

HIGHLIGHT

T Lymphocytes Are Direct, Aryl Hydrocarbon Receptor (AhR)-Dependent Targets of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): AhR Expression in Both CD4⁺ and CD8⁺ T Cells Is Necessary for Full Suppression of a Cytotoxic T Lymphocyte Response by TCDD

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T Lymphocytes Are Direct, Aryl Hydrocarbon Receptor (AhR)-Dependent Targets of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD): AhR Expression in Both CD4⁺ and CD8⁺ T Cells Is Necessary for Full Suppression of Cytotoxic T Lymphocyte Response by TCDD. Kerkvliet, N. I., Shepherd, D. M., and Baecher-Steppan, L. (2002). *Toxicol. Appl. Pharmacol.* 185, 146–152.

The cellular basis for the potent suppression of T cell-mediated immune responses in mice following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is not fully understood. Although activation of the aryl hydrocarbon receptor (AhR) is required, the specific AhR⁺ cells that transduce the suppression have been difficult to identify *in vivo*. The recent availability of AhR^{-/-} mutant mice has provided a resource for novel approaches to investigate the direct targets of TCDD. In our studies, we used an *in vivo* acute graft versus host (GVH) model of T cell immunity to address the direct AhR-dependent effects of TCDD on T cells. In this model, T cells from C57B1/6 mice are injected into C57B1/6 × DBA/2 F1 host mice. The injected T cells recognize the MHC disparity of the host cells, resulting in the generation of an antihost cytotoxic T lymphocyte (CTL) response. By comparing the ability of TCDD to suppress the CTL response of T cells obtained from AhR^{+/+} and AhR^{-/-} C57B1/6 mice, the need for AhR expression in T cells themselves could be assessed. The results of these studies showed that the CTL response of T cells from AhR^{+/+} mice was highly suppressed when the F1 host mice were treated with 15 μg/kg TCDD. TCDD treatment also protected the F1 host mice from the loss of body weight that accompanies the induction of the GVH response. In contrast, when grafted T cells were derived from AhR^{-/-} mice, there was no suppression of the CTL response by TCDD, and the host animals lost significant body weight. Furthermore, when T cells from AhR^{+/+} and

AhR^{-/-} mice were separated into CD4⁺ and CD8⁺ subsets and recombined using one subset from each donor prior to injection into the F1 host, suppression of the CTL response by TCDD was still apparent, but the degree of suppression was significantly reduced when either subset was AhR^{-/-}. These results indicate that direct AhR-dependent effects of TCDD occur in both CD4⁺ and CD8⁺ T cell subsets and both T cell subsets contribute to the full suppression of the CTL response by TCDD. © 2002 Elsevier Science (USA)

The immune system is recognized as one of the most sensitive targets for the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Laboratory animals exposed to low doses of TCDD show altered bone marrow and thymic development, suppressed humoral and T cell-mediated immune responses, and altered resistance to infectious diseases and cancer (Kerkvliet and Burleson, 1994). The immunotoxicity of TCDD has been shown to depend on expression of the Ah receptor (AhR), which acts together with its dimerization partner, AhR nuclear translocator (ARNT), as a ligand-activated transcription factor. AhR/ARNT-binding core sequences, also known as dioxin response elements (DREs), have been identified in the promoter regions of several genes important in cell activation, proliferation, and differentiation (Lai *et al.*, 1996). Mice engineered to express a nonfunctional AhR (AhR^{-/-}) are resistant to TCDD-induced changes in lymphoid development (Thurmond *et al.*, 2000) and to TCDD-induced suppression of immune responses (Vorderstrasse *et al.*, 2001).

Although the data from AhR^{-/-} mice demonstrate the essential role of the AhR in the immunotoxicity of TCDD, the target cells in which AhR activation occurs have not been clearly identified. Identification of the specific target cells has been difficult owing to the complex interplay of many different cell types that serve various roles in initiating, expanding, mediating, and modifying the effector functions involved in an

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immune response. Furthermore, progress has been hampered by the absence of significant effects of TCDD on immune functions *in vitro*. For example, although several T cell-dependent immune responses, including delayed and contact hypersensitivity and the generation of cytotoxic T lymphocytes (CTL), are suppressed by TCDD treatment in the intact animal (Clark *et al.*, 1981; Hinsdill *et al.*, 1980; Kerkvliet *et al.*, 1990, 1996; Vos *et al.*, 1973), the addition of TCDD to mixed lymphocyte cultures or to mixed lymphocyte-tumor cell cultures has no effect on T cell proliferation or the generation of CTL activity (De Krey and Kerkvliet, 1995). Likewise, TCDD did not affect the ability of spleen cells or T cell clones to proliferate or produce cytokines in response to polyclonal T cell stimulation (Lawrence *et al.*, 1996; Prell *et al.*, 1995). The lack of direct effects of TCDD on T cells *in vitro* was not due to absence of AhR expression in T cells, however, the binding of the activated AhR from T cells to a ³²P-labeled DRE oligonucleotide probe could not be detected (Lawrence *et al.*, 1996). These results led to the theory that TCDD affects T cell functions via an indirect mechanism.

Although the absence of *in vitro* effects of TCDD on T cells suggests that T cells may not be direct targets of TCDD, it is possible that tissue culture conditions compensate for or mask such direct effects. In the present studies, we have used a novel *in vivo* approach to definitively test the hypothesis that T cells are direct targets of TCDD via their AhR. We have employed a parent-into-F1 acute graft versus host (GVH) model to examine the generation of a CD4⁺ T cell-dependent CD8⁺ CTL response under the influence of TCDD exposure. In our model, donor cells are obtained from C57B1/6 (H-2^{bb}) mice and injected into F1 host mice derived from crossing C57B1/6 and DBA/2 (H-2^{dd}) mice. The C57B1/6 T cells recognize the H-2^{bd} antigens expressed on the cells in the H-2^{bd} F1 host. Importantly, the T cells of the F1 host will not recognize the injected donor cells as foreign, and thus, the subsequent CTL response is mediated solely by the donor T cells (Kubota *et al.*, 1983; Via and Shearer, 1988).

The hypothesis that TCDD acts directly on T cells via their AhR was addressed by comparing the effects of TCDD on the CTL response generated by AhR^{-/-} and AhR^{+/+} donor T cells following their injection into the F1 host. The results of these studies showed that TCDD suppressed the development of CTL activity only if the donor T cells expressed the AhR, demonstrating for the first time that T cells are directly targeted by TCDD via their AhR. Furthermore, full suppression of the CTL response by TCDD required AhR expression in both CD4⁺ and CD8⁺ T cells, demonstrating that both T cell subpopulations are direct targets of TCDD.

METHODS

Animals. C57B1/6J, DBA/2J, and B6D2F1 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 8 weeks of age. B6-AhR-deficient (AhR^{-/-}) mice, created by deletion of exon 2 of the AhR

gene (Schmidt *et al.*, 1996), were purchased from The Jackson Laboratory and maintained as a breeding colony in our laboratory animal facility. The AhR^{-/-} mice used in these studies were obtained by breeding AhR^{+/-} females with AhR^{-/-} males. Offspring were genotyped by methods previously described (Vorderstrasse *et al.*, 2001). Since the AhR^{-/-} mice have been backcrossed more than 10 generations onto the C57B1/6 background, normal C57B1/6 mice were used the source of control AhR^{+/+} cells. In all experiments, donor cells were obtained from female mice and injected into female F1 recipient mice. DBA/2 mice were used to propagate the P815 mastocytoma cell line, which expresses H-2^d antigens and was used as the target in the CTL assay. All mice were provided with food (Harlan Teklad 8604 rodent diet, Madison, WI) and water *ad libitum* and maintained in accordance with the National Research Council and Society of Toxicology guidelines for the humane use of animals in research.

Experimental protocol. TCDD (99% purity; Cambridge Isotope Laboratories, Woburn, MA) was dissolved in anisole and diluted in peanut oil. A dosing solution of anisole in peanut oil was prepared as a vehicle control. F1 mice were treated with vehicle or 15 µg/kg TCDD by gavage 1 day before the injection of donor T cells. The rationale for exposing the F1 host rather than the T cell donor to TCDD is based on previous studies showing that T cells removed from a TCDD-treated mouse prior to activation are not functionally impaired (Tomar and Kerkvliet, 1991). Body weights of F1 mice were recorded daily following T cell injection. F1 mice were killed by CO₂ overdose and spleens were removed for evaluation of CTL activity.

Preparation of donor T cells. Spleen cell suspensions were prepared from AhR^{+/+} and AhR^{-/-} mice. Splenic T cells were enriched by panning twice on dishes coated with affinity-purified rat anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30–60 min. Flow cytometric analysis showed that the panned cell suspensions contained 47% CD4⁺, 37% CD8⁺, 5% B220⁺, and 1% Mac-1⁺ cells. Purified CD4⁺ or CD8⁺ T cell subsets were obtained using a CELLection biotin binder kit (#115.21; Dynal, Lake Success, NY) according to the manufacturer's instructions. Briefly, spleen cells enriched for T cells by panning were incubated with streptavidin-labeled magnetic polystyrene beads that were coated with biotinylated-anti-CD4 antibody (clone 53-6.7; BD Pharmingen, San Diego, CA) or biotinylated-anti-CD8 antibody (clone RM4-5; BD Pharmingen). Following magnetic selection, the purified T cells were released from the beads using DNase, which cleaves the DNA linker attaching the streptavidin to the beads. The resulting T cell purity was >95% for both T cell subsets with a viability >96%. The purified T cell subsets from AhR^{+/+} and AhR^{-/-} mice were then recombined at a CD4:CD8 ratio of 1.5:1 in the following pairs: CD4⁺(AhR^{-/-}) with CD8⁺(AhR^{+/+}), CD4⁺(AhR^{+/+}) with CD8⁺(AhR^{-/-}), and CD4⁺(AhR^{+/+}) with CD8⁺(AhR^{+/+}) prior to injection into F1 hosts.

Flow cytometry. For flow cytometric analysis, 1 × 10⁶ cells were stained with fluorochrome-labeled antibodies to CD4, CD8, B220 (BD Pharmingen), and CD11b (Mac-1; Caltag, Burlingame, CA). Cells were incubated with rat IgG (Cappel, West Chester, PA) to block nonspecific binding before addition of fluorochrome-conjugated mAb. All mAb were titrated for optimal concentration. Isotype-matched mIgs were used as controls for nonspecific fluorescence. Listmode data were collected from 20,000 to 100,000 unfixed cells using a EPICS XL flow cytometer (Beckman Coulter, Miami, FL). Listmode data were analyzed using WinList software (Verity Software House, Topsham, MA).

CTL assay. Anti-H-2^d donor-derived CTL activity present in the spleen of the F1 host mice was measured in a 6-h ⁵¹Cr-release assay using ⁵¹Cr-labeled P815 tumor cells. Effector to target (E:T) cell ratios ranging from 100:1 to 12.5:1 were tested in duplicate for each sample. The amount of ⁵¹Cr released into the supernatant was measured, and the percentage of cytotoxicity was calculated for each E:T ratio as previously described (De Krey and Kerkvliet, 1995).

Statistics. Results are presented as the mean ± SEM of four or five mice per group. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Analysis of variance was performed with a signif-

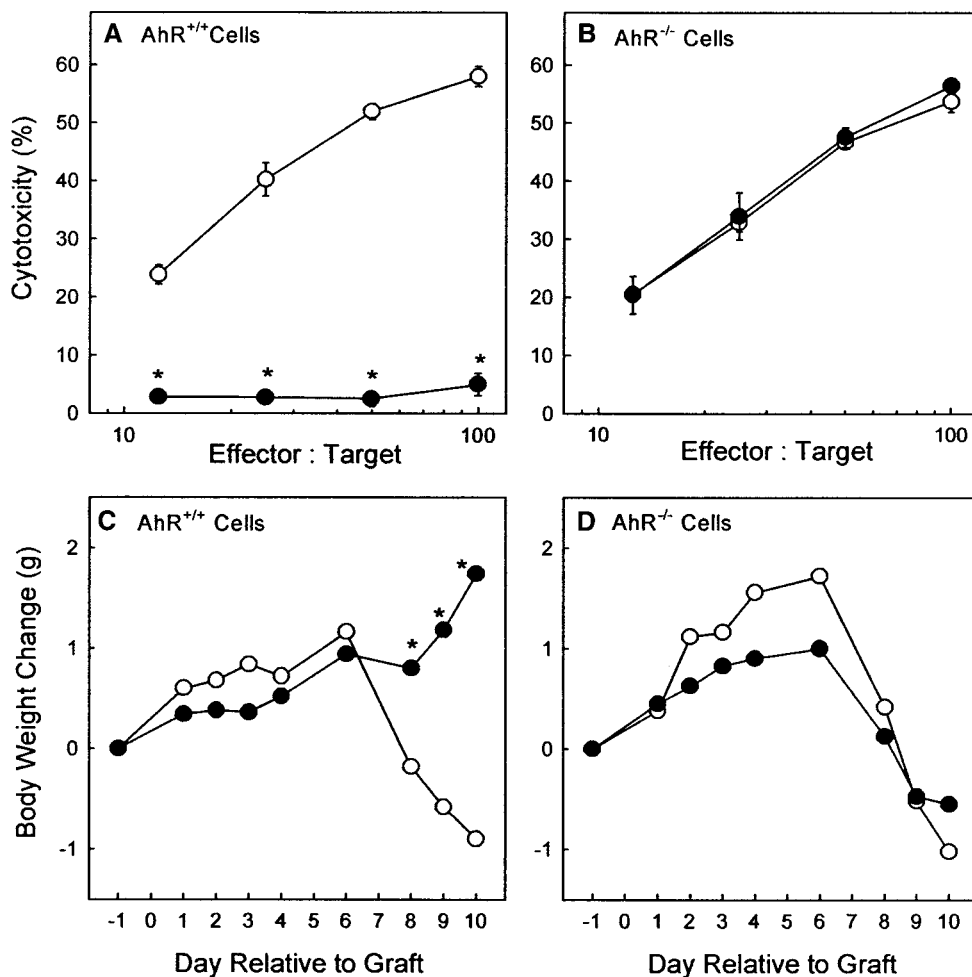


FIG. 1. CTL activity generated in B6D2F1 mice injected with parental T cells from C57B1/6 -AhR^{+/+} or -AhR^{-/-} donors. F1 mice were dosed by gavage with vehicle (open symbols) or 15 $\mu\text{g}/\text{kg}$ TCDD (closed symbols) 1 day before the transfer of donor cells. On day 0, F1 mice were injected with T cell-enriched spleen cells from either AhR^{+/+} or AhR^{-/-} donors. Ten days later the cytotoxic activity in the spleen of host mice was measured in a 6-h ⁵¹Cr-release assay. CTL activity in the spleens of F1 mice that received AhR^{+/+} T cells is shown in A while the response by those that received AhR^{-/-} T cells is shown in B. Body weight data are presented as the change in body weight relative to day -1 for AhR^{+/+} recipients (C) and AhR^{-/-} recipients (D). Data points represent the mean \pm SEM of five mice per treatment. * $p < 0.05$. SEM is not shown in C and D for clarity.

ificance level of $\alpha = 0.05$. Comparisons between means were made using the least significance difference multiple comparison t test, with values of $p < 0.05$ considered to be statistically significant.

RESULTS

Preliminary experiments established that the anti-host CTL response generated by C57B1/6 T cells in B6D2F1 mice was sensitive to suppression by TCDD exposure. Treatment of F1 mice with either 15 or 30 $\mu\text{g}/\text{kg}$ TCDD by gavage 1 day prior to the iv injection of 2×10^7 C57B1/6 spleen cells reduced CTL activity (E:T 100:1) from 55% in controls to less than 10% in both TCDD-treated groups (data not shown). At the same time, vehicle- but not TCDD-treated mice lost body weight, an expected consequence of the induction of the acute GVH response.

Since treatment of the F1 host with TCDD suppressed the generation of CTL activity, studies were designed to address the question of whether AhR expression in the donor T cells was necessary for this suppression to occur. To this end, donor T cells were obtained from AhR^{+/+} and AhR^{-/-} mice for transfer into F1 recipients. The F1 hosts were treated with 15 $\mu\text{g}/\text{kg}$ TCDD 1 day prior to T cell transfer, and CTL activity was measured on day 10. As shown in Fig. 1, A and B, T cells derived from either AhR^{+/+} or AhR^{-/-} mice generated equivalent CTL responses, indicating that the absence of the AhR in the T cells did not alter the baseline responsiveness of the cells. In addition, the vehicle-treated mice lost a significant amount of body weight, correlating with the development of the CTL response (Fig. 1, C and D). As expected, the CTL response in mice that received AhR^{+/+} T cells was highly suppressed by

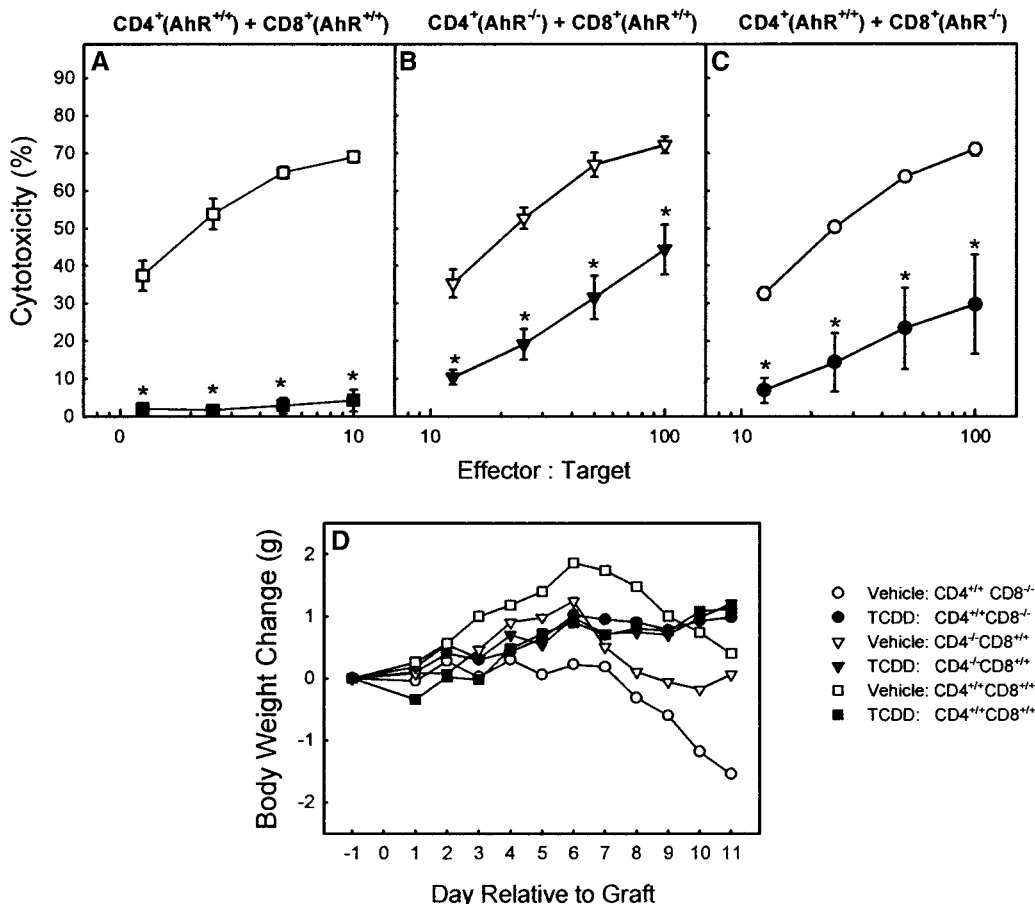


FIG. 2. CTL response in B6D2F1 mice injected with purified CD4 and CD8 T cells with different AhR genotypes. F1 mice were dosed by gavage with vehicle (open symbols) or 15 $\mu\text{g}/\text{kg}$ TCDD (filled symbols) 1 day before the transfer of donor cells. On day 0, F1 mice were injected with T cell combinations of purified CD4 and CD8 splenic T cells from AhR^{+/+} or AhR^{-/-} donors as indicated in A–C. The cytotoxic activity in the spleen of host mice was measured in a 6-h ⁵¹Cr-release assay. Body weight data are presented as the change in body weight relative to day-1 (D). Data represent the mean \pm SEM of four or five mice per treatment. * $p < 0.05$. SEM is not shown in D for clarity.

TCDD exposure (Fig. 1A), and the mice gained, rather than lost, body weight during the experimental time period (Fig. 1C). Notably, and in contrast to AhR^{+/+} T cells, if the T cells did not express the AhR, TCDD treatment did not suppress the CTL response (Fig. 1B). TCDD-treated F1 mice that received T cells from AhR^{-/-} donors also showed the characteristic loss of body weight associated with the unimpeded development of CTL activity (Fig. 1D). Thus, these data clearly demonstrate the critical role of the AhR in the responding donor T cells in the immunosuppressive effect of TCDD.

Since the ability of TCDD to suppress the CTL response in the F1 host was controlled by the AhR status of the donor T cells, it was of interest to determine whether there was a selective or differential effect of TCDD on the CD4⁺ or the CD8⁺ T cell subset. For these studies, F1 hosts were injected with combinations of T cell subsets that were obtained from AhR^{+/+} and AhR^{-/-} mice. Splenic CTL activity was measured on day 11 after the injection of donor cells. As shown in Fig. 2, vehicle-treated F1 mice that received any of the combina-

tions of T cells developed equivalent CTL responses. As expected, TCDD-treated F1 mice that received CD4⁺ and CD8⁺ cells recombined from AhR^{+/+} donors exhibited a highly suppressed CTL response (Fig. 2A). In contrast, TCDD-treated F1 mice receiving either CD4⁺ or CD8⁺ T cells from AhR^{-/-} mice demonstrated an intermediate degree of suppression (Fig. 2B and 2C), which was significantly less compared to the mice that received both subsets from AhR^{+/+} mice. These data demonstrate that expression of the AhR in both CD4⁺ and CD8⁺ T cells subsets is necessary for full suppression of the CTL response and indicate that both T cell subsets are directly affected by TCDD via their AhR. This conclusion was supported by the body weight data (Fig. 2D). Although there was some variation in initial body weight gain among the vehicle-treated groups, the vehicle-treated mice all lost weight after day 7. In contrast, mice in the TCDD-treated groups all gained weight over the same time period. The average weight gain of about 1 g was less than we have observed in several other experiments and precluded detection of a direct correla-

tion between suppression of CTL activity and body weight gain.

DISCUSSION

The results presented here unequivocally demonstrate, for the first time, the requirement for the AhR to be expressed in T cells for TCDD to induce suppression of a T cell-mediated immune response. Although T cell functions *in vivo* have long been known to be sensitive to suppression by TCDD, few direct effects of TCDD on T cells *in vitro* could be demonstrated. Thus, the emerging paradigm was that T cells were indirect targets of TCDD via other AhR-expressing cells in the animal. Alternate targets, such as the endocrine system or antigen-presenting cells, have been investigated, but the results of these studies have failed to provide compelling evidence for an indirect pathway of T cell dysfunction (De Krey and Kerkvliet, 1995; Shepherd *et al.*, 2001). Although the GVH model used in our studies cannot exclude the possibility that TCDD also acts through the AhR in host tissues to influence T cell responses (i.e., a multihit model), the complete lack of effect of TCDD when AhR^{-/-} T cells were injected (Fig. 1B) indicate that a host effect alone is not sufficient to induce suppression of the CTL response. Future studies that compare the effects of TCDD in F1 host mice derived by crossing B6 AhR^{-/-} mice with DBA/2 mice should provide a low AhR-responsive background and minimize any role played by the host. If suppression of the CTL response is less dramatic in these hosts, it will suggest that AhR-expressing host tissues play a role in the suppression of the CTL response.

Several genes known to play a role in the activation and differentiation of T cells have been shown to express putative DREs in their promoter regions, and therefore represent potential targets for TCDD in T cells (Frueh *et al.*, 2001; Lai *et al.*, 1996; Puga *et al.*, 2000a,b). However, only a few genes have been shown to be directly regulated by AhR activation, and the data are primarily derived from experiments with hepatocytes. For example, many Phase I metabolizing enzymes are induced by AhR activation in hepatocytes (Nebert *et al.*, 1993; Okino and Whitlock, 2000; Schmidt and Bradfield, 1996); however, their induction and role in T cell functions have not been studied. On the other hand, the IL-2 gene plays an important role in T cell functions and appears to be directly regulated by AhR activation. Jeon and Esser (2000) reported that the promoter region of the mouse IL-2 gene contained three potential DRE sites that were capable of binding to the ligand-activated AhR and inducing luciferase expression in a reporter system. They also reported that the IL-2 gene was induced in thymocytes after *in vivo* exposure to TCDD and in splenic T cells after additional *in vitro* stimulation with anti-CD3. Similarly, we found that IL-2 production by alloreactive CD8⁺ T cells was transiently elevated by TCDD exposure following the injection of allogeneic tumor cells (Kerkvliet *et al.*, 1996). This early production of IL-2 preceded the premature termination of

CTL response in TCDD-treated mice. Since IL-2 plays an important role in down-regulating immune responses through the induction of activation-induced T cell death (Li *et al.*, 1999); Li, 2000; Van Parijs *et al.*, 1999), inappropriate IL-2 gene expression in T cells could result in premature T cell death and suppression of CTL activity. Adseverin gene expression has also recently been reported to be regulated by AhR activation, specifically in lymphoid tissues (Svensson and Lundberg, 2001; Svensson *et al.*, 2002). Also known as scinderin, adseverin is an actin-binding protein that participates in actin reorganization within cells in response to external signaling (Kwiatkowski, 1999). Increased expression of adseverin in T lymphocytes stimulated with IL-9 suggests that T cells up-regulate adseverin upon activation and/or differentiation (Robbens *et al.*, 1998). Other genes that may be altered by AhR activation in T cells, as well as their specific roles in TCDD's immunotoxicity, await future identification.

Although DRE-mediated transcriptional alterations resulting from the activation of the AhR are well-recognized pathways for TCDD toxicity, non-DRE mechanisms of toxicity have also been proposed. The possibility of a non-DRE-mediated pathway in T cells is important given that Lawrence *et al.* (1996) were unable to detect DRE binding by the murine T cell AhR following TCDD exposure. For example, there are reports that the ligand-bound AhR can physically associate with other transcription factors, such as the Rel A component of NFκB, to induce mutual functional modulation of gene expression controlled by these transcription factors (Tian *et al.*, 1999; Kim *et al.*, 2000). NFκB is widely expressed by cells of the immune system and has been shown to play important roles in both immune system development and function. Similarly, physical association between the AhR and the retinoblastoma tumor suppressor protein, which controls cell cycle progression through G1 (Ge and Elferink, 1998), has been reported as well as the transcriptional repressor COUP-TF (Klinge *et al.*, 2000). In addition, immunophilin-like proteins that preferentially associate with the non-ligand-bound (Meyer and Perdew, 1999) or ligand-activated AhR (Carver and Bradfield, 1997; Ma and Whitlock, 1997) have been described. A possible role for the immunophilin proteins is intriguing given that the potent immunosuppressive drugs cyclosporin A and FK506 exert their effects by forming complexes with immunophilins (Schreiber, 1991).

In summary, the present studies have shown that activation of the AhR in T cells profoundly compromises the ability of the cells to respond to antigenic stimulation. Since activation of the AhR signaling pathway may induce a novel form of immune suppression, deducing this mechanism of action may provide new insights into basic immune regulation and may also reveal new possibilities for immunosuppressive therapies. The acute GVH model using donor T cells from AhR^{+/+} and AhR^{-/-} mice provides an exciting approach for gaining insight into AhR regulation of T cell functions.

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