain encompassing a TAD, although the proteins, p50 and p52, lack this domain, and homodimers of p50 and p52 are believed to repress transcription. Another distinguishing feature of p50 and p52 is that they exist initially as pre-proteins containing a region structurally similar to IκB inhibitor proteins, which is post-translationally cleaved by an ATP-dependent proteosome. The differences and similarities between these proteins describe a transcription factor uniquely capable of responding to a host of different stimuli.

A number of extracellular stimuli activate NF-κB/Rel, culminating in the transcription of numerous genes (Figure 1-3). NF-κB/Rel is induced by three primary types of inducers: (1) damage to the cell by oxidative intermediates or U.V. radiation, (2) factors representing infection of the cell, for example LPS, viral proteins, and dsRNA, and (3) proinflammatory cytokines. One of these stimuli, the potent proinflammatory cytokine TNFα, has been well-characterized to induce the activation of NF-κB/Rel, through events mediated by the transmembrane region of the TNF receptor. Proteins associated with the intracellular region of this receptor, called TRAFs, are activated, which in turn stimulate a protein called NIK or proteins of the MAPKKK family, which in turn activate the IκB kinase (IKK).

In its inactive state, NF-κB/Rel is sequestered in the cytoplasm by an inhibitor protein, a member of a battery of five inhibitor proteins collectively called IκB. Analogous to their NF-κB/Rel counterparts, the IκBs share structurally similar regions functioning to retain NF-κB/Rel in the cytoplasm. IκBα, the predominate IκB protein and best characterized, interacts with NF-κB/Rel at the site of the RHD, blocking the NLS. Structurally, the IκBα inhibitor protein contains a region of seven repeated ankyrin motifs, a C-terminal PEST sequence required for basal turnover, and an N-terminal domain phosphorylated for signal-related turnover.

Fig. 1-3. Activation of NF-κB/Rel. See text for details.

Figure 1-3


TNFα
TNF Receptor
Plasma membrane
NIK
TRAF2
IKK
PO4
PO4
RelA
p50
IκBα
RelA
p50
κB RE
gene

Activation of NF-κB/Rel following extracellular stimulation is initiated by IKK-mediated phosphorylation of two serine moieties on the N-terminal domain of IκBα, targeting it for degradation.

The phosphorylation of IκBα by IKK defines the most critical step of NF-κB/Rel activation. IKK, a complex of three proteins, contains leucine zipper moieties on its catalytic subunits enabling it to phosphorylate serine residues 32 and 36 on IκBα. The phosphorylated IκBα, still in association with NF-κB/Rel, is quickly ubiquitinated and degraded by the 26S proteosome, exposing the NLS on NF-κB/Rel. The NF-κB/Rel rapidly translocates to the nucleus, interacts with sequence motifs in DNA called κB response elements (κB-RE), and promotes gene expression.

Promotion of gene expression by NF-κB/Rel is defined by the following events: DNA binding, coactivator recruitment, and transcription factor down-regulation. NF-κB/Rel binds to κB REs, comprised of the ten base-pair consensus sequence 5'-GGGGYNNCCCY-3', inducing a physical bend in DNA. The NF-κB/Rel then recruits the coactivator CBP/p300 and promotes transcription. Additional phosphorylation of NF-κB/Rel at various sites increases transcriptional activity by strengthening coactivator interaction, but is not involved in translocation or DNA binding. After mediating transcription, NF-κB/Rel is down-regulated in an autoregulatory feedback loop. IκBα transcription is directly up-regulated by NF-κB/Rel, and NF-κB/Rel activity is dampened as newly synthesized IκBα enters the nucleus, re-engaging the transcription factor due to a higher affinity of NF-κB/Rel to IκBα than to the κB RE. In the final step of down-regulation, NF-κB/Rel is exported into the cytoplasm via the NES on IκBα.

NF-κB/Rel in immunity

NF-κB/Rel was originally characterized in the immune system and is considered an essential element in the function of immune responses. Initially, NF-κB/Rel was casually linked to aspects of immune function by analyzing the upstream gene regulatory regions of a host of genes influencing immune functions. The genes established to be induced by NF-κB/Rel were GM-CSF, IL-6, IL-12, TNFα, IL-2 , major histocompatibility (MHC) proteins, adhesion proteins, and iNOS (Baldwin, Jr., 1996). Further identification of the important role of NF-κB/Rel in immunity has been accomplished by inhibiting the activation of NF-κB/Rel by various means. However, the most compelling evidence was derived from studies of animals deficient in various members of NF-κB/Rel.

The deletion of genes that code for the specific members of NF-κB/Rel in mice has allowed for the elucidation of various functions of NF-κB/Rel. The only member of NF-κB/Rel absolutely required for survival is Rel A. Deficiency in Rel A is embryonically lethal, and animals die due to massive liver defects (Beg *et al.*, 1995). Fortunately, the generation of other NF-κB/Rel null animals have produced viable animals, allowing for a comprehensive analysis of function. These NF-κB/Rel deficient animals have permitted researchers to define a number of aspects of immunity under the control of NF-κB/Rel, ranging from the nonspecific innate immune response to the adaptive immune response and from T cells to DC.

Nonspecific immune responses, including inflammation and innate responses, appear to be impacted by NF-κB/Rel. NF-κB/Rel-deficient animals display an inflammatory phenotype (Weih *et al.*, 1996; Donovan *et al.*, 1999; Xia *et al.*, 1997), characterized by an accumulation of neutrophils infiltrating the tissues of the liver and lung (Weih *et al.*, 1995; Weih *et al.*, 1997). Innate responses were reported to be decreased in NF-κB/Rel-knockout animals, thereby increasing the susceptibility of these animals to pathogens. For example, deletion of p50 and RelB led to increased

mortality in animals exposed to *L. monocytogenes* (Sha *et al.*, 1995; Weih *et al.*, 1997; Carrasco *et al.*, 1998).

Both arms of adaptive immunity, humoral and cell mediated, require an intact NF-κB/Rel for normal function. T cell-dependent, but not T cell-independent, humoral responses were reported to be suppressed in animals deficient in c-rel and p52 (Franzoso *et al.*, 1998; Kontgen *et al.*, 1995; Caamano *et al.*, 1998). The delayed-type hypersensitivity response, used to model a cell-mediated response, was suppressed in RelB null animals (Weih *et al.*, 1995). The response to acute LCM virus was also suppressed in these animals, characterized by reduced viral clearance and markedly reduced CD8 T cell numbers (Weih *et al.*, 1997).

In more detailed investigations, T and B cells from NF-κB/Rel-deficient animals were analyzed to determine the role of NF-κB/Rel in specific lymphocyte functions. T cells from knockout animals showed a decrease in mitogen-induced proliferation and survival. T cells from c-rel knockouts were unable to proliferate in response to anti-CD3 and ConA (Kontgen *et al.*, 1995). Additional studies found that NF-κB/Rel was essential for survival of T cells in the thymus, and in p50/p52 knockout mice there was a significant loss of peripheral CD4 and CD8 T cells (Franzoso *et al.*, 1997; Senftleben *et al.*, 2001). These mice also display a profound decrease in B cells (Cariappa *et al.*, 2000; Caamano *et al.*, 1998), due to an apparent block in development at an immature stage (Franzoso *et al.*, 1997). In addition to an influence on B cell maturation, p50 appears to be important for B cell class switching and survival (Weih *et al.*, 1997; Grumont *et al.*, 1998).

As previously noted, NF-κB/Rel knockout mice demonstrate infiltration of neutrophils in various tissues, suggesting that NF-κB/Rel influences neutrophil generation and migration. Animals deficient in RelB, p50, and p52 displayed an increase in the number of neutrophils in the spleen and lymph nodes (Franzoso *et al.*, 1997; Weih *et al.*, 1995; Weih *et al.*, 1996), due to an up-regulation of myelopoiesis in the bone marrow of these animals (Weih *et al.*, 1997). In addition, there was a

parallel decrease in the number of other myeloid cells in RelB deficient animals(Wu *et al.*, 1998), highlighted by a profound loss of splenic and thymic DC (Weih *et al.*, 1995).

In summary, extensive research over the past 15 years has revealed a prominent role for NF-κB/Rel in the function of immune system. The influence of NF-κB/Rel on immune responses has been verified through the creation of NF-κB/Rel null animals. Thus, NF-κB/Rel appears to be necessary for the following functions in the immune system: (1) the development and maturation of B cells (2) the survival of T cells, (3) the hematopoietic development of myeloid cells, notably DC and neutrophils, and (4) the induction of immune responses by influencing antigen presentation and secretion of cytokines.

Hypothesis and Objectives

The studies presented in this dissertation were designed to test the hypothesis that TCDD directly affects APC and molecular signaling pathways, potentially playing a role in TCDD-induced immunotoxicity. This hypothesis developed from numerous observations and reports that demonstrated APCs were targets of TCDD toxicity. The toxic effects of TCDD on APCs were often defined *in vivo*, involving a number of potentially conflicting variables. These include the effects of neighboring cells and circulating cytokines, both able to influence APC function. To eliminate these variables found *in vivo* and to characterize the direct effects of TCDD on APCs, we analyzed the impact of TCDD exposure *in vitro* or *ex vivo* on several molecular signaling pathways as well as important cellular processes of the APC.

In Chapter 2 the status and behavior of the AhR in APC populations of macrophages, monocytes and DC was characterized. The AhR is an essential factor

in TCDD-induced immune toxicity and is thought to mediate toxicity through alterations in transcription. In Chapter 3, the effects of TCDD on molecular signaling by NF-κB/Rel, a crucial transcription factor involved in APC function and normal immunity, was examined in a DC line. NF-κB/Rel, in particular, was studied due the similarities between NF-κB/Rel null animals and *in vivo* models describing TCDD immunotoxicity. Chapter 4 addresses the impact of TCDD on the production of nitric oxide, a potent oxidant produced by macrophages and a possible mediator of immunotoxicity. In Chapter 5, we assessed the direct effects of TCDD on the DC processes of development, maturation and survival, to identify and characterize toxic effects induced by TCDD.

Chapter 2

Status and Behavior of the Aryl Hydrocarbon Receptor in Antigen-Presenting Cells

Authors:

Carl E. Ruby

B. Paige Lawrence

Nancy I. Kerkvliet

Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor that promotes gene expression by binding to dioxin response elements (DRE). Numerous studies using congenic and knockout mice have revealed that the AhR mediates the toxic effects of the environmental contaminant 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD has been shown to produce a spectrum of toxic pathologies in numerous tissues, with the immune system being a sensitive target. Thus, we sought to determine the status and behavior of the AhR in murine antigen-presenting cells (APC), crucial in the initiation and maintenance of antigen-specific immune responses. Using macrophage, monocyte and dendritic cell (DC) lines, representing several of the populations of APC, we examined the status and behavior of the murine AhR in resting and lipopolysaccaride-activated cells. Additionally, we extended this examination of the AhR to include dendritic cells isolated from *ex vivo* culturing and from Flt3L-treated mice. Our results demonstrate that each of the APC examined expressed detectable AhR, but TCDD only increased AhR binding to DRE motifs in the RAW264.7 macrophage cell line. These data suggest that macrophages may represent a potential direct target of TCDD toxicity through AhR-DRE mediated events.

Introduction

TCDD, the prototypic ligand for the AhR, is a widespread environmental contaminant inducing a host of biochemical and pathological toxicities. The toxic conditions induced by TCDD are primarily mediated by the AhR, a ligand-activated basic helix-loop-helix (bHLH) transcription factor. The AhR is found in its

unliganded form in the cytoplasm as a multiprotein complex containing two heat shock protein 90s (Chen and Perdew, 1994) and an immunophilin protein (Meyer and Perdew, 1999). Activation of the AhR following ligand binding is characterized by shedding of the accessory proteins, translocation to the nucleus, and dimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT), a homologous bHLH protein (Rowlands and Gustafsson, 1997; Schmidt and Bradfield, 1996). The AhR-ARNT heterodimer interacts with specific regions of DNA called dioxin response elements (DRE) in regulatory regions and promotes the transcription of gene products (Schmidt and Bradfield, 1996). A number of gene products are influenced directly by AhR promotion including the cytokine IL-2 (Jeon and Esser, 2000) and CYP1A1 xenobiotic metabolizing enzyme (Schmidt and Bradfield, 1996). TCDD toxicity is considered to be mediated by the AhR through effects on gene expression.

The immune system is a sensitive target of TCDD toxicity, evident in the suppression of adaptive immune responses in mice exposed to low doses of TCDD (Kerkvliet and Burleson, 1994). APC constitute a population of cells that are essential in the activation of naive T cells. Three populations of cells function as APC: activated B cells, monocytes/macrophages, and DC. Of these cells, mouse B cells have been shown to be a direct target of TCDD toxicity (Sulentic *et al.*, 1998), and further examination of B cell populations revealed that these cells expressed AhR capable of binding DRE motifs in response to TCDD exposure (Masten and Shiverick, 1996; Williams *et al.*, 1996). Although B cells have been well-characterized for the status and function of the AhR in mice, the remaining APC populations have not been thoroughly evaluated.

We sought to determine the status and behavior of the AhR in populations of murine macrophages, monocytes and DC. Resting and LPS-activated murine APC lines were analyzed by RT-PCR, immunoblotting and electrophoretic mobility shift assay (EMSA). Additionally, we examined AhR expression and behavior in bone

marrow-derived DC and splenic DC from Flt3L-treated mice. The results demonstrate that the APC populations studied expressed AhR protein, but TCDD only induced binding of the AhR to DRE motifs in the macrophage cell line, RAW264.7. Furthermore, LPS-activated macrophage and DC lines appeared to exhibit an increase of AhR binding to DRE motifs in the absence of TCDD. The data presented here identify macrophages as a potential direct target of TCDD toxicity, via DRE mediated events.

Materials and Methods

Reagents

TCDD (≥99% pure) was purchased from Cambridge Isotope Laboratories Inc. (Woburn, MA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Anti-murine AhR antibodies (3-14B) were either provided by Dr. Alan Poland, which were raised against synthetic peptides corresponding to the N-terminal of the C57Bl/6 mouse liver AhR (A. Poland, personal communication) or were purchased from Affinity Bioreagents (Golden, CO). Lipopolysaccaride (*E. coli*) was purchased from Sigma (St. Louis, MO). Flt3L was a gift from Immunex (Seattle, WA), and GM-CSF was purchased from Pharmingen (San Diego, CA).

Cell culture

The cell lines RAW264.7 and WEHI 3 (ATCC, Rockville, MD) were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Na-pyruvate, 1 mM non-essential amino acids and 50 μg/mL gentamicin. Hepa1c1c7 cells (from James Whitlock, Stanford University) were maintained in MEMα supplemented with 10% FBS and 100 U/mL of penicillin. DC2.4 cells (Shen *et. al.*,

1997) (from Ken Rock, Dana Farber Cancer Institute, MA) were cultured in DMEM containing 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 10 mM Na-pyruvate and 50 μg/mL gentamicin. Cells from each cell line were cultured to about 90% confluency, and exposed to 10^{-9} M TCDD or vehicle (0.01% DMSO) and 1 μg/mL LPS for two hours prior to harvesting.

Generation of DC from bone marrow (BMDC)

Preparations of DC were made by an adaption of the protocol originally described by Inaba et al. (1992a) under aseptic conditions. Briefly, femurs from C57Bl/6 mice were flushed with BMDC media (DMEM, 20% FBS, 100 U/mL gentamicin and 2 mM L-glutamine). The cells flushed from the femurs were centrifuged briefly at low speed to pellet any debris, and were transferred to a fresh tube and washed. The red blood cells and dead cells were removed by gradient centrifugation. Purified bone marrow cells were then washed twice and plated out at a density of 5 X 10^5 cells/mL in tissue culture flasks (Costar, Cambridge, MA). Cells were cultured in BMDC media with 10 ng/mL GM-CSF for three days at 37°C in 5% CO_2. Following the initial incubation, cultures were swirled to dislodge nonadherent cells and the supernatant was removed and replaced with fresh media. Two days, later culture media was replaced and, after a total of seven days *ex vivo*, the DC were visible as clumps of loosely adherent cells. Cells were harvested by vigorous pipeting to dislodge loosely adherent cells. The harvested cells were analyzed by flow cytometry for the expression of DC markers, CD11c, CD86 and MHC II (data not shown).

Flt3L treatment and preparation of splenic DC

Balb/c mice were injected for nine days with 100 μM human Flt3L (Immunex, Seattle, WA) subcutaneously. On day ten mice were euthanized by CO_2 and the spleens were removed aseptically. Spleens were processed by pressing the

spleen between the frosted ends of two microscope slides to yield a single-cell suspension. Erythrocytes were removed by hypotonic lysis in water. Cells were washed once in Hanks buffered saline solution (HBSS), 5% fetal bovine serum, 20 mM HEPES, 50 μg/mL gentamicin.

Cell staining and flow cytometry

Cells were incubated in 96-well plate on ice with saturating concentrations of mAb. Nonspecific mAb binding was blocked by pre-incubating the cells with rat, mouse or hamster IgG. All cell preparations were stained with antibodies to CD11c, MHC II and CD86 for the selective analysis on DC. Appropriately labeled isotype controls were used to determine nonspecific fluorescence. Listmode data were collected on a Coulter XL flow cytometer and analyzed by WinList software (Verity Software House, Inc., Topsham, ME). Cell viability was determined by propidium iodide exclusion. For cell surface evaluation 20,000 viable cells were analyzed.

Sorting

Cells were sorted by their expression of CD11c to a purity of 95% or greater on a Cytomation Mo-Flo high speed sorter according to the manufacturer (Ft. Collins, CO).

RNA preparation and RT-PCR

Cells were grown to 95% confluency and harvested. Briefly, RNA was extracted from about 5 X 10^6 cells lysed in Solution D (4 M guanididium thiocyanate, 0.25 M sodium citrate, 0.5% sarcosyl). Cells were passed through a 26 G needle to shear DNA and 2 M sodium acetate was added. Phenol:chlorloform:isoamyl acetate was added to the lysate, incubated on ice for 15 minutes and centrifuged at 10,000 rpm for 10 minutes. Isopropanol was added to precipitate RNA overnight at -80°C. RNA was pelleted, dried and resuspended in

water. cDNA was synthesized from RNA purified from 1 X 10^6 cells using oligo(dT) primers, RNasin and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer. PCR was preformed for 35 cycles using the DNA Engine (PTC-200) from MJ Research (Watertown, MA). DNA PCR products were run on a 2% EtBr/agarose gel and visualized by UV transilluminator. All cDNA samples were initially analyzed for the expression of the housekeeping gene β2-microglobulin to determine relative amounts and integrity of mRNA and degraded DNA amplification products were discarded. Primers for AhR, as previously described by Yamaguchi *et al.* (1997) and for β2-microglobulin were designed using RightPrimer (BioDisk Software, San Francisco, CA) to span introns and allow discrimination between amplified genomic DNA and cDNA PCR products.

Whole cell lysates and sub-cellular fractionation

Whole cell lysates were prepared by incubating cells in 10 mM Tris (pH 7.4), 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL leupeptin for 20 minutes at 4°C. Samples were centrifuged at 15,000 rpm in a microcentrifuge, 4X SDS-PAGE sample buffer was added to the supernatant, and samples were boiled for 5 minutes.

Nuclear extracts were prepared using an adaption of the protocol by Dyer and Herzog (1995). Briefly, cell pellets were resuspended in sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM Mg Acetate, 1 mM Dithiothreitol, 0.5 mM Phenylmethylsulfonyl Fluoride) with 0.5% [vol/vol] IGEPAL by gentle pipetting and centrifuged. The nuclei were washed twice in sucrose buffer without IGEPAL. Nuclei were then resuspended in Buffer A (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), incubated on ice for 30 minutes, and centrifuged at 15,000 rpm in a microcentrifuge at 4°C. Nuclear lysates were stored at -80°C.

Immunoblotting.

Cell extracts were subjected to SDS-PAGE by the method of Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol with a Genie Electroblotter (Idea Scientific Inc., Minneapolis, MN). Membranes were blocked overnight at 4°C in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk (NFDM). Antibodies were diluted in TBS containing 1% NFDM and the membranes were incubated with primary antibodies for at least 1.5 hours at room temperature at 1 μg/mL (3-14B) or at a concentration recommended by the manufacturer. HRP-conjugated secondary antibodies, donkey anti-rabbit IgG (Amersham Pharmacia, Piscataway, NJ) were used according to the manufacturer's instructions. Following each antibody treatment, blots were washed at least three times in TBS containing 0.05% Tween 20. Antibody complexes were visualized by chemiluminescence (Pierce, Rockford, IL).

DNA binding assay

Electrophoretic mobility shift assays (EMSA) were used to assess sequence specific binding of nuclear AhR to DNA (Lawrence *et al.*, 1996). Briefly, a synthetic 26-base pair consensus DRE probe (upper strand 5'-GATCTGGCTCTTCTCACGCAACTCCG-3' and lower strand 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') was labeled with α-[^{32}P]dATP using Klenow fragment (Invitrogen, Carlsbad, CA) and used for DNA binding assays. Nuclear extracts were prepared as described above. Samples (10 μg or 5 X 10^5 cell equivalents) were incubated with 10X HED buffer (0.25 M HEPES, 15 mM EDTA, 10 mM DTT), 4 μg poly dI-dC (Amersham Pharmacia, Piscataway, NJ), 120 mM NaCl, and 100,000 cpm of ^{32}P-labeled DRE for 20 minutes at room temperature. Anti-AhR was added to the reaction mixture and incubated for 10 minutes at room temperature. Products of the binding reaction were separated on a 5%

polyacrylamide gel in 0.5% TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Results

The cell lines RAW264.7, WEHI 3, and DC2.4, representing the APC populations of macrophages, monocytes, and DC respectively, were used to determine the distribution and behavior of the AhR in APCs. These cell lines, derived from C57Bl/6 mice, are considered sensitive to TCDD toxicity due to their allelic expression of AhR^{bb}. Expression of AhR in these cell lines was initially investigated by RT-PCR and then by immunoblotting. Cells were also activated with LPS, because it has been shown that activation of human monocytes and macrophages increased AhR mRNA levels when compared to resting cells (Hayashi *et al.*, 1995).

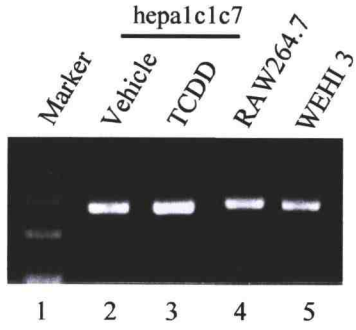
RNA from RAW264.7 and WEHI 3 cells was examined using AhR-specific primers. As seen in Figure 2-1A, RT-PCR analysis demonstrated RAW264.7 and WEHI 3 cells lines expressed AhR message. Hepa1c1c7 cells were used as a positive control (Fig. 2-1A) and message integrity was verified with β2-microglobulin specific primers (data not shown).

Expression of AhR protein in resting and LPS-activated APC exposed to TCDD or vehicle control was analyzed from whole cell lysates and visualized by immunoblot, as shown in Figure 2-1B. The three APC lines expressed AhR protein, and there appeared to be no visible difference in AhR expression between resting and LPS-activated cells. The appearance of two smaller bands corresponding to AhR protein in vehicle and TCDD-treated WEHI3 cells (Fig. 2-1B) are a by-product of the anti-AhR used, as reported by the manufacturer.

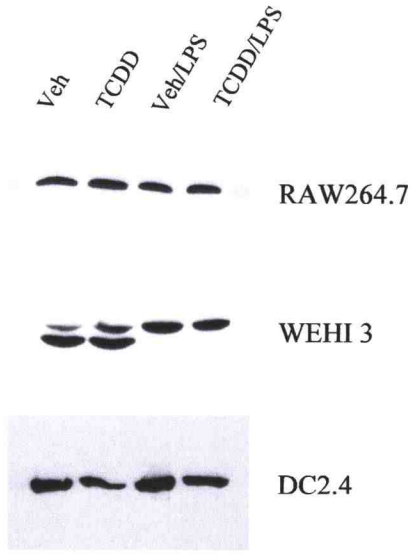
Fig. 2-1. Macrophage and monocyte cell lines expressed AhR. (A) AhR message was amplified from hepa1c1c7, RAW264.7 and WEHI 3 cDNA libraries as described in the *Materials and Methods*. (B) Whole cell lysates (50 μg) from RAW264.7, WEHI 3 and DC24 cell treated with 10^{-9} M TCDD or vehicle control (0.01% DMSO) and 1 μg/mL LPS were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-AhR antibodies and HRP-secondary antibodies. Bound antibodies were detected with chemiluminescent reagents. Data are representative of two or more independent experiments.

Figure 2-1

A.


hepa1c1c7
Marker
Vehicle
TCDD
RAW264.7
WEHI 3
1
2
3
4
5

B.


Veh
TCDD
Veh/LPS
TCDD/LPS
RAW264.7
WEHI 3
DC2.4

Following ligand binding and translocation to the nucleus, the AhR interacts with specific regions in DNA called DREs and promotes transcription of gene products. We examined the ability of the AhR to bind to DRE motifs in DNA following exposure with TCDD. Nuclear lysates from resting or LPS-activated cells, exposed to TCDD or vehicle control, were analyzed by EMSA to confirm AhR interaction with DREs. To identify accurately the binding of the AhR to DRE motifs, hepa1c1c7 cells were used as a control, as seen in Figure 2-2A. Detectable amounts of basal AhR-DRE complexes were observed only in RAW264.7 and DC2.4 cells (Fig. 2-2B, lane 1 and 9), but not in WEHI 3 cells. Exposure to TCDD increased AhR-DRE interactions in RAW264.7 cells (Fig. 2-2B, lane 2), but not in the other two cell lines. LPS activation of the APC cell lines increased AhR-DRE interactions in the absence of TCDD in RAW264.7 and DC2.4 cells (Fig. 2-2B lane 3 and 11). However, TCDD did not appear to increase AhR-DRE complexes in an additive fashion in these activated cells (Fig. 2-2B, lane 4 and lane 12).

Analysis of bone marrow-derived DC (BMDC) and splenic DC from Flt3L-treated mice allowed for further characterization of the presence and behavior of the AhR in DC. BMDC and Flt3L DC from the spleens of Balb/c mice treated for nine days with Flt3L were purified by sorting for the expression of the DC surface marker CD11c. Cell lysates from hepa1c1c7 cells, a positive control for AhR, and from sorted BMDC and Flt3L DC, were examined for the expression of AhR as visualized by immunoblotting. The two DC populations studied expressed AhR, and the difference in the migration of the bands corresponding to AhR protein was due to the strains of mice used (Fig. 2-3). The BMDC were generated from C57Bl/6 mice that have been shown to express an AhR with a molecular mass of 95 kDa, and the Flt3L splenic DC were harvested from AhR^{bb} Balb/c mice, expressing a 109 kDa AhR.

Binding of the AhR to DRE motifs in DC was analyzed by EMSA. Nuclear lysates from purified BMDC and Flt3L DC were treated with TCDD or vehicle

Fig. 2-2. DNA binding analysis of nuclear protein extracts from APC lines treated with vehicle or TCDD. Binding of the AhR to ^{32}P-labeled DRE was analyzed by EMSA. Nuclear lysates (5 μg) from (A) hepa1c1c7 cells treated with 10^{-9} M TCDD or vehicle control (0.01% DMSO) for two hours and supershifted with increasing amounts anti-AhR antibody, as a positive control. (B) RAW264.7, WEHI 3 and DC2.4 cells were treated with 10^{-9} M TCDD or vehicle control and 1 μg/mL LPS for two hours. The arrow identifies the AhR-DRE complexes. Data are representative of at least two independent experiments.

Figure 2-2

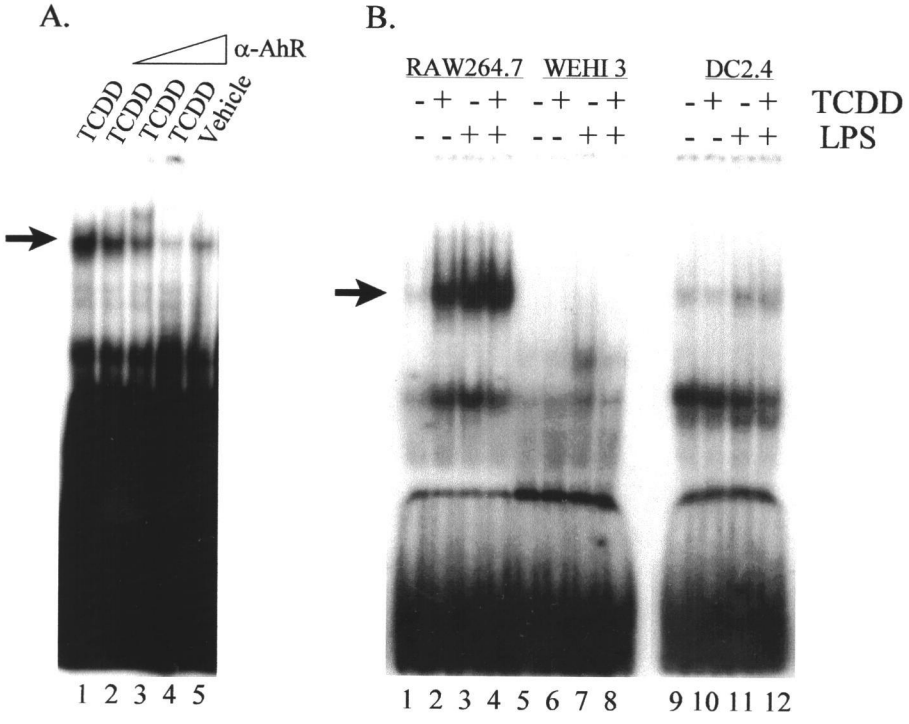
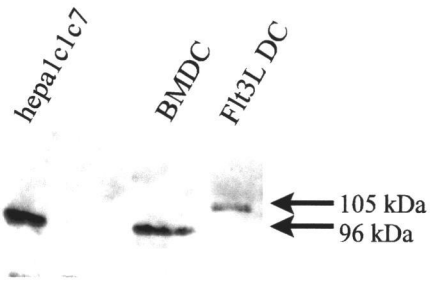

A.
α-AhR
TCDD
TCDD
TCDD
TCDD
Vehicle
1 2 3 4 5
B.
RAW264.7
WEHI 3
DC2.4
- + - + - + - + - + - +
TCDD
- - + + - - + + - - + +
LPS
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 2-3. Expression of AhR in BMDC and splenic DC from Flt3L-treated mice. BMDC and DC from the spleens of two Flt3L-treated mice were purified by sorting and treated with 10^{-9} M TCDD or vehicle control (0.01% DMSO). Hepa1c1c7 cells were used as a positive control. Whole cell lysates (1 X 10^6 cell equivalents for BMDC, and 5 X 10^5 cell equivalents for hepa1c1c7 and Flt3L DC,) were separated by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-AhR antibodies and HRP- secondary antibodies. Bound antibodies were detected with chemiluminescent reagents. Data are representative of two independent experiments.

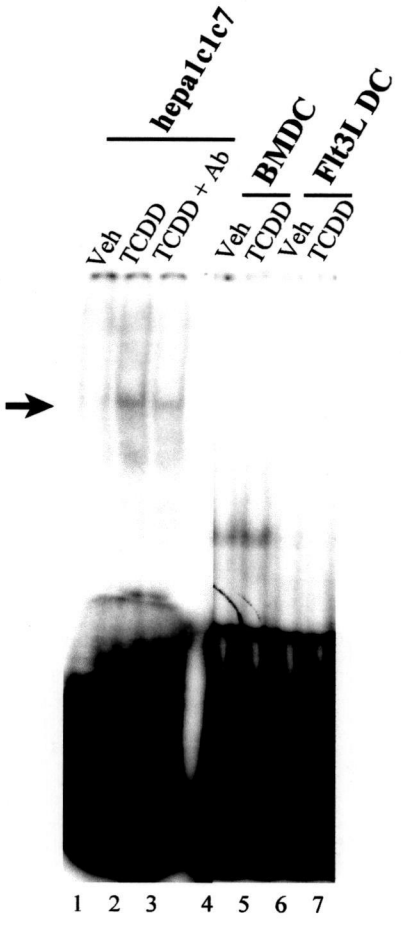
Figure 2-3



control and analyzed following an incubation of two hours at 37°C. Hepa1c1c7 cells were used as a positive control for AhR interaction with DRE (Fig. 2-4, lane 2). We were unable to detect any AhR-DRE complexes in BMDC or Flt3L DC, neither in the vehicle nor TCDD-exposed cells (Fig. 2-4, lanes 4, 5, 6, and 7).

Fig. 2-4. DNA binding analysis of nuclear protein extracts from BMDC and splenic DC from Flt3L-treated mice. Binding of the AhR to ^{32}P-labeled DRE was analyzed by EMSA. Nuclear lysate (10 μg) from hepa1c1c7 cells treated with 10^{-9} M TCDD or vehicle control (0.01% DMSO) for two hours, and antibodies to AhR was added to supershift the AhR as a control for AhR-DRE complexes. Nuclear lysates from purified BMDC and splenic DC from Flt3L-treated mice were treated with 10^{-9} M TCDD or vehicle control for two hours and 10 μg of nuclear lysate was analyzed by EMSA. The arrow identifies the AhR-DRE complexes.

Figure 2-4


hepa1c1c7
BMDC
Flt3L DC
Veh
TCDD
TCDD + Ab
Veh
TCDD
Veh
TCDD
1 2 3 4 5 6 7

Discussion

The AhR has been identified as the primary mediator of TCDD-induced immunotoxicity. AhR-knockout animals exposed to TCDD generated normal immune responses to sheep red blood cells and P815 tumor cells (Vorderstrasse *et al.*, 2001), and did not exhibit thymic atrophy, a hallmark of TCDD immunotoxicity (Staples *et al.*, 1998). The importance of the AhR in TCDD-induced immune suppression prompted us to evaluate the status and behavior of the AhR in APCs, which are essential in activating naive T cells. The results show that murine APCs, represented by macrophage, monocyte, and DC lines, express AhR protein. In addition, we show for the first time that bone marrow-derived DC and Flt3L-generated DC also express AhR. Although all APCs examined expressed AhR and in some cases had detectable levels of basal AhR-DRE binding, TCDD exposure led to increased AhR-DRE complexes only in the macrophage cell line, RAW264.7.

Although monocytes are precursors of macrophages, the differences between the AhR-DRE binding in these cells may be due to maturation state. In rats, monocytes in the peripheral blood and peritoneal cavity were shown to express less CYP1A1 following TCDD exposure compared with mature macrophage populations. These differences between monocytes and macrophages appeared to be related to the increased level of AhR protein expressed in macrophages (Germolic *et.al.*, 1995). Macrophage populations appear be directly targeted by TCDD through increased AhR-DRE interactions observed in these studies, that could promote or inhibit various gene products, including CYP1A1 and cyclooxygenase 2 (Kraemer et al., 1996).

Our studies also revealed that activation of with LPS led to increased AhR-DRE binding in the RAW264.7 and DC2.4 cells. This finding is in agreement with prior observations that cellular activation in the absence of a exogenous ligand increases AhR-DRE interactions in activated splenocytes (Crawford *et al.*, 1997).

Activation of the RAW264.7, WEHI 3 and DC2.4 cell lines did not alter the expression of the AhR protein, in contrast to B cells which increase AhR expression (Marcus *et al.*, 1998; Sulentic *et al.*, 1998). One explanation for the lack of increases in AhR protein levels in our studies, is most likely due to the short activation time of two hours used compared to longer activation times in the previous studies. Another explanation for the differences in AhR expression may be the expression of the AhR is regulated differently in B cells.

These studies demonstrated that binding of the AhR to DRE motifs in DC did not appear to be induced by TCDD. Although, we were able to detect AhR-DRE interactions by EMSA of the DC2.4 cell line, we did not detect AhR binding to DREs in the other two DC types BMCD and splenic. To verify these results the exposure time of X-ray films exposed to the dried EMSA gels was lengthened, ensuring low level AhR binding was not overlooked (data not shown). One possible explanation could be related to AhR repressor (AhRR) protein expression. The AhRR is a member of the bHLH/PAS transcription family and competitively binds to ARNT and the resulting heterodimer interacts with DRE sequences, thereby blocking the activity of the AhR (Minura *et.al.*, 1999; Gradin *et. al.*, 1999). The increased presence or the AhRR in DC could account for the lack of increased AhR-DRE interactions in TCDD-exposed DC, and allow for an increase in undimerized AhR, potentially leading to non-DRE mediated toxicity. Indeed, a number of reports have identified possible mechanisms of AhR-mediated toxicity not requiring binding to DRE motifs. Exposure to TCDD has been shown to rapidly increase Ca^{++} concentration and protein phosphorylation independent of DRE mediated events, but dependent on the AhR (Enan *et al.*, 1998; Clark *et al.*, 1991). Moreover, the AhR has also been shown to interfere with the signaling of other transcription factors including the estrogen receptor (Klinge *et al.*, 2000) and Rb (Ge and Elferink, 1998; Elferink *et al.*, 2001). Interestingly, we have shown that TCDD suppresses the

activation of NF-κB/Rel in DC2.4 cells, apparently through an interaction between the AhR and Rel A (Ruby, submitted 2002; Tian *et al.*, 1999; Kim *et al.*, 2000).

In conclusion, we have characterized the status and behavior of the AhR in APC populations of macrophages, monocytes, and DC. While the AhR was detected in all of the cell types representing APC, TCDD only increased AhR-DRE binding in the macrophage cell line. These data suggest all APC populations are potential direct targets of TCDD, but that toxicity may be induced by different pathways of AhR signaling.

Chapter 3

2,3,7,8 Tetrachlorodibenzo-*p*-dioxin Suppresses TNFα and Anti-CD40 Induced Activation of NF-κB/Rel in Dendritic Cells: p50 Homodimer Activation is Not Affected

Authors:

Carl E. Ruby

Mark Leid

Nancy I. Kerkvliet

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Abstract

2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCDD) suppresses many immune responses, both innate and adaptive. Suppression is mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor. The AhR mediates TCDD toxicity presumably through alteration of transcriptional events, either by promoting gene expression or potentially by physically interacting with other transcription factors. Another transcription factor, NF-κB/Rel, is involved in several signaling pathways in immune cells, and is crucial for generating effective immune responses. DC, considered to be the "pacemakers" of the immune system, are recently recognized as targets of TCDD and are also dependent on NF-κB/Rel for activation and survival. In these studies we investigated if TCDD would alter the activation of NF-κB/Rel in dendritic cells (DC). The dendritic cell line, DC2.4, was exposed to TCDD prior to treatment with TNFα or anti-CD40, and NF-κB/Rel activation was measured by EMSA and immunoblotting. TCDD suppressed the binding of NF-κB/Rel to its cognate response element in TNFα- and anti-CD40-treated cells and blocked translocation to the nucleus. The AhR was shown to associate with Rel A, following co-immunoprecipitation, and appeared to block their binding to DNA. Interestingly, p50 homodimers freely bound to DNA. These results suggest that TCDD may alter the balance between NF-κB/Rel heterodimers and transcriptional inhibitory p50 homodimers in DC, leading to defects in the DC and suppression of the immune response.

Introduction

The immune system is a sensitive target of TCDD, a widespread environmental contaminant that induces a host of biochemical and pathological

effects (Kerkvliet and Burleson, 1994). Numerous studies linking TCDD exposure to an increase in susceptibility to various pathogens, and suppression of humoral and cell mediated immune responses in mice, verify the impact of TCDD on the immune system (House *et al.*, 1990; Kerkvliet and Brauner, 1987; Kerkvliet and Baecher-Steppan, 1988). TCDD toxicity is primarily mediated by the aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helix transcription factor (reviewed in Rowlands and Gustafsson, 1997). Binding of TCDD to AhR in the cytoplasm initiates shedding of two heat shock protein 90s and an immunophillin protein complexed with AhR, allowing rapid translocation of AhR into the nucleus. The activated AhR, following dimerization with ARNT, promotes the expression of various genes through an interaction with specific regions of DNA called dioxin response elements (DRE). In contrast to the direct influence on gene expression via the DRE, the AhR might also alter gene expression through indirect means as seen in the increased expression of the transcription factor AP-1 (Puga *et al.*, 1992). Additional affects of the AhR on transcription factors include cross talk with the estrogen receptor (Klinge *et al.*, 2000) and physical association with retinoblastoma protein (Ge and Elferink, 1998) and Rel A (Tian *et al.*, 1999).

The transcription factor NF-κB/Rel is intimately involved in the immune system (reviewed in Baldwin, Jr., 1996; Ghosh *et al.*, 1998). NF-κB/Rel consists of a family of five proteins p50, p52, Rel A, RelB and c-rel, forming various DNA-binding homo- and heterodimeric complexes. The Rel proteins, Rel A, RelB, and c-rel, share a conserved NH_2 terminus identified as the Rel homology domain and a nonconserved COOH terminus containing a transcriptional activation domain. In contrast, p50 and p52 lack transcriptional activation domains, forming homodimer complexes that may inhibit transcription (Lernbecher *et al.*, 1993). Activation of NF-κB/Rel is regulated by a battery of inhibitor proteins called IκBs that block the nuclear localization signal, leading to cytoplasmic sequestration. Phosphorylation of the inhibitor protein on specific serine moieties directs its degradation, allowing NF-

κB/Rel to translocate to the nucleus and influence gene expression. The immune system is dependent on NF-κB/Rel for the transcription of some of its most critical genes, including cytokines and signaling proteins. Moreover, the dependence of the immune system on NF-κB/Rel is apparent in mice with deleted NF-κB/Rel. Such knockout mice display a wide range of immune defects from suppressed humoral and cell mediated immunity to a profound loss of DC (Wu *et al.*, 1998; Doi *et al.*, 1997; Sha *et al.*, 1995).

TCDD-exposed mice show many similarities to NF-κB/Rel knockout mice. However apart from the role of the AhR, the biochemical and cellular mechanisms underlying TCDD immunotoxicity have yet to be elucidated. A potential mechanism of toxicity could be via alterations in the transcription factor NF-κB/Rel. DC have recently been shown to be a target of TCDD toxicity (Vorderstrasse and Kerkvliet, 2001; Shepherd *et al.*, 2001), providing us with a highly relevant model to study this potential mechanism of TCDD immunotoxicity. DC are integral in the regulation of the immune system as the most potent antigen-presenting cell, inducing and maintaining immune responses (Banchereau and Steinman, 1998). Furthermore, on a molecular level, DC rely on NF-κB/Rel to mediate their differentiation, maturation and survival (Oyama *et al.*, 1998; Verhasselt *et al.*, 1999; Rescigno *et al.*, 1998). In the studies reported here, we have investigated the effects of TCDD on the activation of NF-κB/Rel in DC by TNFα and anti-CD40, widely shown to activate NF-κB/Rel (Baldwin, Jr., 1996; Ghosh *et al.*, 1998). We utilized a DC cell line, DC2.4, and employed DNA binding assays and various other immunoblot techniques to determine the activation of NF-κB/Rel. Our results show that TCDD induces suppression of DNA binding and nuclear translocation of NF-κB/Rel in TNFα- and anti-CD40-activated DC2.4 cells. This suppression appeared to be mediated predominantly by an association between the AhR and Rel A, inhibiting the binding of NF-κB/Rel heterodimers to DNA. In contrast p50 homodimers were unaffected by TCDD. These results suggest that TCDD may alter the balance between NF-

κB/Rel heterodimers and transcriptional inhibitory p50 homodimers in DC, leading to defects in the DC and suppression of the immune response.

Materials and Methods

Reagents and antibodies

Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Recombinant mTNFα was purchased from Preprotech (London, UK). TCDD (≥99% pure) was purchased from Cambridge Isotope Laboratories Inc. (Woburn, MA). All other reagents and cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Anti-CD40 (FGK45.5) antibodies were provided by Dr. Tony Vella (Oregon State University, Corvallis, OR). Anti-murine AhR antibodies (3-14B) were provided by Dr. Alan Poland (NIOSH, Morgantown, WV) and were raised against synthetic peptides corresponding to the N-terminal of the C57Bl/6 mouse liver AhR (A. Poland, personal communication). Antibodies against Rel A, RelB, c-rel, p50, p52, IκBα, and actin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-phospho-IκBα was purchased from Cell Signaling Technologies (Beverly, MA). The pcDNA3/βmAhR-FLAG construct was a gift from Dr. Gary Perdew (Pennsylvania State University, University Park, PA). Dexamethasone was purchased from Sigma (St. Louis, MO).

Cell culture

The cell line DC2.4, derived from C57Bl/6 mice, was provided by Dr. Kenneth L. Rock (Division of Lymphocyte Biology, Dana Farber Cancer Institute, Boston, MA) (Shen *et al.*, 1997). DC2.4 cells were maintained in DMEM medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 10 mM Na-pyruvate, and 50 μg/mL gentamicin. Cultures were grown to 75% confluency,

treated with TCDD (10^{-9} M) for 24 hours, and activated with TNFα (10 ng/mL) or anti-CD40 (25 μg/mL) for two hours before harvesting. Dexamethasone (10^{-9} M) controls were treated for two hours prior to the addition of TNFα and harvested after two hours.

Whole cell lysates and sub-cellular fractionation

Whole cell lysates were prepared by incubating DC2.4 cells in 10 nM Tris (pH 7.4), 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL leupeptin for 20 minutes at 4°C. Samples were centrifuged at 15,000 rpm in a microcentrifuge, 4X SDS-PAGE sample buffer was added to the supernatant, and placed in boiling water for 5 minutes.

Nuclear and cytoplasmic extracts were prepared as described (Dyer and Herzog, 1995). Briefly, cell pellets were resuspended in sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 2 mM Mg acetate, 1 mM DTT, 0.5 mM PMSF) with 0.5% [vol/vol] IGEPAL nonionic detergent by gentle pipetting and centrifuged. To the cytoplasmic fraction, 0.22 volumes of 5X cytoplasmic extraction buffer (0.15 M HEPES, 0.7 M KCl, 0.015 M $MgCl_2$) was added. The cytoplasmic fraction was then centrifuged at 15,000 rpm in a microcentrifuge, and the supernatant was transferred to a fresh tube containing 25% vol/vol glycerol and stored at -80°C. The nuclei were washed twice in sucrose buffer without IGEPAL. Nuclei were then resuspended in low salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and then one volume of high salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM KCl, 0.2 mM EDTA 1% IGEPAL , 0.5 mM DTT, 0.5 mM PMSF) was carefully added in 1/4 increments. Nuclei were incubated on ice for 30 minutes, diluted 1:2.5 with diluent (25 mM HEPES, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, 0.5 PMSF) and centrifuged at 15,000 rpm in a microcentrifuge at 4°C. Nuclear lysates were stored at -80°C.

DNA binding assay

Electrophoretic mobility shift assays (EMSA) were used to assess sequence specific binding of DC2.4 nuclear NF-κB/Rel to DNA (Dyer and Herzog, 1995). Briefly, a synthetic 20-bp consensus κB-RE probe (upper strand 5'-GATCGGCAGGGGAATTCCCC-3' and lower strand 5'-GATCGGGGAATTCCCCTGCC-3') was labeled with α-[^{32}P]dATP using Klenow fragment (Invitrogen, Carlsbad, CA) and used for DNA binding assays. Nuclear extracts were prepared as described above. Samples (5 μg) were incubated with binding buffer (12 mM HEPES, pH 7.3; 4 mM Tris-HCl, pH 7.5; 100 mM KCl; 1 mM EDTA; 20 mM DTT; 1 mg/mL bovine serum albumin), 4 μg poly dI-dC (Amersham Pharmacia, Piscataway, NJ) and 100,000 cpm of ^{32}P-labeled κB-RE for 20 minutes at room temperature. For supershift analysis antibodies to Rel A, RelB, c-rel, p50 and p52 were added to the reaction mixture according to the manufacturer's instruction (Santa Cruz Biotech, Santa Cruz, CA) and incubated for 10 minutes at room temperature. Samples were analyzed on a 5% polyacrylamide gel in 0.5% TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and visualized by autoradiography.

Immunoblotting

Cell extracts were subjected to SDS-PAGE by the method of Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol using a Genie Electroblotter (Idea Scientific Inc., Minneapolis, MN). Membranes were blocked overnight at 4°C in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk (NFDM). Antibodies were diluted in TBS containing 1% NFDM and the membranes were incubated with primary antibodies for at least 1.5 hours at room temperature. The primary antibodies, anti-Rel A, anti-RelB, anti-c-rel, anti-actin, anti-IκBα, and anti-phospho-IκBα, were used according to the manufacturers'

instructions. HRP-conjugated secondary antibodies, donkey anti-rabbit IgG and goat anti-mouse IgG, were used according to the manufacturers' instructions. Following each antibody treatment, blots were washed three times in TBS containing 0.05% Tween 20. Antibody complexes were visualized by chemiluminescence (Pierce, Rockford, IL).

Transient transfections

DC2.4 cells were transfected at 80% confluency in 25 cm^2 tissue culture flasks by a lipofectamine procedure as specified by the manufacturer (Invitrogen, Carlsbad, CA). The cells were transfected with pcDNA3/βmAhR-FLAG (Gary Perdew, Pennsylvania State University, University Park, PA) . Transfection efficiency was determined to be about 35% by intracellular staining and analysis by FACS (data not shown). The transfected cells were harvested with trypsin-EDTA and washed once in PBS.

Intracellular staining and flow cytometry

DC2.4 cells were collected and washed in cold PAB (PBS, 1% fetal bovine serum, 0.1% sodium azide) and then washed in PAB-0.05% saponin. Cells were treated with mouse IgG or goat IgG to block nonspecific binding and then appropriate purified antibodies were added, followed by the addition of fluorochrome-conjugated streptavidin and secondary antibodies. FITC-labeled anti-actin antibody was used as a positive control (Sigma, St. Louis, MO). For each sample, at least 10,000 events were collected as listmode data. Listmode data were collected on a Coulter XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME).

Co-Immunoprecipitation

Cytosolic lysate isolated from pcDNA3/βmAhR-FLAG transfected cells was immunoprecipitated with streptavidin magnetic beads (Dynal, Oslo, Norway) coated with biotinylated anti-FLAG antibody (Sigma, St. Louis, MO). As a control, magnetic beads without anti-FLAG antibody were used. Cytosol in buffer I (0.32 M sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 10 nM Tris-HCl, pH8.0, 1 mM DTT, 0.5 mM PMSF, 2 mM MgAc, and 0.5% IGEPAL) was rotated for two hours with 25 μL of anti-FLAG magnetic beads at 4°C. The beads were washed in fresh buffer I four times. The beads were then resuspended in 2X SDS sample buffer and incubated in boiling water for five minutes. The samples were analyzed for Rel A, RelB and c-rel by immunoblotting.

Results

TCDD decreases the binding of NF-κB/Rel in TNFα and anti-CD40 treated DC2.4 cells

The binding of TNFα to CD120 (TNFR) and ligation of CD40 by CD154 are two events that lead to activation of NF-κB/Rel in DC (Baldwin, Jr., 1996; Ghosh *et al.*, 1998). The TNFR and CD40 signal activation of NF-κB/Rel, via proteins associated with the NH_2 terminus of the receptors called TRAFs. These TRAFs initiate the phosphorylation of IκBs by activating IκB kinases (Rothe *et al.*, 1995), leading to translocation of NF-κB/Rel into the nucleus and binding to κB response elements in DNA. To determine if TCDD altered the activation of NF-κB/Rel, DC2.4 cells were exposed to TCDD or vehicle control for 24 hours, and then activated for two hours with TNFα or anti-CD40. NF-κB/Rel activation was measured by EMSA.

We identified at least two bands corresponding to NF-κB/Rel binding in DC2.4 cells treated with TNFα and anti-CD40 (Fig. 3-1A and 3-1B). TNFα or anti-CD40 treatment both increased the intensity of the NF-κB/Rel bands (Fig. 3-1A, lanes 1 v. 3 and Fig. 3-1B, lanes 1 v. 3) verifying the capacity of TNFα and anti-CD40 to activate NF-κB/Rel in these cells. TNFα induced greater activation of NF-κB/Rel compared to anti-CD40 in several independent experiments. DC2.4 cells exposed to TCDD and then treated with TNFα demonstrated a decrease in the intensity of the bands corresponding to NF-κB/Rel binding DNA when compared to vehicle treated controls (Fig. 3-1A, lanes 3 v. 4). We also observed a decrease in NF-κB/Rel binding in DC2.4 cells exposed to TCDD and activated for two hours with anti-CD40 (Fig. 3-1B, lane 3 and 4). In Fig. 3-1B (lanes 1 and 2), treatment with TCDD in the absence of an activation signal increased the intensity of the upper band corresponding to NF-κB/Rel binding to DNA. However this phenomenon proved to be inconsistent, as seen in Fig. 3-1A, (lane 1 v. lane 2) as well as in additional experiments. As a control, we treated cells with dexamethasone, shown previously to suppress NF-κB/Rel activation (Auphan *et al.*, 1995). In Fig. 3-1C (lanes 1 and 2) dexamethasone suppressed TNFα induced activation of NF-κB/Rel. Thus, TCDD appeared to inhibit, NF-kB/Rel DNA binding to a similar extent dexamethasone inhibited binding.

Antibody-supershift analysis of NF-κB/Rel binding in TNFα and anti-CD40 activated DC2.4 cells

The composition of the NF-κB/Rel dimers that corresponded to the two bands seen in the previous EMSA (Fig. 3-1A and 3-1B) were characterized by antibody-supershift. The upper band from both TNFα and anti-CD40 activated cells was supershifted by antibodies to Rel A, RelB, p50, and to a lesser extent c-rel in anti-CD40 activated cells (Fig. 3-2A and 3-2B). In contrast, the lower band was

Fig. 3-1. TCDD suppresses NF-κB/Rel activation. (A) Nuclear extracts from cells exposed to 10^{-9} M TCDD or DMSO (0.01%) for 24 hours and then treated with 10 ng/mL TNFα for two hours were used for EMSA. A κB-RE sequence was used to detect the κB-binding activity. (B) Nuclear extracts from cells exposed to 10^{-9} M TCDD or DMSO (0.01%) for 24 hours and then treated with 25 μg/mL anti-CD40 (FGK45.5) for two hours were used for EMSA. (C) Nuclear extracts from cells treated with 10^{-6} M dexamethasone (Dex) one hour prior to treatment with 1 ng/mL TNFα were used as a control (lanes 1 and 2). The results are representative of at least two or more separate experiments.

Figure 3-1

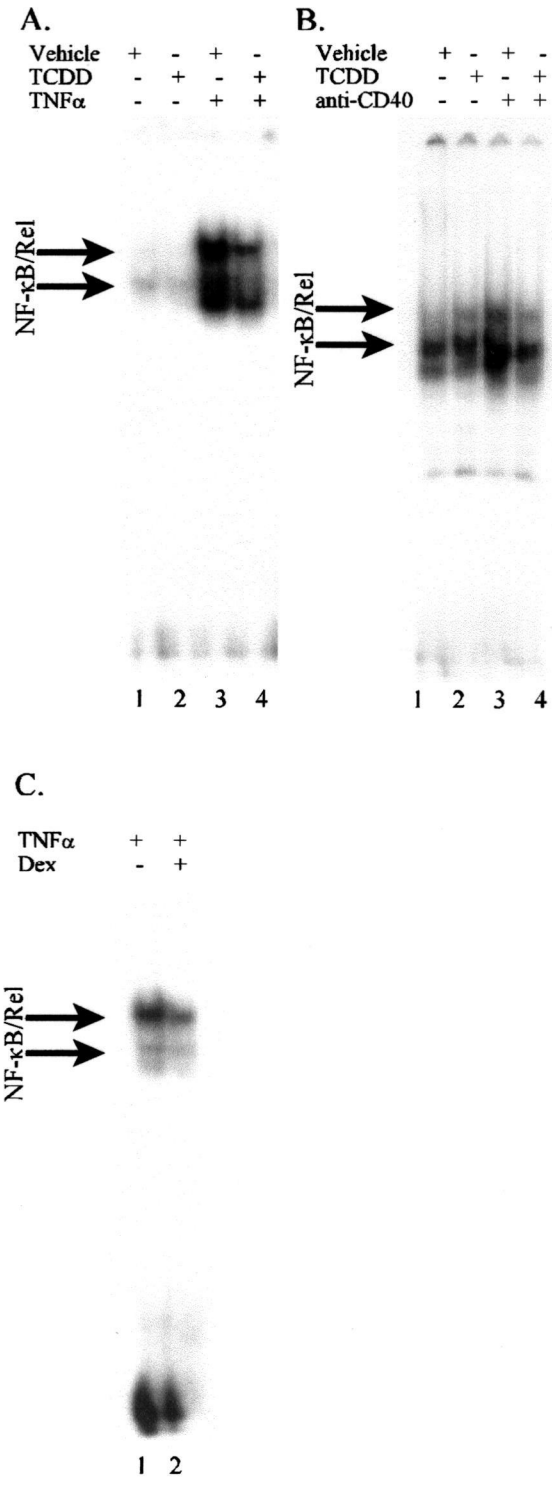
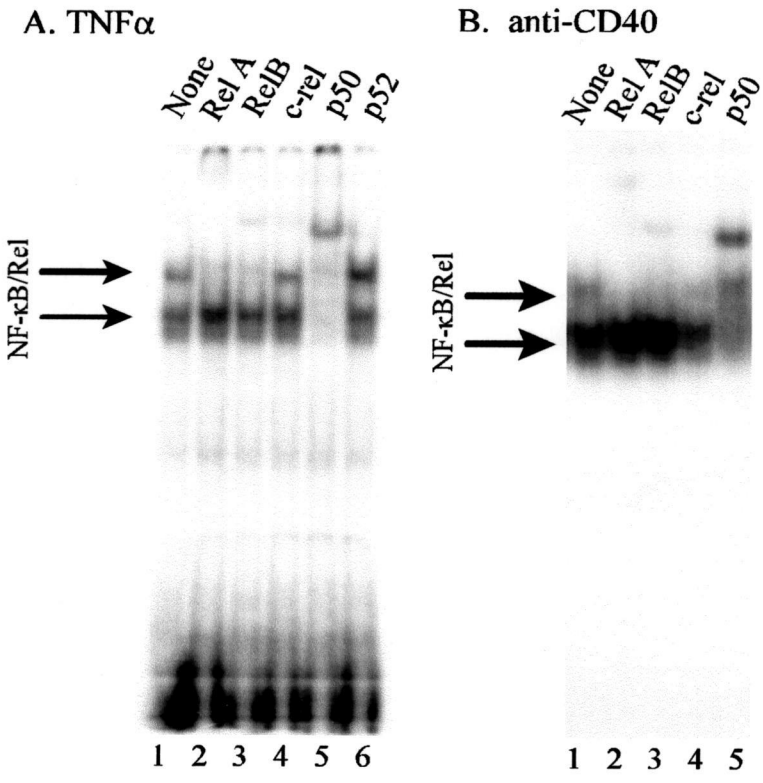

A.
Vehicle + - + -
TCDD - + - +
TNFα - - + +
NF-κB/Rel
1 2 3 4
B.
Vehicle + - + -
TCDD - + - +
anti-CD40 - - + +
NF-κB/Rel
1 2 3 4
C.
TNFα + +
Dex - +
NF-κB/Rel
1 2

Fig. 3-2. Supershift analysis of nuclear lysates from DC2.4 cells. Nuclear extracts from cells treated with (A)10 ng/mL TNFα and (B) 25 μg/mL anti-CD40 were incubated with specific antisera against the different members of the NF-κB/Rel family before EMSA.

Figure 3-2


A. TNFα
None
Rel A
RelB
c-rel
p50
p52
NF-κB/Rel
1 2 3 4 5 6
B. anti-CD40
None
Rel A
RelB
c-rel
p50
NF-κB/Rel
1 2 3 4 5

supershifted only by antibodies to p50 (Fig. 3-2A lane 5 and Fig. 3-2B lane 5). These data indicate that the lower band likely corresponds to p50 homodimer binding and the upper band is consistent with Rel/p50 heterodimer binding.

TCDD decreased levels of NF-κB/Rel in the nucleus following TNFα activation

An important step in NF-κB/Rel activation is translocation of the transcription factor to the nucleus. Following the proteolysis of IκBα, the cytoplasmic inhibitor protein, NF-κB/Rel translocates to the nucleus where it induces gene expression. In order to determine a possible explanation to account for the decreased binding of NF-κB/Rel in TCDD exposed cells, we analyzed the ability of NF-κB/Rel to translocate to the nucleus following TNFα activation. Nuclear NF-κB/Rel protein was visualized by immunoblot from DC2.4 cells exposed to TCDD for 24 hours and activated for two hours with TNFα. In Fig. 3-3, the levels of Rel A, RelB and to a lesser extent c-rel protein were decreased in the nucleus of TCDD-exposed cells activated with TNFα compared to vehicle controls. We additionally demonstrated dexamethasone treatment decreased the level of Rel A proteins, in the nucleus following activation with TNFα (data not shown). The effect of TCDD on NF-κB/Rel appears to occur upstream of translocation.

TCDD does not alter levels of cellular NF-κB/Rel protein

Because TCDD treatment decreased NF-κB/Rel binding to DNA and reduced levels of Rel proteins in the nucleus, a possible mechanism for these effects could be a reduction in NF-κB/Rel protein expression. To determine if TCDD alters the expression of NF-κB/Rel, cellular levels of NF-κB/Rel in DC2.4 cells exposed to TCDD or a vehicle control for 24 hours were visualized by immunoblot. Protein levels of NF-κB/Rel in cells exposed to TCDD for 24 hours were unaltered when compared to vehicle controls (Fig. 3-4). In addition, there was no observable effect

Fig 3-3. TCDD suppresses translocation of NF-κB/Rel. Nuclear extracts from DC2.4 cells treated with 10^{-9} M TCDD or vehicle controls 24 hours prior to treatment with 10 ng/mL TNFα for 2 hours were separated on SDS-PAGE and immunoblotted with antibodies specific to Rel A, RelB and c-rel. Gels were loaded with 25 μg of nuclear extract. Results are representative of three separate experiments.

Figure 3-3

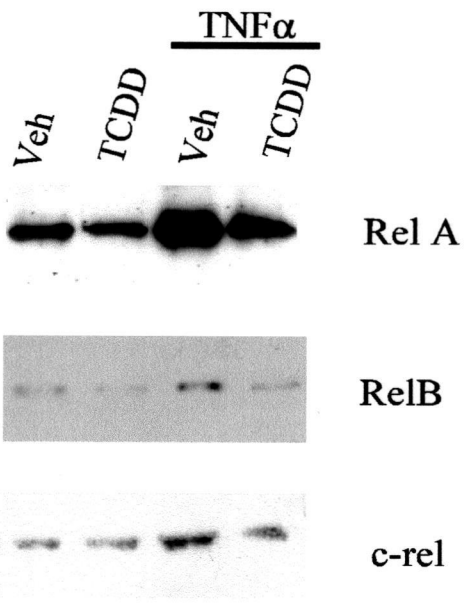
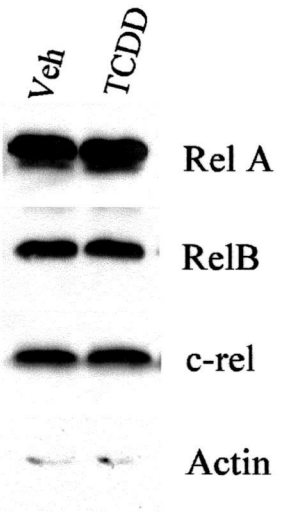


Fig. 3-4. Protein levels of NF-κB/Rel are not altered by TCDD. Whole cell extracts from DC2.4 cells treated with 10^{-9} M TCDD or vehicle control were separated by SDS-PAGE and immunoblotted with antibodies specific to Rel A, RelB or c-rel. Results are representative of two or more separate experiments.

Figure 3-4



of TCDD on NF-κB/Rel in intracellular staining using FACS analysis (data not shown). Protein expression of Rel proteins is not likely affected by TCDD.

TCDD does not alter phosphorylation or proteolysis of IκBα

NF-κB/Rel is sequestered in the cytoplasm by IκBα which, when phosphorylated at specific serine residues, earmarks it for destruction (Karin and Ben Neriah, 2000). IκBα degradation permits translocation and subsequent binding of NF-κB/Rel to DNA. Thus the level of expression of IκBα is a pivotal element in the activation of NF-κB/Rel. This was demonstrated following dexamethasone treatment, which increases expression of IκBα leading to suppression of NF-κB/Rel activation (Auphan *et al.*, 1995). It is possible that TCDD alters either the expression or the phosphorylation of IκB leading to decreased NF-κB/Rel translocation. We measured the levels of IκBα and levels of the phosphorylated form of IκBα to determine if TCDD altered these upstream events. Cellular lysates from DC2.4 cells, exposed to TCDD or vehicle for 24 hours and then stimulated with TNFα at various times, were analyzed by immunoblotting.

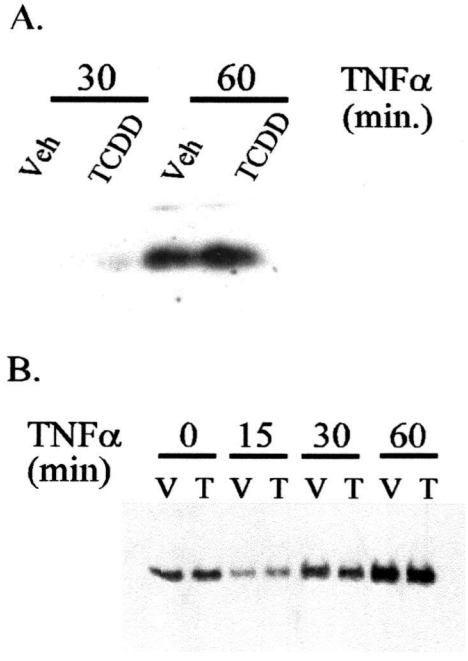
The phosphorylated form of IκBα was difficult to detect at times earlier than 30 minutes following TNFα treatment. In Fig. 3-5A, cells exposed to TCDD and activated with TNFα for 30 or 60 minutes did not display any differences in the phosphorylation of IκBα when compared to vehicle controls. In addition, TCDD altered neither total protein levels of IκBα (Fig. 3-5B lane 1 v. lane 2) nor the proteolysis of IκBα at 15, 30, and 60 minutes following TNFα treatment (Fig. 3-5B). TCDD apparently does not alter IκBα expression, phophorylation, and destruction.

Rel A and RelB interact with AhR in transfected DC2.4 cells

A potential mechanism to explain the suppression of NF-κB/Rel by TCDD is through an association between the AhR and the NF-κB/Rel protein Rel A, originally

Fig. 3-5. Proteolysis of IκBα is not altered by TCDD. Cellular extracts from DC2.4 cells treated with 10^{-9} M TCDD or vehicle 24 hours prior to treatment with 10 ng/mL TNFα for (A) 30 and 60 minutes or (B) 0, 15, 30, and 60 minutes were separated by SDS-PAGE and immunoblotted with antibodies specific for the phosphorylated form of IκBα (A) or IκBα (B). Results are representative of three separate experiments.

Figure 3-5



described in hepa 1c1c7 cells (Tian *et al.*, 1999). To determine if the AhR interacted with the proteins of NF-κB/Rel in DC2.4 cells, we immunoprecipitated the AhR and probed for NF-κB/Rel proteins. Cells of the immune system have been shown to express significantly less AhR than the hepa 1c1c7 cells used in the afore-mentioned study (Lawrence *et al.*, 1996; Ruby unpublished results). To overcome this deficiency, DC2.4 cells were transfected with an expression vector containing the murine AhR protein fused to a FLAG epitope (Meyer *et al.*, 1998). Cells were transiently transfected for 24 hours and analyzed by immunoblot to characterize AhR distribution and activity. Overexpression of the AhR led to increased AhR-DRE interactions visualized by EMSA, and the addition of TCDD did not appear to alter the levels of AhR-DRE binding complexes in these cells (data not shown).

Transfected cells were incubated with or without TNFα for two hours, and lysates from the cells were immunoprecipitated with magnetic beads coated with anti-FLAG antibodies. Control beads were used to determine nonspecific binding. As shown in Fig. 3-6, Rel A co-immunoprecipitated with transfected AhR. In addition, the amount of Rel A that co-immunoprecipitated with transfected AhR in TNFα-treated cells was increased compared to untreated controls. The RelB protein, in numerous experiments, demonstrated an interaction with AhR that was slightly above background levels, while c-rel failed to co-immunoprecipitate with AhR. Thus Rel A appears to be the dominant NF-κB/Rel protein to interact with the AhR.

Transfected AhR preferentially decreased the binding of NF-κB/Rel

In order to verify that the AhR, possibly through an interaction with Rel proteins, suppressed NF-κB/Rel binding to DNA, we overexpressed the AhR in DC2.4 cells and measured NF-κB/Rel activation by EMSA. DC2.4 cells were transfected with FLAG-AhR and following a two hour incubation with TNFα, nuclear lysates were generated and analyzed by EMSA. The overexpression of AhR led to a striking loss of the top band (Fig. 3-7, lanes 3, 4, and 5), previously

Fig. 3-6. Rel A co-immunoprecipitates with the Ah receptor. DC2.4 cells transiently transfected with FLAG-AhR for 24 hours were treated with or without 10 ng/mL TNFα for 2 hours. Cells were lysed and extracts incubated with magnetic beads coated with antibodies specific to FLAG or uncoated control bead. Precipitates were separated by SDS-PAGE and immunoblotted with antibodies specific to Rel A, RelB, and c-rel. Results are representative of three separate experiments.

Figure 3-6

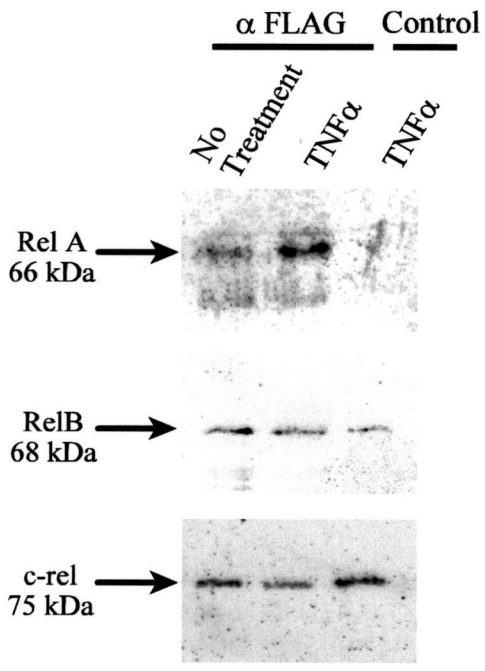
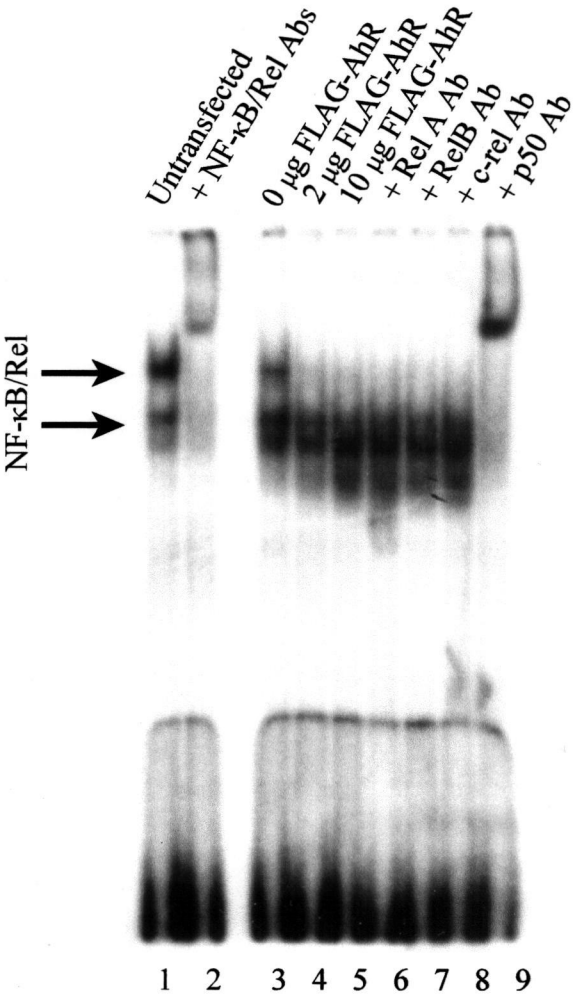

α FLAG
Control
No Treatment
TNFα
TNFα
Rel A
66 kDa
RelB
68 kDa
c-rel
75 kDa

Fig. 3-7. Overexpression of AhR blocks Rel/p50 heterodimer binding. Nuclear lysates from DC2.4 cells untransfected (lane 1) or transfected with 0 μg, 2 μg, or 10 μg of FLAG-AhR DNA for 24 hours and with TNFα for 2 hours (lanes 3-5) were analyzed by EMSA. Supershift analysis of untransfected cells with antibodies specific for NF-κB/Rel (lane 2) and cells transfected with 10 μM FLAG-AhR for 24 hours and with 10 ng/mL TNFα for 2 hours using specific antibodies to Rel A, RelB, c-rel and p50 (lanes 6-9). Results are representative of three separate experiments.

Figure 3-7


Untransfected
+ NF-κB/Rel Abs
0 μg FLAG-AhR
2 μg FLAG-AhR
10 μg FLAG-AhR
+ Rel A Ab
+ RelB Ab
+ c-rel Ab
+ p50 Ab
NF-κB/Rel
1 2 3 4 5 6 7 8 9

identified by supershift analysis to consist of Rel A/p50 or RelB/p50 heterodimer binding to DNA (Fig. 3-2). In contrast, the intensity of the lower band appeared to be largely unaffected by overexpression of the AhR. The lower band was supershifted with the addition of antibodies to p50, but not by antibodies specific for Rel A, RelB or c-rel (Fig. 3-7). These data demonstrate that overexpression of AhR selectively inhibits the binding of Rel/p50 heterodimers, but does not alter the binding of p50 homodimers.

Discussion

The results from this study demonstrate a suppression of NF-κB/Rel activity by TCDD in a dendritic cell line. We have shown that TCDD exposure decreased NF-κB/Rel translocation to the nucleus and binding to DNA in DC2.4 cells activated with either TNFα or anti-CD40 that may be mediated by a physical association between the AhR and Rel A or RelB proteins. These data agree with previously published results (Tian *et al.*, 1999) establishing the ability of TCDD to suppress NF-κB/Rel in the hepatoma cell line, hepa1c1c7, by means of a physical interaction between the AhR and Rel A. The activation of the AhR in TNFα treated cells also appears to be blocked due to the association between the AhR and Rel A. We have also shown that p50 homodimer activation is not altered by the AhR, and resulting in a predominance of NF-κB/Rel binding consisting of primarily of p50 homodimers. This finding is in partial agreement with a study done by Puga et. al. (2000) who reported a selective increase in p50 homodimer binding to DNA in the hepa1c1c7 cells following exposure to 5 nM TCDD.

Interestingly our results appear to contrast with those of Sulentic et. al. (2000), and Gollapudi et. al. (1998) who reported that TCDD activates NF-κB/Rel in B-cells and HIV-infected promonocytes. Apart from obvious differences in TCDD

dose and cell type between our study and the previous studies, a major difference was that the latter studies analyzed the level of NF-κB/Rel in relatively inactive or resting cells. This is an important point because NF-κB/Rel activity is markedly increased in activated cells, and activated NF-κB/Rel plays a critical role in the induction of an immune response (Sha, 1998). Our studies included the use of TNFα and anti-CD40, both potent activators of dendritic cells and NF-κB/Rel. It was only following these activation stimuli that we observed suppression NF-κB/Rel binding to DNA by TCDD, and these stimuli have been shown to be critical in the function and survival of dendritic cells (Rescigno *et al.*, 1998; Miga *et al.*, 2001).

The suppression of NF-κB/Rel and the shift in Rel/p50 heterodimer and p50 homodimer balance shown in this study could be a potential mechanism of TCDD-induced immunotoxicity. Effective immune responses are dependent on DC, and DC differentiation, maturation and survival are dependent on NF-κB/Rel activity (Oyama *et al.*, 1998; Verhasselt *et al.*, 1999; Rescigno *et al.*, 1998). Furthermore, alterations in DC function have been shown to lead to immune suppression (Woods *et al.*, 2000). Work done recently in our laboratory has shown that TCDD exposure significantly reduces the number of splenic DC in mice (Vorderstrasse and Kerkvliet, 2001; Shepherd *et al.*, 2001), and the suppression of NF-κB/Rel conceivably explains this phenomenon. DC development from stem cells in the bone marrow and their maturation rely on the activity of NF-κB/Rel as demonstrated in NF-κB/Rel knockout mice (Wu *et al.*, 1998). The capacity of TCDD and the AhR to suppress the activation of NF-κB/Rel in DC could derail development and maturation reducing the number of DC in the spleens of TCDD-exposed mice. Survival of the DC, another event critical in the generation of immune responses, is dependent on the ligation of CD40 and TRANCE, both signaling through NF-κB/Rel (Josien *et al.*, 2000). Blocking the function of one or both these molecules leads to unproductive DC - T cell interactions and premature termination of the immune response (Miga *et al.*, 2001). Thus immune suppression could be induced through a

sequence of events beginning with the decrease in NF-κB/Rel binding in DC by TCDD, and culminating in defective DC development, maturation or survival.

Depending on cell type, the p50 homodimer of NF-κB/Rel has been shown to inhibit rather than promote transcription (Lernbecher *et al.*, 1993). The inhibitory property of p50 homodimers may be related to their ability to bind to DNA but failure to introduce a substantial "flexture" or bending of DNA important in promoting transcription (Kuprash *et al.*, 1995). DNA-bound p50 homodimers are also unable to recruit a coactivator complex containing CBP or p/CAF, severely impairing their capability to promote transcription (Sheppard *et al.*, 1999). Our data show that while the AhR can block Rel/p50 heterodimer activity, it appears to have no effect on p50 homodimer binding, thereby shifting the balance between these protein complexes bound to DNA. This phenomenon could also lead to suppression of gene expression. We did not see a significant increase in p50 homodimer binding following TCDD treatment, but the ratio of p50 homodimers to transactivating Rel/p50 heterodimers could be altered allowing the p50 homodimers to out-compete the Rel/p50 heterodimers.

In summary, we have found that TCDD suppressed TNFα– and anti-CD40-induced activation of NF-κB/Rel in the dendritic cell line, DC2.4. This suppression may result from an association between the AhR and the NF-κB/Rel proteins Rel A and to a lesser extent RelB. Overexpresssion of the AhR did not influence p50 homodimer binding to DNA, suggesting that inhibitory p50 homodimers do not associate with the AhR. This phenomenon allows for unobstructed binding of p50 homodimers to DNA and possible induction of secondary suppressive effects on transcription. Thus, TCDD may affect the function and survival or both of the DC, an important professional antigen-presenting cell, that could lead to extensive immune defects.

Chapter 4

Effect of 2,3,7,8 Tetrachlorodibenzo-*p*-Dioxin on Nitric Oxide Production in RAW264.7 Cells

Authors:

Carl E. Ruby

Nancy I. Kerkvliet

Abstract

2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCDD), the prototypic Ah receptor ligand, induces a host of immunomodulatory phenomena, including suppression of cell- mediated and humoral immune responses. In addition, TCDD has been shown to induce oxidative stress mediated by increased production of superoxide and nitric oxide (NO). We examined the direct effects of TCDD on the ability of macrophages (RAW264.7) to produce NO, a potential immune suppressant. We measured concentrations of NO, as nitrite, and the expression of inducible nitric oxide synthase (iNOS) in RAW264.7 cells exposed to TCDD and treated with lipopolysaccaride (LPS), to induce production of NO. Our results indicate that direct TCDD exposure fails to influence significantly the production of NO and the expression of the iNOS in LPS-treated RAW264.7 cells.

Introduction

The ubiquitous environmental contaminant TCDD has been shown to alter many facets of the immune response. For example, TCDD suppresses the humoral immune response in mice to sheep red blood cells (Davis and Safe, 1988; Kerkvliet *et al.*, 1990) and inhibits cytotoxic T lymphocyte development and expansion in response to allografted tumor (Kerkvliet *et al.*, 1996). TCDD represents the prototypic ligand for the Ah receptor (AhR), a ligand-activated transcription factor known to mediate the toxic effects of TCDD on immune responses (Staples *et al.*, 1998; Vorderstrasse *et al.*, 2001), but the specific mechanisms of immunotoxicity remains unknown.

TCDD-induced oxidative stress, an emerging area of research, may represent a potential mechanism of toxicity. A number of studies have established the ability of

TCDD, to induce oxidative stress in mice and rats leading to toxicity ranging from DNA damage to lipid peroxidation (Shertzer *et al.*, 1998; Hassoun *et al.*, 1998; Alsharif *et al.*, 1994). TCDD exposure increases the production of the reactive oxygen species, superoxide, in peritoneal exudate cells, brain tissue and hepatic cells (Hassoun *et al.*, 2001; Hassoun *et al.*, 1998; Alsharif *et al.*, 1994), and preliminary studies in our laboratory has suggested that the reactive nitrogen species, nitric oxide (NO), is also increased in murine peritoneal exudate cells following TCDD exposure (Moos and Kerkvliet, unpublished results).

NO is synthesized by converting L-arginine to L-citrulline with the involvement of the cofactor NADPH by a battery of enzymes called nitric oxide synthases (NOS) of which there are three isoforms. The inducible isoform, iNOS, is capable of producing large quantities of NO and is rapidly induced in macrophages, where it was initially characterized (Gross and Wolin, 1995). NO production in macrophages is directly linked to iNOS expression. The transcriptional regulatory domain of iNOS, located 400 base pairs upstream of the transcriptional start site, contains numerous interferon-γ response elements, two TNFα response elements, an IL-6 response element and two NF-κB/Rel binding sites (Xie and Nathan, 1994).

Overproduction of NO induced by TCDD represent a viable mechanism for immune suppression by TCDD. NO is an uncharged hydrophobic gas with a high diffusion rate in biological systems allowing it to diffuse quickly over a large area (Gross and Wolin, 1995) and potentially affecting many cells. Overproduction of NO has been shown to alter the level and function of cytokines (Hill *et al.*, 1996; Peng *et al.*, 1995). Moreover, NO is capable of inhibiting the development of T cells in the thymus (Tai *et al.*, 1997) and in the spleen (Okuda *et al.*, 1996), it a potent immunotoxic molecule. We investigated the effects, that TCDD may have on the *in vitro* production of NO in macrophages. We exposed the macrophage cell lines RAW264.7 and P388.D1 to TCDD and treated with LPS to induce NO production. NO production, measured as nitrite, and expression of iNOS, responsible for

synthesis of NO, were assayed. The results demonstrated that NO production and iNOS expression following LPS treatment were not significantly altered in TCDD-exposed RAW264.7 cells.

Materials and Methods

Reagents

Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). TCDD (≥99% pure) was purchased from Cambridge Isotope Laboratories Inc. (Woburn, MA). All other reagents and cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Anti-murine iNOS antibody was purchased from Caymen Chemicals (Ann Arbor, MI). Lipopolysaccaride (*E. coli*) was purchased from Sigma (St. Louis, MO).

Cell culture

The cell lines RAW264.7 and P388.D1 were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 10 mM Na-pyruvate, 1 mM non-essential amino acids and 50 μg/mL gentamicin. RAW264.7 and P388.D1 cells were plated in 24-well plates at 5 X 10^5 cells/well and exposed to10^{-9} M TCDD for 24 hours. LPS was then added to the wells at 1 μg/mL, and the plates were incubated for 0 to 48 hours prior to analysis of NO production. Cells used for preparation of whole cell lysates were cultured to about 50% confluency in 75 cm^2 culture flasks, exposed to 10^{-9} M TCDD or vehicle (0.01% DMSO) for 24 hours, and then treated with 1 μg/mL LPS for 24 hours.

Determination of NO

NO production was evaluated by the colorimetric Greiss reaction (Green *et al.*, 1982). The assay detects concentrations of nitrite (NO_2), the stable oxidative metabolite of NO (Neilly *et al.*, 1994; al Ramadi *et al.*, 1992; Beckerman *et al.*, 1993). Briefly, 50 μL aliquots of media from cultured RAW 264.7 cells were mixed with an equal volume of Greiss reagent (1.32% sulfanilamide in 60% acetic acid/0.1% N-1-naphthyl-ethylene diamine-HCl) and incubated at room temperature for 10 minutes. The absorbance at 562 nm was measured in an automated microtiter plate reader (Bio-Tek Instruments, Winooski, VT), and NO_2 was quantitated using $NaNO_2$ as a standard.

Whole cell lysates

Whole cell lysates were prepared by incubating RAW264.7 cells in 10 mM Tris (pH 7.4), 0.5% Triton X-100, 1 mM EDTA, 1 mM DTT, 3 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL leupeptin for 20 minutes at 4°C. Samples were centrifuged at 15,000 rpm in a microcentrifuge, 4X SDS-PAGE sample buffer was added to the supernatant, and samples were boiled for 5 minutes.

Immunoblotting.

Cell extracts were subjected to SDS-PAGE by the method of Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 25 mM Tris (pH 8.3), 192 mM glycine, 20% methanol with a Genie Electroblotter (Idea Scientific Inc., Minneapolis, MN). Membranes were blocked overnight at 4°C in TBS (25 mM Tris, pH 7.4, 150 mM NaCl) containing 5% nonfat dry milk (NFDM). Antibodies against iNOS were diluted according to the manufacturer's directions in TBS containing 1% NFDM, and the membranes were incubated with primary antibody for at least 1.5 hours at room temperature. HRP-conjugated secondary antibodies, donkey anti-rabbit IgG (Amersham Pharmacia,

Piscataway, NJ) was used according to the manufacturer's instructions. Following each antibody treatment, blots were washed three times in TBS containing 0.05% Tween 20. Antibody complexes were visualized with chemiluminescent reagents (Pierce, Rockford, IL).

Results and Discussion

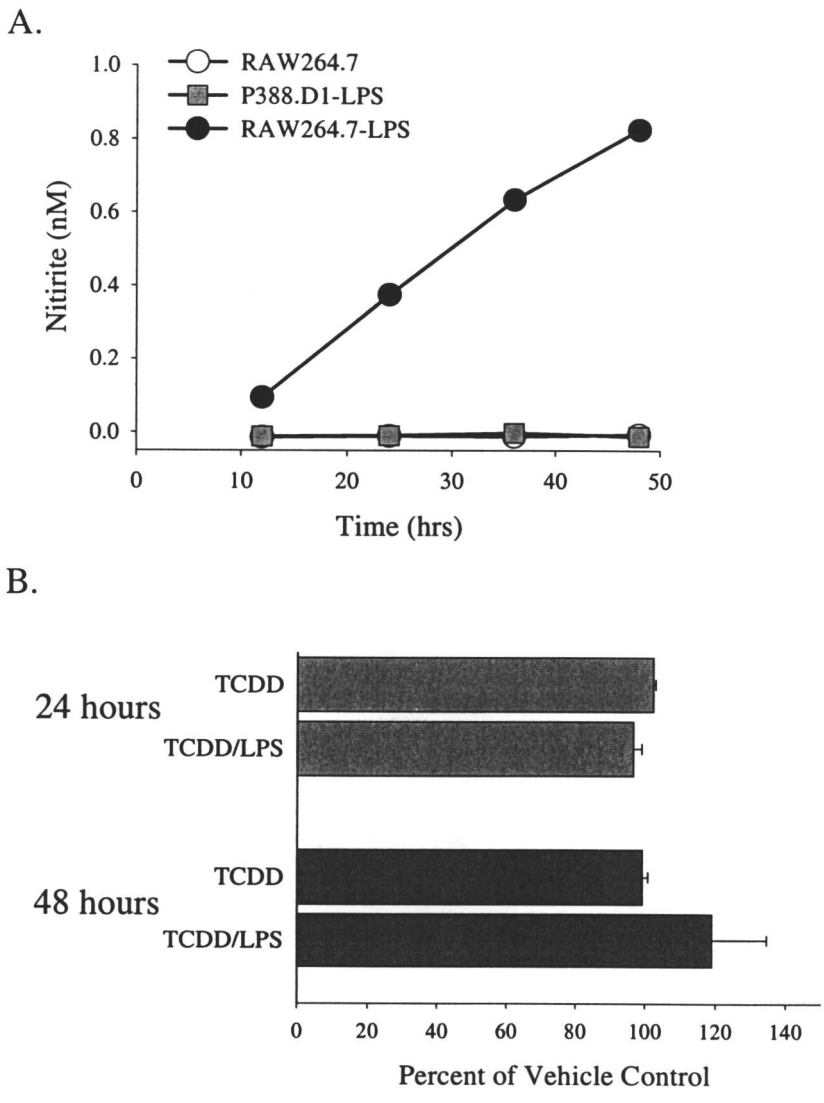
The murine macrophage cell lines RAW264.7 and P388.D1 were used to test the effects of TCDD on the production of NO. The RAW264.7 cell line, has been extensively used to analyze NO production in macrophages, and we recently demonstrated RAW264.7 cells are responsive to TCDD as measured by an increase in AhR binding to DRE motifs in DNA, making these cells an good model to determine if TCDD alters NO production (Ruby, Chapter 2, this thesis). The cell line P388.D1 was used as a negative control because it has been shown to be deficient in the expression of iNOS (Le Page *et al.*, 1996). RAW264.7 and P388.D1 cell cultures were treated with LPS and TCDD for up to 48 hours and culture supernatants were analyzed to determine the effects of TCDD on NO production.

NO production increased over time in RAW264.7 cultures treated with LPS for up to 48 hours, but not in cultures of the macrophage cell line P388.D1 (Fig. 4-1A). Production of NO was observed in LPS treated RAW264.7 cells, but not in untreated RAW264.7 cells and P388D.1 cells. The production of NO in RAW264.7 cells exposed to TCDD for 24 hours, and then treated with LPS for 24 or 48 hours was not significantly different from vehicle controls, as summarized in Fig. 4-1B.

The production of NO in macrophages is predominantly carried out by iNOS, the high output isoform of NOS. We examined the effects of TCDD on the expression of iNOS in RAW264.7 cells exposed to TCDD and stimulated with

Fig. 4-1. The production of NO in the form of nitrite in macrophage cell lines. (A) Production of nitrite in RAW264.7 cells and P388.D1 cells cultured with 1 μg/mL LPS for 12, 24, 36 and 48 hours. (B) RAW264.7 macrophages treated with 10^{-9} M TCDD or 0.01% DMSO (vehicle control) and cultured in 24-well culture plates with media. After 24 hours, cells were treated with 1ug/mL LPS and incubated for an additional 24 or 48 hours. Supernatants were analyzed for NO by the Greiss method (see Material and Methods). Five replicate wells for each experiment. The bar graph represents seven separate experiments.

Figure 4-1


A.
RAW264.7
P388.D1-LPS
RAW264.7-LPS
Nitirite (nM)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time (hrs)
B.
24 hours
TCDD
TCDD/LPS
48 hours
TCDD
TCDD/LPS
0
20
40
60
80
100
120
140
Percent of Vehicle Control

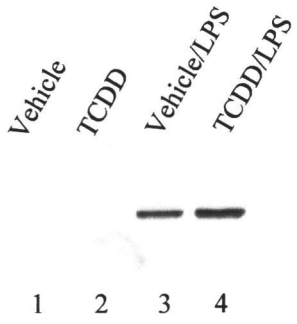
LPS for 24 hours. Levels of iNOS were visualized by immunoblot. In the initial analysis, TCDD alone did not increase iNOS expression, but TCDD appeared to increase the level of iNOS protein in LPS-treated cells compared to vehicle controls (data not shown). These effects proved to be insignificant following numerous experiments. A representative blot of the effect of TCDD on the expression of iNOS is shown in Fig. 4-2A. Fig. 4-2B summarizes seven independent experiments, measuring optical density between TCDD-exposed cells and their vehicle controls. Based on the results of these studies TCDD does not appear to directly influence the production of NO by RAW264.7 macrophages.

Our results are in agreement with a recent study showing that TCDD failed to increase NO production, measured as nitrosylhemoglobin in blood in rats (Glover *et al.*, 2000). In contrast, our data differ from other results in our laboratory describing increased NO production measured from peritoneal exudate cells (PEC) from mice exposed to TCDD in vivo and then cultured *ex vivo* with LPS (Moos and Kerkvliet, unpublished results). These experiments involved an *in vivo* exposure to TCDD, implicating the involvement of other factors found in the animal that may be necessary for increased NO production. Such factors could include proinflammatory cytokines such as TNFα and IL-1β that are increased in TCDD-exposed mice under inflammatory conditions (Moos *et al.*, 1994). TNFα and IL-1β are known to enhance the production of NO in macrophages by synergizing with other stimuli, including LPS (MacMicking *et.al.*, 1997). Alternatively, neutrophils co-cultured with macrophages have been shown to promote increased expression of iNOS and an up-regulation of NO production (Sheth *et al.*, 2000). TCDD has been shown to enhance neutrophil responses following SRBC-induced inflammation (Kerkvliet and Oughton, 1993; Moos *et al.*, 1994), and TCDD increases the numbers of neutrophils circulating in the blood and residing in the spleen of mice challenged with P815 tumor cells (Choi and Kerkvliet, unpublished results). The increased production of NO seen in TCDD-exposed PEC may depend on the combination of proinflammatory

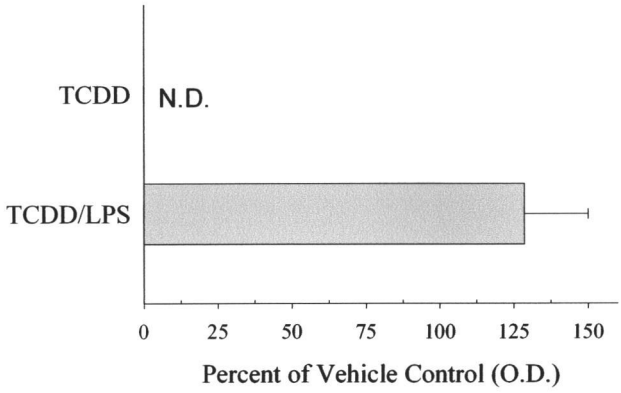
Fig. 4-2. Effects of TCDD on the expression of iNOS in LPS-treated macrophages. (A) RAW264.7 cells were exposed to 10^{-9} M TCDD or 0.01% DMSO (vehicle control) and then cultured in the presence or absence of 1 μg/mL LPS for 24 hours. Whole cell lysates were separated by PAGE and visualized by immunoblot using antibodies to iNOS. (B) Summary of iNOS protein levels, determined by densitometry, from seven independent experiments.

Figure 4-2

A.


Vehicle
TCDD
Vehicle/LPS
TCDD/LPS
1
2
3
4

B.


TCDD
N.D.
TCDD/LPS
0
25
50
75
100
125
150
Percent of Vehicle Control (O.D.)

cytokines or interactions with neutrophils or both, and thus account for differences between *in vitro* and *in vivo* responses.

In conclusion, our results show that *in vitro* exposure to TCDD does not significantly increase the production of NO by RAW264.7 macrophages. These results were strengthened when *in vitro* TCDD exposure also failed to increase iNOS protein levels in these cells. The studies presented here demonstrate the lack of a direct effect of TCDD on the production of NO in macrophages, suggesting that the oxidative stress found in TCDD-exposed animals might not be dependent on NO or may be induced indirectly by cytokine dysregulation.

Chapter 5

2,3,7,8 Tetrachlorodibenzo-*p*-Dioxin Directly Enhances Dendritic Cell (DC) Expression of Major Histocompatibility Class II (MHC II) and Costimulatory Molecules and Sensitizes DC to Deletion

Authors:

Carl E. Ruby

Castle J. Funatake

Nancy I. Kerkvliet

Abstract

The ubiquitous environmental contaminant, 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), induces immune dysfunction, characterized by suppression of adaptive immune responses. Recent studies have shown that exposure of mice to TCDD results in a significant decrease in the number of splenic dendritic cells (DC) and alterations in the expression of costimulatory molecules on the remaining DC. DC represent the most potent antigen-presenting cell, initiating immune responses through the activation of naive T cells. In the present studies, the direct effects of TCDD on DC development, maturation and survival were investigated. DC were generated in vitro by culturing mouse bone marrow cells with GM-CSF, in the presence or absence of TCDD. DC maturation and death were induced with TNFα and anti-CD95 antibodies, respectively. The results indicates that TCDD did not affect the generation and development of immature DC from bone marrow precursors. Instead, TCDD directly enhanced the TNFα-induced maturation of cultured DC, measured as an increase in the percent of DC expressing MHC II, CD86, and CD40. In addition, TCDD was shown to sensitize TNFα-treated DC to CD95-mediated death. These results suggest that TCDD directly alters the maturation and survival of DC, potentially contributing to altered T cell-dependent immune responses in TCDD-exposed animals.

Introduction

TCDD induces a broad spectrum of immune dysfunction at low doses, establishing the immune system as a sensitive target of TCDD toxicity (Kerkvliet and Burleson, *et. al.*, 1994). Sensitivity of the adaptive immune response to TCDD is seen in the suppressed induction of cytotoxic T lymphocyte activity in response to

P815 tumor allograft and in the reduced antibody responses to sheep red blood cells (Kerkvliet *et al.*, 1996; Kerkvliet *et al.*, 1990). The activation of T cells that mediate these adaptive immune responses rely on antigen-presenting cells (APC) to present antigen via major histocompatibility (MHC) molecules, to provide costimulation, and to secrete various cytokines. These functions of APCs represent potential targets for TCDD induced immunotoxic effects.

Dendritic cells (DC), the most potent APC, originate from hematopoietic stem cells (HSC) found in the bone marrow and differentiate in the presence of the growth factors GM-CSF and Flt3L (reviewed by Banchereau *et. al.*, 2000). The resulting immature DC migrate out of the bone marrow to lymphoid and non-lymphoid tissues, where they monitor the local environment for antigens. These immature DC express constitutive levels of costimulatory molecules and are highly phagocytic. In response to antigen uptake and concurrent exposure to inflammatory cytokines, the DC undergo maturation. Maturation is characterized by a number of changes in the DC, culminating in an increase surface expression of major histocompatibility class II molecules (MHC II) and the costimulatory molecules CD86 and CD40 (Winzler *et al.*, 1997). The mature DC then migrate to the T cell regions in the spleen or to the draining lymph nodes where they present antigen to naive T cells. Following antigen presentation, the generation of functional effector T cells is contingent on the ability of the DC to express costimulatory molecules, to secrete cytokines and to maintain physical association with the T cell (Banchereau *et. al.*, 2000).

Recently, DC have been revealed to be a target of TCDD toxicity *in vivo*. Splenic DC numbers were significantly reduced in mice exposed to TCDD, and the remaining DC in these animals expressed altered levels of accessory proteins (Vorderstrasse and Kerkvliet, 2001). In addition, our laboratory has reported that DC express the Ah receptor and TCDD suppresses activation of the transcription factor NF-κB/Rel in a DC line (Ruby, Chapters 2 and 3 of this thesis). NF-κB/Rel dependent transcription has been shown to be involved in various aspects of DC

development and antigen presentation (Weih *et al.*, 1995; Yoshimura *et al.*, 2001). Based on these findings, we hypothesized that TCDD would directly affect the DC, by influencing the processes of development, maturation, or survival.

In order to characterize the direct effects of TCDD on DC, we used an *ex vivo* model that generates large numbers of DC from HSC in the bone marrow (Inaba *et al.*, 1992a). Using this model, we were able to eliminate variables found *in vivo,* including the effect of neighboring cells and circulating cytokines that could influence the effects of TCDD on DC. The processes of DC development, maturation and survival were evaluated by flow cytometric analysis of activation markers and apoptotic phenotype. The results of these studies demonstrate that TCDD directly enhanced TNFα-induced maturation of DC and enhanced CD95-mediated apoptosis and death. These effects on DC may contribute to the suppression of adaptive immune responses seen in TCDD-exposed mice.

Materials and Methods

Reagents and antibodies

TCDD (≥99% pure) was purchased from Cambridge Isotope Laboratories Inc. (Woburn, MA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). All other reagents and cell culture supplies were purchased from Invitrogen (Carlsbad, CA). Dexamethasone (Dex) and 4-6-diaminodino-2-phenylindole (DAPI) was purchased from Sigma (St. Louis, MO). Murine recombinant GM-CSF (specific activity 1.0 X 10^4 U/μg) and matrix metalloproteinase inhibitor (MPI) were obtained from Pharmingen (San Diego, CA). Murine recombinant TNFα (specific activity 1.1 X 10^4 U/μg) was obtained from PeproTech (London, U.K.). FITC-Annexin V was purchased from R&D Systems (Minneapolis, MN) and 7-amino-actinomycin D (7-

AAD) was purchased from Calbiochem (San Diego, CA). Anti-CD95 (Jo-2) and crosslinking anti-hamster IgG were purchased from Pharmingen (San Diego, CA).

Generation of DC from bone marrow

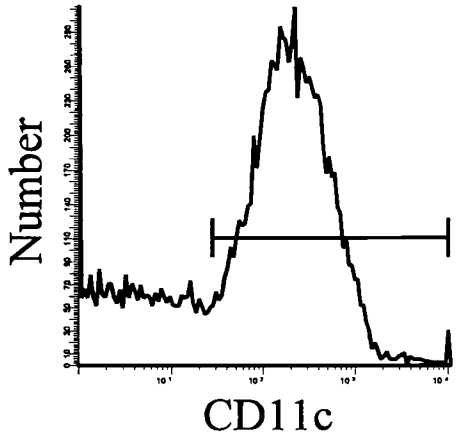
DC cultures were prepared by aseptic technique by an adaption of the protocol as previously described (Inaba *et. al.,* 1992a; Inaba *et. al.* 1992b). Briefly, femurs from C57Bl/6 mice were flushed with BMDC media (DMEM, 20% FBS, 100 μg/mL gentamicin and 2 mM L-glutamine). The resulting cell suspension was centrifuged briefly at low speed to pellet debris, and the cells were transferred to a fresh tube and washed. The red blood cells and dead cells were removed by gradient centrifugation with Lympholyte M (Cedarlane Laboratories, Ontario, Canada). Purified bone marrow cells were then washed twice and plated at a density of 5 X 10^5 cells/mL in six- or 24-well tissue culture plates (Costar, Cambridge, MA). Cells were cultured in BMDC media with 10 ng/mL GM-CSF for three days at 37°C in 5% CO_2. Following an initial three day incubation, the cultures were swirled to dislodge non-adherent cells, and the supernatant was aspirated off and replaced with fresh media. Two days later the process was repeated and, after a total of seven days ex vivo, the DC were visible as clumps of loosely adherent cells. Cells were harvested by vigorous pipeting to dislodge the loosely adherent cells. The harvested cells were analyzed by flow cytometry for the expression of the DC marker CD11c (Figure 5-1).

Cell staining and flow cytometry

Cells were incubated in wells of a 96-well plate on ice with saturating concentrations of mAb. Nonspecific mAb binding was blocked by pre-incubating the cells with rat, mouse or hamster IgG (Jackson ImmunoResearch, West Grove, PA). All cell preparations were stained with antibodies to CD11c for the selective analysis of DC. In various experiments cells were stained with mAb to MHC II, CD86, CD40, CD54, LFA-1, CD95 and/or FasL (Pharmingen, San Diego, CA).

Fig. 5-1. CD11c staining of bone marrow-derived DC. Bone marrow cells from C57Bl/6 mice were cultured with 10 ng/mL GM-CSF for seven days. DC were harvested and stained for CD11c. An electronic gate was set based on the high levels of staining for this DC marker as shown. Expression of cell surface molecules was subsequently evaluated by gating on these CD11c high expressing cells.

Figure 5-1



Appropriately labeled isotype controls were used to determine nonspecific fluorescence. Listmode data were collected on a Coulter Epics XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME). Cell viability was determined by propidium iodide exclusion. For cell surface evaluation, 20,000 viable cells were analyzed.

Determination of apoptosis and death of DC

DC were cultured as previously described and treated on day 6 with 10 ng/mL of TNFα. One day later the media was replaced with media containing reduced serum (5%) and lacking GM-CSF. Cultures were then treated with TCDD or vehicle control (0.01% DMSO). DC were harvested after treatment in culture, incubated in a 96-well plate on ice, and stained with mAb to CD11c as previously described. Apoptosis was measured by annexin V binding and 7-AAD uptake. DC were placed in binding buffer (0.1 M HEPES, M NaCl, 2.5 mM $CaCl_2$) and stained with 0.1 μg FITC-conjugated annexin V and 2 μg 7-AAD. Cells were incubated for 10 minutes at room temperature in the dark prior to flow analysis.

Staining of nuclei for chromatin condensation

Cells were harvested and 5 X 10^4 cells were deposited on slides with a Cytospin centrifuge (Shandon Southern Products, Cheshire, UK). After air drying the cells were fixed in 3.7% formaldehyde for ten minutes and stained with 1 μg/mL DAPI for two minutes. Cells were rinsed twice in PBS and water, and dried. Coverslips were mounted with Mounting Media (1 mg/mL p-phenylenediamine in 90% glycerol/PBS), sealed, and cells were viewed through a Zeiss fluorescence microscope to verify nuclear condensation and confirming apoptosis.

Statistical analysis

For all the experiments, a Student's *t* test was used to compare means of vehicle-treated groups to TCDD-exposed groups. For analysis, values of $p \leq 0.05$ were considered significant and expressed as follows: $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Results

Effects of TCDD exposure on the development of immature DC

To model the generation and development of DC *ex vivo*, we cultured bone marrow from C57Bl/6 mice with GM-CSF for up to six days. Under these conditions, the HSC present in the bone marrow develop into immature DC, identified by the expression of the DC marker CD11c, and low level expression of CD86 and MHC II. In order to determine if TCDD alters the development of DC, bone marrow cultures were treated with TCDD or vehicle control. Developing DC were then analyzed at days 1-3 and days 4-6 for the expression of CD11c, CD86, and MHC II. As shown in Figure 5-2, TCDD did not significantly alter the number of $CD11c^+$ cells generated in culture, and development of DC, represented by the percent of $CD11c^+$ cells co-expressing MHC II and CD86, was similar in cultures exposed to TCDD compared to vehicle control cultures (Fig. 5-3). Thus, it appears that TCDD does not directly alter the generation and development of immature DC.

TCDD alters the expression of MHC II, costimulatory, and accessory molecules on DC following TNFα treatment

The process of DC maturation is characterized by an up-regulation of the expression of MHC II and costimulatory molecules on the DC. One of the most

Fig. 5-2. Effect of TCDD on the generation of DC numbers. Bone marrow from C57Bl/6 mice were cultured with 10 ng/mL GM-CSF and exposed to 10^{-8} M TCDD or vehicle control (0.01% DMSO). Cultures were harvested, counted, and analyzed for the percent of cells expressing CD11c on (A) days 1-3 and (B) days 4-6.

Figure 5-2

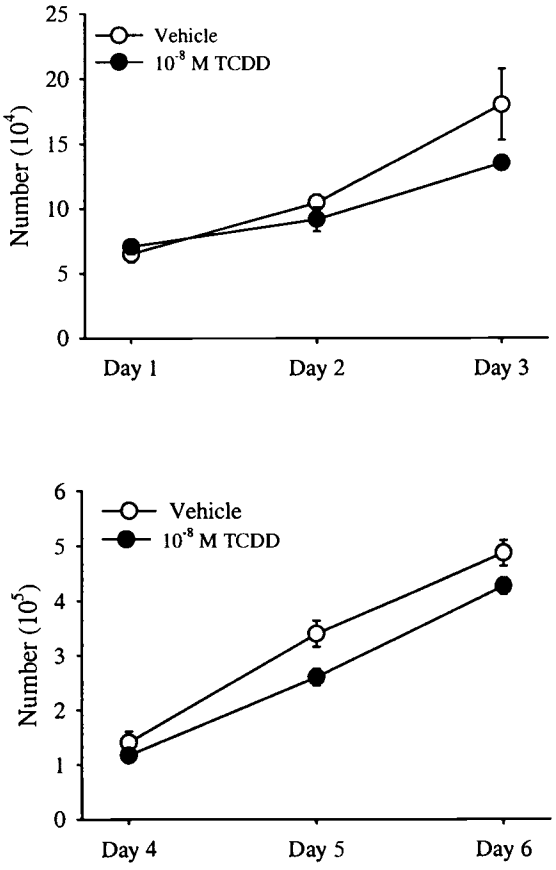
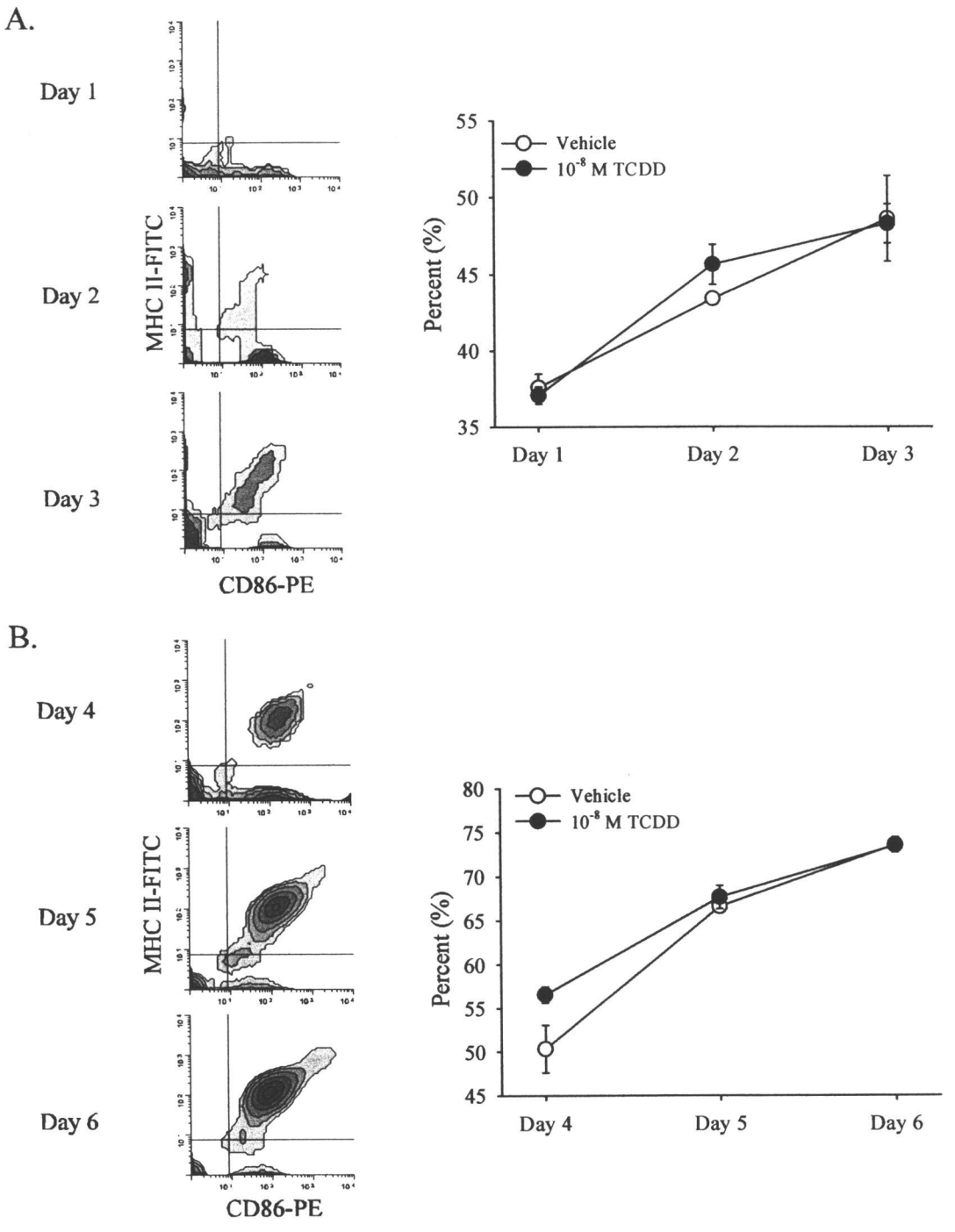

Vehicle
10⁻⁸ M TCDD
Number (10⁴)
25
20
15
10
5
0
Day 1
Day 2
Day 3
Vehicle
10⁻⁸ M TCDD
Number (10⁵)
6
5
4
3
2
1
0
Day 4
Day 5
Day 6

Fig. 5-3. Effect of TCDD on the development of DC. Bone marrow from C57Bl/6 mice were cultured with 10 ng/mL GM-CSF and exposed to 10^{-8} M TCDD or vehicle control (0.01% DMSO). DC cultures were harvested, counted, and analyzed for the co-expression of MHC II and CD86, measured as percent (%), on $CD11c^{+}$ DC on (A) days 1-3 and (B) days 4-6.

Figure 5-3


A.
Day 1
Day 2
Day 3
MHC II-FITC
CD86-PE
Vehicle
10-8 M TCDD
Percent (%)
55
50
45
40
35
Day 1
Day 2
Day 3
B.
Day 4
Day 5
Day 6
MHC II-FITC
CD86-PE
Vehicle
10-8 M TCDD
Percent (%)
80
75
70
65
60
55
50
45
Day 4
Day 5
Day 6

potent factors that stimulates DC maturation is the inflammatory cytokine TNFα (Winzler *et al.*, 1997; Zhang *et al.*, 1997). Immature DC cultured for five days were exposed to TCDD or vehicle control one day prior to treatment with TNFα. In addition some cultures were treated with Dex, a potent inhibitor of DC maturation, as a control (Matyszak *et al.*, 2000; Piemonti *et al.*, 1999). After a total of seven days in culture, $CD11c^+$ cells were analyzed for the expression of MHC II, CD86, CD40, CD54, and LFA-1 by flow cytometry.

Cultures treated with TNFα demonstrated an increase in the percent of DC expressing high levels of MHC II and CD86, as defined by the labeled gates (Fig. 5-4). Exposure to TCDD increased the percent of TNFα-treated DC expressing high levels of MHC II^{high} and $CD86^{high}$ in a dose-dependent fashion (Table 5-1). Dexamethasone suppressed significantly induction of MHC II^{high} and $CD86^{high}$ on TNFα-treated DC.

In other studies TCDD increased the percent of TNFα-treated DC expressing CD40, a costimulatory molecule found predominantly on mature DC (Banchereau *et al.*, 2000) (Fig. 5-5 and Table 5-2). Additionally, these TCDD-exposed DC exhibited an increase in the expression of the adhesion molecule CD54, measured as median channel fluorescence (MCF). In contrast to the increases in costimulatory molecule expression by TCDD, the percent of DC expressing LFA-1, another adhesion molecule, was significantly reduced following TCDD treatment.

Effects of TCDD on CD95L expression

The protein CD95L is up-regulated on mature DC, and is capable of generating apoptotic signaling in T cells. Since TCDD appeared to enhance the maturation of DC , exposure could also increase CD95L expression and lead to enhanced T cell deletion (Lu *et al.*, 1997). *In vivo*, FasL is often proteolytically

Fig. 5-4. Effect of TNFα on the expression of MHC II and CD86 on DC. Day 6 DC cultures were treated with 10 ng/mL TNFα and harvested following 24 hour incubation. DC were analyzed by flow cytometry. Representative histograms depict MHC II and CD86 expression on $CD11c^+$ cells. Solid grey line (vehicle), solid black line (TNFα), dotted line (isotype control). Regions were set based on high levels of MHC II and CD86 staining.

Figure 5-4

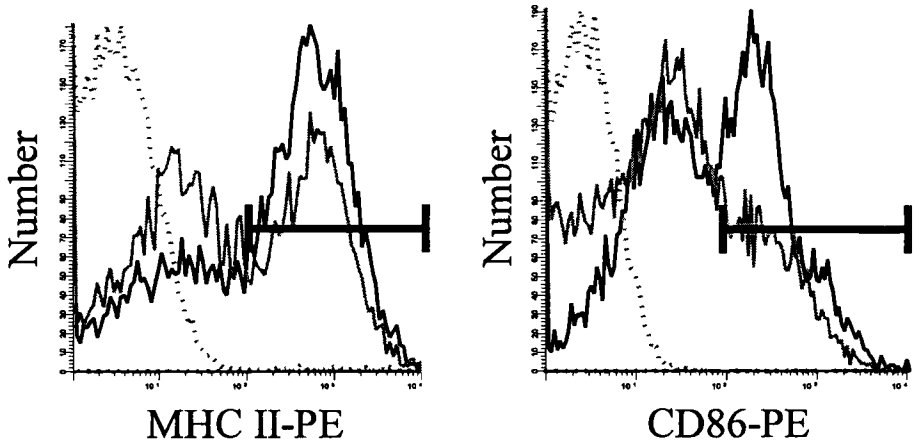


Table 5-1

Number and Expression of MHC II^{high} and CD86^{high} on TNFα treated Bone Marrow-Derived DC[a]

	Vehicle	10^{-9}M TCDD	10^{-8}M TCDD	10 nM Dex
CD11c^{+} %	51.99 (3.04)	58.86 (1.65)	57.53 (6.67)	41.11 (1.72)*
# (10^{6})	1.36 (0.10)	1.26 (0.07)	1.22 (0.15)	1.40 (0.21)
MHC II^{high} %	41.15 (2.48)	50.12 (2.25)*	55.69 (0.98)**	22.78 (0.94)**
M.C.F.	158.01 (22.02)	174.23 (11.01)	218.81 (13.49)	230.81 (21.33)**
CD86^{high} %	44.68 (1.18)	51.87 (1.15)**	56.63 (0.97)**	21.03 (0.02)**
M.C.F.	267.21 (10.75)	249.38 (12.24)	277.93 (14.64)	371.82(48.68)**

[a] Data represent the mean ± (SEM) of 3 wells per group. Data were analyzed as described in *Material and Methods*.

Fig. 5-5. Effect of TCDD on the expression of CD40, CD54, and LFA-1 on TNFα–treated DC. Day 5 DC cultures were exposed to TCDD (10^{-9} M and 10^{-8} M) or vehicle control (0.01% DMSO) and on day 6 treated with 10 ng/mL TNFα. After seven days total in culture, DC were harvested and analyzed by flow cytometry. Representative histograms depict CD40, CD54, and LFA-1 expression on $CD11c^+$ cells. Solid grey line (vehicle), solid black line (10^{-8} M TCDD), dotted line (isotype control).

Figure 5-5

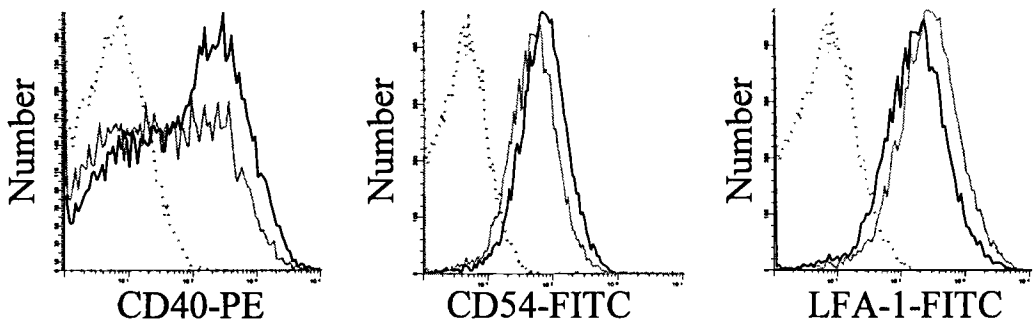


Table 5-2

Number and Expression of CD40, CD54 and LFA-1 on TNFα treated Bone Marrow-Derived DC[a]

	Vehicle	10^{-9}M TCDD	10^{-8}M TCDD	10 nM Dex
CD11c+ %	51.99 (3.04)	58.86 (1.65)	57.53 (6.67)	41.11 (1.72)*
# (10^6)	1.36 (0.10)	1.26 (0.07)	1.22 (0.15)	1.40 (0.21)
CD40 %	42.56 (2.34)	50.56 (1.04)*	55.17 (3.29)*	24.60 (1.60)**
M.C.F.	47.06 (1.54)	49.55 (3.63)	55.98 (4.67)	27.49 (1.71)**
CD54 %	91.85 (0.69)	93.01 (0.66)	93.38 (0.56)	88.38 (0.50)
M.C.F	60.69 (3.21)	66.64 (2.92)	76.82 (0.92)**	44.88 (2.10)**
LFA-1 %	51.05 (1.75)	41.33 (0.89)**	41.15 (0.92)**	68.03 (1.54)**
M.C.F.	34.95 (0.94)	32.84 (1.36)	34.00 (0.71)	35.66 (0.43)

[a] Data represent the mean ± (SEM) of 3 wells per group. Data were analyzed as described in *Material and Methods*.

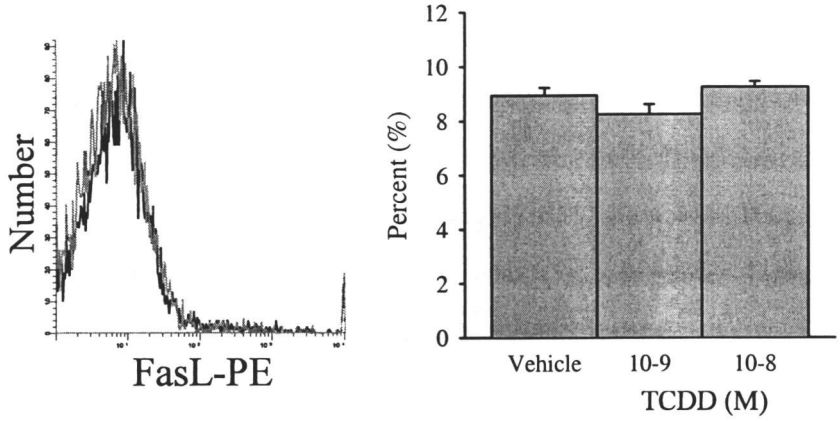
cleaved removing the extracellular portion of the protein making it difficult to measure FasL on the surface of DC (Kayagaki *et al.*, 1995). We used a matrix metalloproteinase inhibitor (MPI) in culture to block proteolytic modifications to FasL, allowing for more accurate measurements of FasL expression. DC cultured for five days were treated with TCDD or vehicle control for one day prior to treatment with TNFα and MPI, and after a total of seven days in culture DC were analyzed by flow cytometry. DC treated with MPI and TNFα expressed higher levels of FasL protein when compared to cell treated with TNFα alone, verifying the effectiveness of the MPI to protect against protein modification (data not shown). However, as shown in Figure 5-6, TCDD did not alter the expression of FasL on DC .

TCDD exposure increases the susceptibility of DC to anti-CD95 apoptotis and death

Our laboratory has previously reported a loss of splenic DC in TCDD-exposed mice, but we were unable to determine if the loss was from increased deletion or from emigration out of the spleen (Vorderstrasse and Kerkvliet, 2001). To clarify the ability of TCDD to influence the deletion of DC, we tested the hypothesis that TCDD would lead to increased sensitivity of DC to CD95-mediated apoptotic death. Following ligation by FasL, CD95 has been well established to induce apoptotic death in T cells and B cells (Nagata and Golstein, 1995; Nagata, 1999). Ligation of CD95 has also been shown to induce apoptosis in DC, both mature and immature (Matsue *et al.*, 1999; Ashany *et. al.*, 1999; McLellan *et al.*, 2000). To address the hypothesis that TCDD enhances apoptosis, DC cultured for six days were treated with TNFα for one day to induce maturation, and were then exposed to TCDD or vehicle control for one day. On day eight, the DC cultures were treated with anti-CD95 (Jo-2), a known agonistic antibody shown to induce apoptosis in other cell types (Ogasawara *et al.*, 1995). After a total of nine days apoptosis and death of DC were analyzed by flow cytometry.

Fig. 5-6. Effect of TCDD on the expression of FasL on TNFα-treated DC. Day 5 DC cultures were exposed to TCDD (10^{-9} M and 10^{-8} M) or vehicle control (0.01% DMSO) and on day 6 treated with 10 μM matrix metalloproteinase inhibitor and10 ng/mL TNFα. After seven days total in culture, DC were harvested and analyzed by flow cytometry. Representative histograms depict FasL expression on $CD11c^+$ cells. Solid grey line (vehicle), solid black line (10^{-8} M TCDD), and dotted line (isotype control).

Figure 5-6



Apoptosis and death were measured by binding of annexin V and the uptake of 7-AAD, as defined by our controls. Using 0.1 mM H_2O_2 to induce apoptosis and heating cultured DC (65°C) for two minutes to induce necrosis, we were able to identify cells that were apoptotic and dead, and we established defined gates for apoptotic ("A") and dead ("D") populations of cells (Fig. 5-7A) (Gardner *et al.*, 1997).

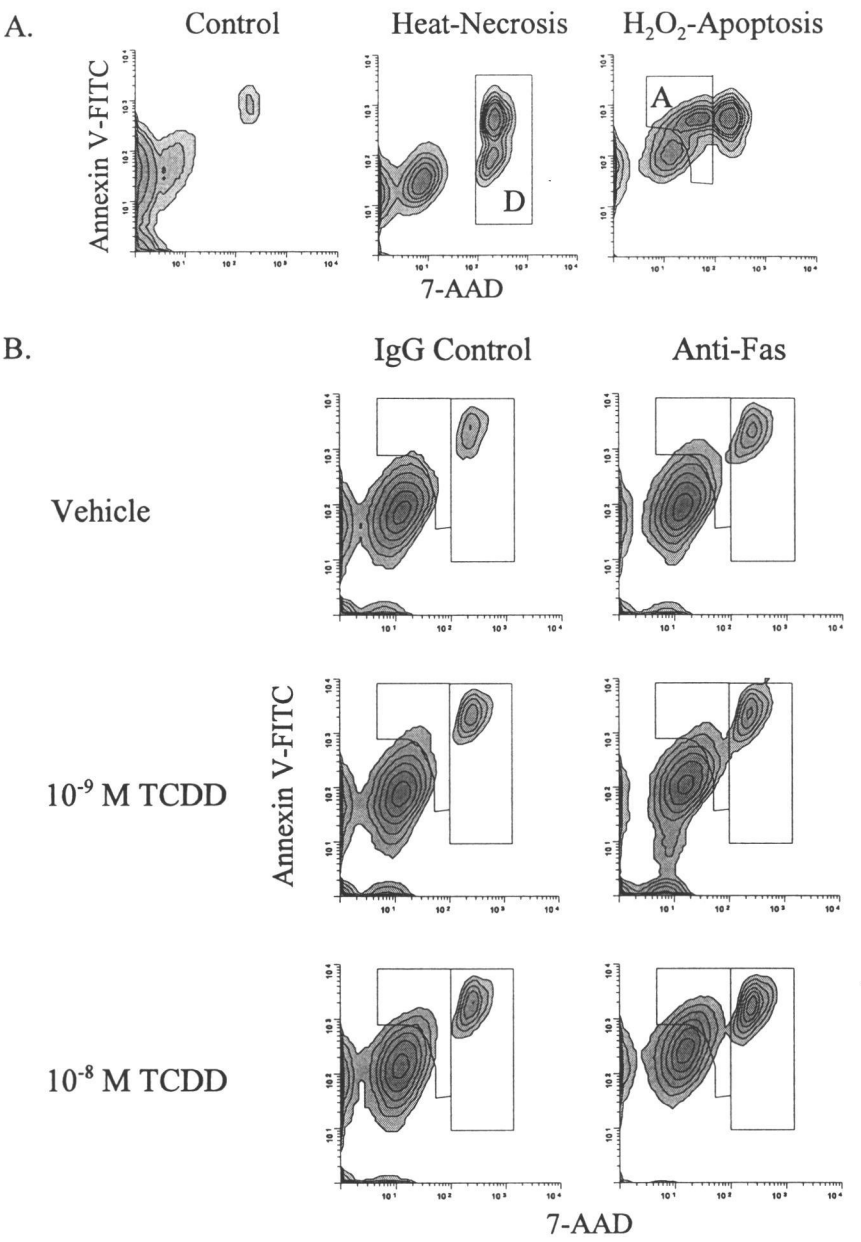
DC cultures exposed to various doses of TCDD or vehicle control were analyzed for apoptosis and death following treatment with agonistic anti-CD95 for 24 hours. This time point was found to be optimal for measuring apoptosis following a preliminary time course experiment (data not shown). As shown in Figure 5-7B, TCDD increased the percent of DC undergoing apoptosis, identified in Region A, and increased the percent of DC that stained for high levels of 7-AAD (Region D), when compared to vehicle controls. The increase in anti-CD95 apoptosis and death appeared to be dose-dependent, although the low dose (10^{-9} M) of TCDD had only a small effect compared to the high dose (10^{-8} M) (Fig. 5-7C). Interestingly, in some experiments TCDD alone at the highest dose induced a small, but significant increase in apoptosis and death when compared to vehicle/Ham IgG control. DAPI staining confirmed the apoptotic process (data not shown).

TCDD does not alter the expression of CD95 on DC

One mechanism which TCDD could increased anti-CD95 induced cell death is by increasing expression of CD95. TNFα-matured day 7 DC cultures were exposed to TCDD or vehicle control for 24 hours and analyzed for the expression of CD95 by flow cytometry. As seen in Figure 5-8 TCDD did not alter CD95 expression on DC compared to vehicle controls.

Fig. 5-7. Effect of TCDD on anti-CD95 mediated apoptosis and death of TNFα-treated DC. (A) DC cultures were treated with 0.1 mM H_2O_2 for six hours to induce apoptosis, or heated for two minutes at 65°C to induce necrosis to define apoptosis and death. DC were harvested, stained with annexin V, 7-AAD and antibodies to CD11c to identify DC, and analyzed by flow cytometry. Regions were established from these controls to identify apoptotic cells "A" and dead cells "D". (B) Day 6 DC cultures were treated with 10 ng/mL TNFα. On day 7 media was replaced with media containing reduced serum (5%) and lacking GM-CSF. The cultures were exposed to TCDD (10^{-8} or 10^{-9} M) or vehicle control (0.01% DMSO) for one day, then anti-CD95 (Jo-2) was added at 1 μg/mL, and treated with 2 μg/mL crosslinking antibody. After a total of nine days in culture, DC were harvested and stained with annexin V, 7-AAD and antibodies to CD11c to identify DC. DC were analyzed by flow cytometry, and apoptotic and dead DC were defined by regions set previously using necrosis and H_2O_2 controls. (C) Graphical representation of the effect of TCDD on anti-CD95 mediated apoptosis and death of TNFα-treated DC. Vehicle control (open bars); 10^{-9} M TCDD (grey bars); 10^{-8} M TCDD (black bars).

Figure 5-7


A.
Control
Heat-Necrosis
H_2O_2-Apoptosis
Annexin V-FITC
A
D
7-AAD
B.
IgG Control
Anti-Fas
Vehicle
10^{-9} M TCDD
10^{-8} M TCDD
Annexin V-FITC
7-AAD

C.

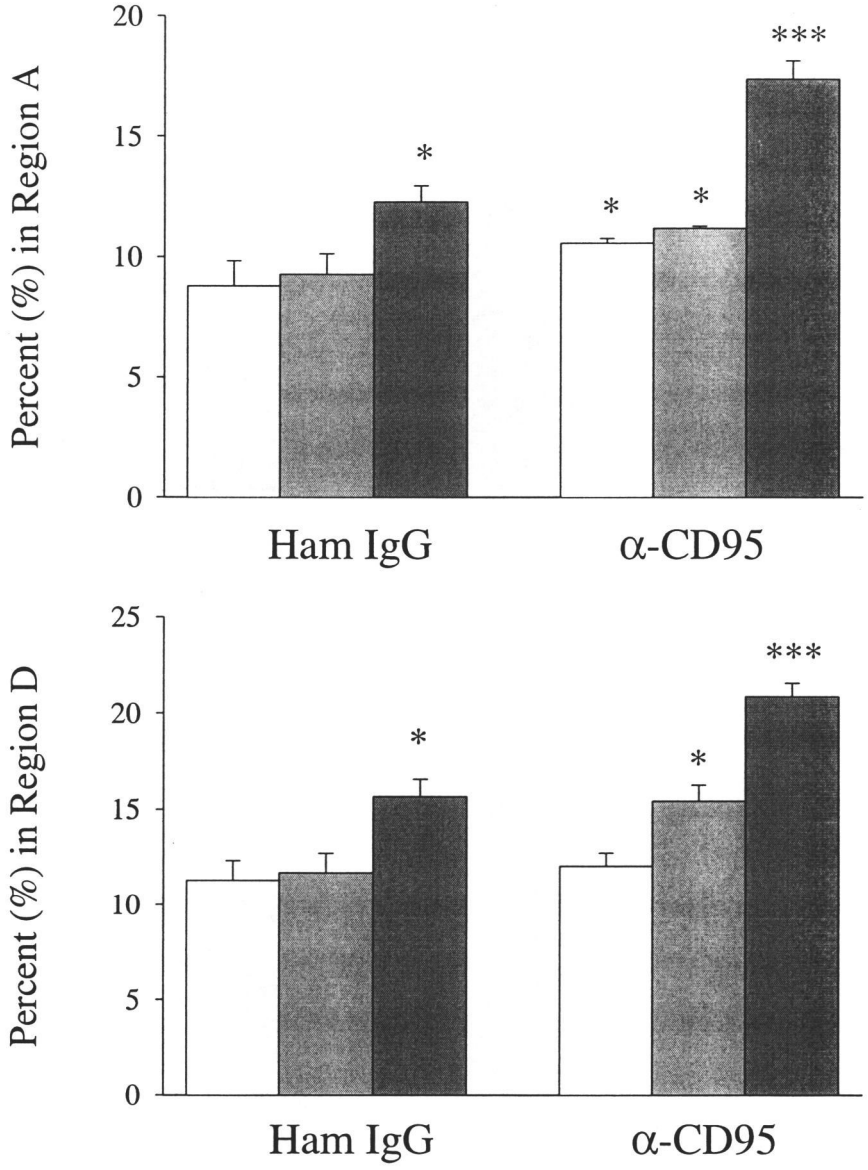
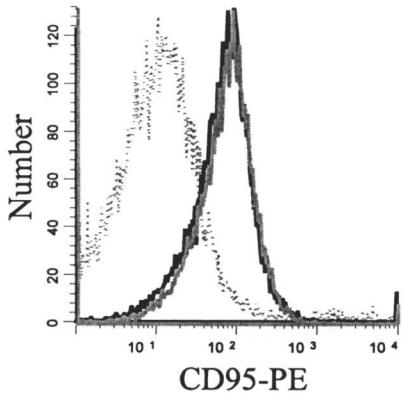

Percent (%) in Region A
20
15
10
5
0
*
*
*
***
Ham IgG
α-CD95
Percent (%) in Region D
25
20
15
10
5
0
*
*
***
Ham IgG
α-CD95

Fig. 5-8. Effect of TCDD on the expression of CD95 on TNFα-treated DC. Day 6 DC cultures were treated with 10 ng/mL TNFα and exposed to TCDD (10^{-9} M and 10^{-8} M) or vehicle control (0.01% DMSO) and on day 7. After eight days total in culture, DC were harvested and analyzed by flow cytometry. Representative histograms depict CD95 expression on $CD11c^+$ cells. Solid grey line (vehicle), solid black line (10^{-8} M TCDD), and dotted line (isotype control).

Figure 5-8



Discussion

The purpose of these studies was to evaluate the direct effects of TCDD on DC development, maturation and survival. These processes parallel the theorized discrete stages of DC differentiation: immature, mature, and apoptotic (Winzler *et al.*, 1997). Numerous studies have demonstrated that TCDD exposure suppresses adaptive immune responses, which rely on antigen presentation primarily mediated by DC. The critical position occupied by the DC in the generation of immune responses establishes the DC as a potential target of TCDD toxicity. The studies presented here demonstrate that TCDD directly targets DC by enhancing maturation and decreasing survival.

We first investigated if TCDD altered the generation of immature DC from bone marrow precursors. The development of DC has been shown to depend on the transcription factor NF-κB/Rel protein RelB, as by the profound loss of DC in the spleen and thymus of RelB null mice (Weih *et al.*, 1995). A more detailed study using RelB null bone marrow chimeric mice showed RelB is an indispensable molecule directing the development of splenic DC (Wu *et al.*, 1998). In a previous study, we have observed that TCDD suppresses the activation of NF-κB/Rel in a DC line (Ruby, Chapter 3 of this thesis). Thus, TCDD could possibly target the generation and development of DC. However, our results demonstrate that TCDD had no significant effect directly on the generation and development of DC, as measured by the expression of CD11c and coexpression of MHC II and CD86.

There are several potential expatiations for why TCDD failed to alter the generation and development of DC. TCDD may have failed to alter DC development due to the overabundance of the growth factor GM-CSF in culture. Indeed, excess GM-CSF enabled bone marrow from RelB deficient mice to develop into functionally viable DC (DiMolfetto *et al.*, 1997). Additionally, several other transcription factors, besides RelB, influence the development of DC. For example, animals deficient in the

transcription factors Ikaros and PU.1 had diminished DC numbers in the spleen and thymus (Wu *et al.*, 1997; Anderson *et al.*, 2000). Finally, TCDD might target only more mature cells, as seen when B cells are exposed to TCDD. The primary toxic effect of TCDD on B cells was reported to be limited to the cells entering and within the mature B cell subpopulation (Thurmond and Gasiewicz, 2000; Luster *et al.*, 1988).

DC maturation, characterized by the expression of high levels of MHC II and costimulatory molecules, correlates with the ability of the DC to activate naive T cells (Larsen *et al.*, 1992; Inaba *et al.*, 1994). In the studies here TCDD appears to inappropriately enhance DC maturation, as seen in the increased expression of MHC II and other costimulatory molecules. These results are in agreement with previous studies done in our laboratory demonstrating an increased expression of costimulatory molecules on splenic DC from TCDD-exposed mice (Vorderstrasse and Kerkvliet, 2001). Interestingly, these studies also showed that TCDD exposure decreased the expression of the adhesion molecule, LFA-1, verifying our finding that TCDD decreased LFA-1 expression on TNFα-treated BMDC *in vitro*. At the present time the biological relevance of the decreased LFA-1 expression in not known.

The ability of TCDD to directly enhance maturation of DC appears contradictory to the immunosuppressive activities of TCDD, however this phenomenon could compromise DC function. Maturation of DC increases the expression of MHC and costimulatory molecules, but conversely it reduces the ability of the DC to take up antigen (Sallusto *et al.*, 1995). Therefore, inappropriate DC maturation could potentially suppress antigen presentation through decreased antigen uptake. Another possible suppressive effect of enhanced DC maturation is an increase in apoptosis. In the absence of a survival signals usually received from activated T cells, mature DC have been shown to be committed to death as the final stage of DC differentiation (De Smedt *et al.*, 1998; Winzler *et al.*, 1997). Thus, the

ability of TCDD to enhance the maturation of DC could have a potential downside in decreased antigen uptake or increased apoptosis.

Survival of DC is crucial in the maintenance of immune responses, as seen when a decrease in DC persistence was shown to lead to immune dysfunction (Miga *et. al.*, 2001). Our results suggest that TCDD exposure sensitizes DC to CD95-mediated deletion. These findings are consistent with and may help explain previous observations demonstrating a decrease of splenic DC numbers in TCDD-exposed mice (Vorderstrasse and Kerkvliet, 2001; Shepherd *et al.*, 2001). The increase in apoptosis and death of TCDD-exposed DC also represent a potential mechanism for TCDD-induced immune suppression, as DC survival is correlated with normal immune responses. For example, DC grown in culture with TRANCE, a DC survival factor, and then injected into mice produced a more vigorous T cell response compared to untreated DC (Josien *et al.*, 2000).

Apoptosis is a prelethal event that involves both extracellular and intracellular proteins, many of which could be targeted by TCDD in the DC. We demonstrated that TCDD increases susceptibility of TNFα-matured DC to undergo CD95-mediated apoptosis, however, TCDD did not increase the expression of CD95 on these cells. These findings imply that TCDD is affecting intracellular events and proteins involved in initiating apoptosis. A critical intracellular event in CD95-apoptosis involves the activation of caspases, which lead to DNA fragmentation (Los *et. al.*, 1995) and influence mitochondrial permeability (Li *et. al.*, 1998). TCDD has been shown to increase caspase activity in T cells both *in vitro* and *in vivo* (Kikuchi *et. al.*, 2001; Kamath *et. al.*, 1999). Another protein, FLIP (FLICE-like inhibitor protein), inhibits caspase 8 activation (Scaffidi *et al.*, 1999) by blocking CD95-apoptosis (Irmler *et. al.*, 1997) in DC (Leverkus *et al.*, 2000; Ashany *et al.*, 1999). Thus, the reduced resistance of DC to CD95-apoptosis in this study could ultimately be due to TCDD-mediated alterations in FLIP.

In conclusion, DC maturation and survival appear to be directly affected by TCDD, verifying the effects seen in DC exposed to TCDD in vivo. The enhanced maturation and increased susceptibility to apoptosis in DC could represent potential mechanisms of TCDD-induced immunotoxicity. These direct effects on DC may alter T cell activation or survival, and thereby initiate or exacerbate TCDD-induced immune suppression.

Chapter 6

Summary and Conclusion

The studies presented here were designed to test the hypothesis that TCDD directly influences antigen presenting cells (APCs). The underlying objectives of these studies were the identification and characterization of molecular signaling pathways and cellular processes targeted by TCDD toxicity. Our results describe the effects of TCDD on the signaling pathways of the AhR and NF-κB/Rel, and the cellular processes of DC maturation and survival.

On the molecular level, we characterized the distribution and behavior of the AhR in APCs and have shown that TCDD influences the transcription factor NF-κB/Rel. The AhR has been recognized as the principal factor in TCDD-induced toxicity, by altering gene expression either directly through the DRE, or by a number of secondary mechanisms that have been recently reported. Both DC and macrophages were shown to express detectable levels of AhR. However, TCDD increased the binding of the AhR to DRE in a macrophage cell line, but failed to induce AhR binding in DC. These results precipitated investigations into the identification of other potential mechanisms of TCDD toxicity in DC. The transcription factor NF-κB/Rel, an important factor in antigen presentation, was identified in a DC line as a target of TCDD. TCDD suppressed NF-κB/Rel from binding to its cognate response element, κB-RE, in stimulated cells, which appeared to involve a physical association between the AhR and NF-κB/Rel. In more detailed examinations, transcriptionally inhibitory p50 homodimer binding to the κB-RE was unaltered by the AhR suggesting TCDD preferentially suppressed NF-κB/Rel in DC.

We also investigated the effects of TCDD on processes of DC development, maturation and survival. The evidence from our studies suggest that TCDD sensitizes DC to apoptosis, and this increased susceptibility to deletion, might be a consequence

of TCDD enhancing DC maturation. These finding appear to parallel previous results in the laboratory showing an alteration in DC maturation and a profound loss of splenic DC in TCDD-exposed mice. In light of these direct effects of TCDD on the survival of DC, TCDD may influence immunity by targeting the DC.

The direct effects of TCDD on APC observed in these studies represent potential toxic mechanisms of TCDD-induced immunotoxicity. One potential mechanism is the ability of TCDD to sensitize DC to apoptosis. DC survival correlates with the initiation of T-cell meditated immune responses. Therefore the increased deletion of DC by TCDD may result in failure to generate an immune response.

Another potential mechanism of toxicity is the ability of TCDD to enhance the maturation of DC, possibly leading to inappropriate activation of T cells. Activated T cells undergo deletion in the absence of survival signals supplied by the interaction between the T cell and the DC. In light of TCDD increasing the susceptibility of DC to apoptosis, the duration and strength of this interaction may be insufficient for T cell survival, leading to activation induced cell death.

The suppression of NF-κB/Rel represents the final potential mechanism of TCDD immunotoxicity. NF-κB/Rel is essential for normal immunity and occupies a central role in the responsiveness of many immune cell populations. Furthermore, a number of immunotoxic conditions found in TCDD-exposed mice, including thymic atrophy, suppressed adaptive immune responses and loss of DC, have also been described in NF-κB/Rel deficient mice. The suppression of this transcription factor by TCDD not only in DC, but in other immune cells could result in immune suppression.

In conclusion, the research presented here provides evidence that DC are directly targeted by TCDD through molecular signaling pathways and various cellular processes. These findings support several potential mechanisms of TCDD-induced immunotoxicity While we were unable to completely reveal the exact biochemical or cellular mechanisms of TCDD immune suppression, these findings provide clues to uncovering the truth.

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