

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Affects the Number and Function of Murine Splenic Dendritic Cells and Their Expression of Accessory Molecules

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Primary T cell-mediated immune responses are highly susceptible to suppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure, yet direct effects of TCDD on T cells have been difficult to demonstrate. Since the activation of naive T cells has been shown to be initiated primarily by dendritic cells (DC), these cells represent a potential target for TCDD immunotoxicity. In this report, we have examined the influence of TCDD exposure on splenic DC phenotype and function in the absence of antigenic stimulation. Results showed that DC from TCDD-treated mice expressed higher levels of several accessory molecules including ICAM-1, CD24, B7-2, and CD40, whereas the expression of LFA-1 was significantly reduced. These effects were dose-dependent and persisted for at least 14 days after exposure. The effects were also dependent upon the aryl hydrocarbon receptor (AhR), as similar effects were observed in AhR^{+/+} C57Bl/6 and Balb/c mice but not in AhR^{-/-} mice. When DC from TCDD-treated mice were cultured with allogeneic T cells, the proliferative response and production of IL-2 and IFN- γ by the T cells were increased. Production of IL-12 by the DC was likewise enhanced in comparison to cells from vehicle-treated mice. Interestingly, however, the number of DC recovered from TCDD-treated mice was significantly decreased. Taken together, these results suggest that, in the absence of antigen, TCDD provides an activation stimulus to DC that may lead to their premature deletion. Since the survival of DC has been shown to influence the strength and duration of the immune response, these results suggest a possible novel mechanism for TCDD-induced immune suppression. © 2001 Academic Press

Key Words: dendritic cells; TCDD; IL-12; accessory molecules; adhesion molecules; costimulatory molecules; immunotoxicity.

The immune system is recognized as one of the most sensitive targets for the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental contaminant and prototypic ligand for the aryl hydrocarbon receptor (AhR) (reviewed by Kerkvliet and Burleson, 1994). In mice, TCDD-induced immune dysfunction is characterized by a profound suppression

of T and B lymphocyte effector function, as evidenced by defects in production of antibodies, generation of cytotoxic T cells, and development of delayed-type hypersensitivity responses (House *et al.*, 1997; Kerkvliet *et al.*, 1996; Lundberg *et al.*, 1991). Interestingly, immune suppression is observed only if TCDD exposure occurs early in the generation of an immune response, suggesting that early events in T helper cell activation may be altered (Kerkvliet *et al.*, 1996). However, because T cell responses are mostly unaffected following *in vitro* exposure to TCDD (Lang *et al.*, 1994; Lawrence *et al.*, 1996; DeKrey and Kerkvliet, 1995), it is questionable whether TCDD affects T cells directly. Rather, the cells responsible for the activation of T cells may be the target of TCDD.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) for activation of naive T cells (reviewed by Banchereau and Steinman, 1998). Located throughout the body, immature DC express constitutive levels of costimulatory molecules and are specialized for capturing antigen. When exposed to antigenic stimuli, LPS, or inflammatory cytokines, DC are induced to migrate to the spleen or draining lymph nodes and to undergo a maturation process. As they mature, DC downregulate phagocytic activity, increase expression of adhesion and costimulatory molecules and the major histocompatibility complex (MHC), and begin to produce cytokines. In this mature state, DC activate T cells by presenting antigen in the context of MHC and by providing necessary costimulation via accessory molecules and cytokines. Among the important accessory molecules expressed by DC are adhesion molecules such as ICAM-1 (CD54) and LFA-1 (CD11a), which function to maintain prolonged contact between the DC and T cell during activation, and costimulatory molecules such as B7-1 (CD80), B7-2 (CD86), CD24, and CD40, which signal T cells to proliferate and differentiate (Inaba *et al.*, 1994; Cella *et al.*, 1996; Liu *et al.*, 1992; Enk and Katz, 1994; VanKooten and Banchereau, 1997). DC also produce important cytokines such as IL-12, a cytokine that promotes the differentiation of TH1 vs TH2 cells (Koch *et al.*, 1996; Gately *et al.*, 1998).

The potential for TCDD to influence DC in terms of their ability to activate T cells has not been previously examined. Therefore, in the studies reported here, we provide an initial

characterization of the temporal and dose-related effects of TCDD on the expression of adhesion and costimulatory molecules on DC that are important in T cell activation. In addition, the functional ability of DC from TCDD-treated mice to stimulate T cell proliferation and cytokine production *in vitro* was evaluated.

MATERIALS AND METHODS

Animal treatments. Male C57Bl/6 and female DBA/2 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Female Balb/c mice were purchased from B&K Universal, Inc. (Kent, WA). AhR knockout mice (Fernandez-Salguero *et al.*, 1995), which were of C57Bl/6 \times 129/Sv mixed genetic background, were generously provided by Dr. A. Silverstone (State University of New York Health Science Center, Syracuse, NY). Male mice were housed singly, and female mice were housed five or six per cage. Animals were maintained in front of a laminar flow unit, in accordance with National Research Council guidelines. Mice were used at 7–12 weeks of age and were killed by CO₂ overdose.

TCDD exposure. TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil. The vehicle control consisted of an equivalent amount of anisole in peanut oil. Mice were treated with TCDD or vehicle by gavage. Except in dose–response studies, TCDD was given at 15 μ g/kg, a dose previously shown to suppress immune responses in mice (Kerkvliet *et al.*, 1996).

Reagents and antibodies. Spectral Red streptavidin was obtained from Southern Biotech (Birmingham, AL). Biotinylated-anti-CD11c as well as various fluorochrome-conjugated antibodies to B7–1 (CD80), B7–2 (CD86), CD8 α , ICAM-1 (CD54), LFA-1 (CD11a), and MHCII (I-A^b) were obtained from PharMingen (San Diego, CA). Antibodies to CD24 were purchased from PharMingen or were produced in our laboratory using the J11 d hybridoma (American Type Culture Collection, Rockville, MD). Antibodies to CD40 were purchased from PharMingen and from Southern Biotech. For ELISA, IL-2, and IFN- γ , antibody pairs were purchased from PharMingen, and antibodies to the p40 subunit of mouse IL-12 were purchased from Genzyme (Cambridge, MA).

Preparation of DC. DC were enriched from spleens using the method of Swiggard *et al.* (1992) with modifications as described in Inaba *et al.* (1997). Briefly, splenic tissue was digested with collagenase D (Boehringer Mannheim, Indianapolis, IN) at 37°C for 45–60 min to release DC from the capsule and to increase recovery. Cell suspensions were then diluted in Ca-/Mg-free HBSS and pelleted. Recovered cells were spun over a BSA gradient (1.080 g/ml) and cells in the low-density fraction were collected. These freshly isolated DC-enriched preparations were then stained for flow cytometric analysis. For mixed leukocyte reaction (MLR), the low-density spleen cells were further enriched for DC by overnight culturing based on their property of transient adherence to plastic (Swiggard *et al.*, 1992). In this procedure, the low-density cells were cultured in plastic dishes for 90 min to allow for the DC to adhere. The nonadherent contaminating cells were then washed away, and the remaining adherent cells were left in culture overnight. DC become nonadherent during this culture period and were collected from media the following day. Purity after this final enrichment was greater than 80% CD11c^{hi} cells.

Flow cytometry. Cells were incubated in 96-well plates with saturating concentrations of mAb, which were titrated for optimal concentration. Nonspecific mAb binding was blocked by preincubating cells with rat and/or hamster IgG. All cell preparations were stained with an antibody to CD11c to allow selective analysis of DC (Crowley *et al.*, 1990). Typically, 10–25% of the low-density spleen cells expressed high levels of CD11c (CD11c^{hi}). In various experiments, cells were also stained with mAb to B7–1, B7–2, CD40, CD24, ICAM-1, LFA-1, CD8 α , or MHCII. Appropriately labeled isotype controls were used to determine nonspecific fluorescence. Listmode data were

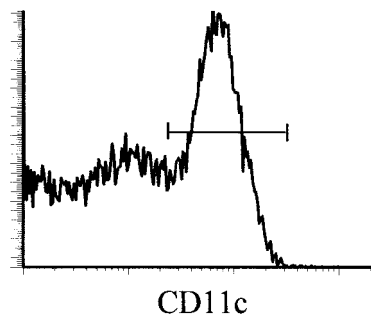


FIG. 1. CD11c staining on low-density spleen cells. Spleens were digested with collagenase and enriched for DC by density gradient centrifugation. The low-density fraction was stained for CD11c and a region was set based on high levels of staining for this DC marker. Expression of accessory molecules was subsequently evaluated by gating on these CD11c^{hi} cells.

collected on a Coulter Epics XL flow cytometer and analyzed using WinList software (Verity Software House, Inc., Topsham, ME). For cell surface molecule evaluation, 10,000 viable CD11c^{hi} cells were analyzed (Fig. 1). A viable cell gate was established based on propidium iodide exclusion. Electronic subtraction during data collection was performed using single color stains to compensate for spectral overlap of fluorochromes. For some samples, WinList software was also used for compensation during data analysis. Cells staining positively for each marker were defined by setting a region to exclude $\geq 95\%$ of the isotype control, whereas the median channel fluorescence (MCF) was calculated based on a region that encompassed all of the cells in the histogram. Due to the heterogeneous staining pattern of CD8 α , MCF was calculated only for the cells in the region defined as positive by the isotype control.

Mixed leukocyte reaction. For each experiment, a pool of T cells was enriched from spleens of untreated female DBA/2 mice by nonadherence to nylon wool (Hathcock, 1991). Resulting cell suspensions typically contained 50% CD4⁺ cells and 20% CD8⁺ cells. T cells (3×10^5) were plated in triplicate in 96-well tissue culture plates with various numbers of stimulatory DC obtained from vehicle- or TCDD-treated C57Bl/6 mice. T cell proliferation was measured by incorporation of ³H-TdR, which was added (1 μ Ci/well) during the last 20 h of culture.

Cytokine analysis. MLR culture supernatants were analyzed for cytokines using antibody sandwich ELISA techniques. The secondary biotinylated antibodies were complexed with avidin–peroxidase and visualized with 2,2'-azinobis[3-ethylbenzthiazoline-6-sulfonic acid] as substrate. Absorbance was read at 405 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

Statistical analysis. For most experiments, a Student's *t* test was used to compare the mean of the vehicle-treated group to the mean of the TCDD-treated group. Where indicated, ANOVA was performed using SAS statistical software (SAS Institute, Inc., Cary, NC), and comparisons between means were made using the least significant difference (LSD) multiple comparison *t* test. ANOVA was also used for analysis of IL-2 production across multiple experiments. Due to nonconstant variance, the analysis of the 28-h IL-2 data was performed on log-transformed data.

RESULTS

Effect of TCDD Exposure on Expression of Accessory Molecules on DC

A number of cell surface proteins play important roles in the function of DC. We initially examined the expression of several of these accessory molecules on splenic DC from C57Bl/6

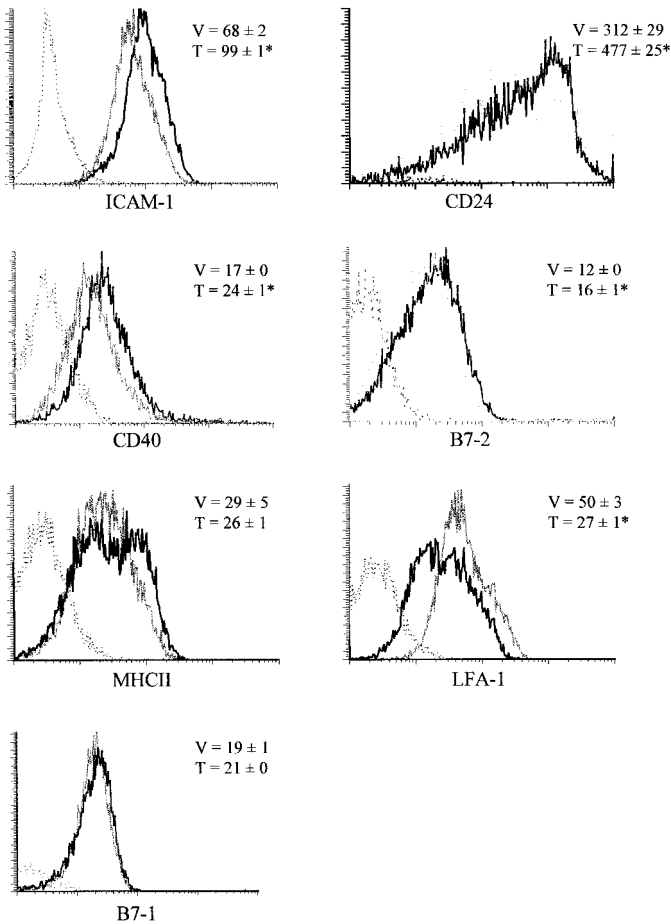


FIG. 2. Effect of TCDD on DC accessory molecule expression 3 days postexposure. C57Bl/6 mice were treated with vehicle control or 15 $\mu\text{g}/\text{kg}$ TCDD. Three days later, spleens were removed, digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. Representative histograms depict accessory molecule expression on all CD11c^{HI} cells. Solid gray line (vehicle), solid black line (TCDD), dotted line (isotype control). The majority of cells stained for the CD24 isotype control are on the baseline and thus are not resolved from the y axis. MCF was determined based on the fluorescence of the entire peak. Values shown are the MCF \pm SEM for vehicle (V)- and TCDD (T)-treated groups ($n = 3/\text{group}$) except for B7-2, in which the TCDD group represents two animals from day 3 and two from day 4 following TCDD exposure. *Different from vehicle ($p \leq 0.05$). Data are representative of three to seven independent experiments.

mice 3 days after treatment with an immunosuppressive dose (15 $\mu\text{g}/\text{kg}$) of TCDD. As shown in the representative histograms in Fig. 2, TCDD exposure significantly increased the expression of ICAM-1, CD24, CD40, and B7-2 as defined by MCF. Although changes in some markers (e.g., B7-2) were small, similar results were seen in three or more independent studies. The expression of MHC class II (MHCII) was not affected in terms of MCF, although TCDD clearly altered the staining pattern of this marker. In contrast to other accessory molecules, LFA-1 expression was consistently decreased on DC isolated from TCDD-treated mice (Fig. 2), while B7-1 expression was not altered.

The dose-dependence of the observed effects of TCDD on the expression of accessory molecules was evaluated on splenic DC isolated from mice treated with different doses of TCDD and euthanized 3 days later. As shown in Fig. 3, all doses of TCDD augmented the expression of ICAM-1 on the cell surface. Similarly, the expression of CD24 was dose-dependently enhanced by TCDD, with significance at the 15 $\mu\text{g}/\text{kg}$ dose. In contrast, the fluorescence of LFA-1 on the cells was significantly decreased at all doses of TCDD tested.

Time Course of Effects of TCDD on DC Phenotype and Cell Number

Since the immunotoxic effects of dioxins are known to persist for weeks after exposure (Kerkvliet and Baecher-Steppan, 1988; Kerkvliet and Brauner, 1987), we also examined DC 7 and 14 days after TCDD exposure to determine if changes in surface molecule expression persisted beyond day 3. As shown in Fig. 4, TCDD exposure increased the expression of ICAM-1, CD24, CD40, and B7-2 and suppressed the expression of LFA-1 on day 7. Similar effects were seen on day 14 (data not shown). Moreover, TCDD appeared to affect the expression of CD24 more dramatically at these later time points. In vehicle-treated mice, CD24 fluorescence was heterogeneous and suggestive of two populations of cells with low and high levels of CD24 expression. In the TCDD-treated mice the peak was more uniform and clearly shifted to the right; the overall intensity of CD24 staining was threefold higher compared to the vehicle-treated mice.

As shown Table 1, the number of DC isolated from the spleen of TCDD-treated mice was markedly reduced 7 and 14 days after exposure, an effect not seen on day 3. Specifically, relative to vehicle controls, the total number of CD11c^{HI} cells recovered in the low-density spleen cell fraction was reduced by 44% on day 7 and by 41% on day 14 in TCDD-treated mice. To evaluate the possibility that TCDD exposure increased the density of the DC, causing them to separate into the high-density cell fraction during density gradient centrifugation, we also examined the cells in the high-density BSA fraction. However, no increase was seen in the percentage or number of CD11c^{HI} cells in this fraction (data not shown). Additionally, TCDD treatment did not alter the total number of spleen cells recovered prior to density separation, suggesting a true loss of DC from the spleen.

DC from Balb/c Mice are Sensitive to TCDD

The C57Bl/6 mouse used in the previous studies is the prototypic TCDD-responsive mouse strain that possesses an AhR genotype that confers high sensitivity to the toxic effects of TCDD. In order to determine if DC would be similarly affected in other mice that possess a sensitive AhR genotype, we examined DC from Balb/c mice. As shown in Table 2, exposure to TCDD for 3 or 7 days caused the same pattern of effects in the Balb/c mice as in C57Bl/6 mice, increasing the

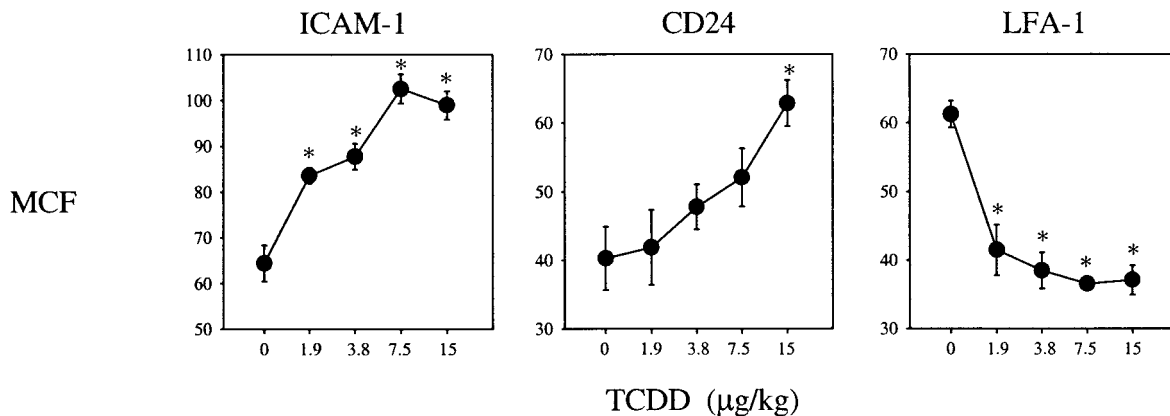


FIG. 3. Dose–response effect of TCDD on DC accessory molecule expression. C57Bl/6 mice were treated with vehicle control or indicated dose of TCDD. Three days later, spleens were removed, digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. MCF represents the staining on all CD11c^{HI} cells. Data points represent the mean MCF \pm SEM ($n = 3$ /treatment group). Data were analyzed by ANOVA and LSD. *Different from vehicle ($p \leq 0.05$).

expression of ICAM-1, CD24, CD40, and B7-2 and decreasing the expression of LFA-1 on DC. Additionally, TCDD decreased the numbers of DC recovered from the spleen of Balb/c mice at both time points.

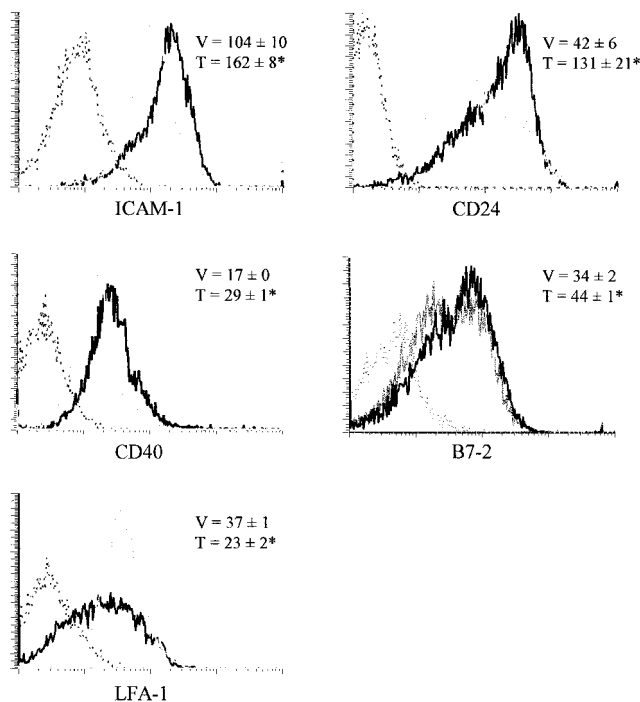


FIG. 4. Effect of TCDD on DC accessory molecule expression 7 days postexposure. C57Bl/6 mice were treated with vehicle control or 15 μ g/kg TCDD. Seven days later, DC were isolated from spleens and analyzed by flow cytometry as described in Fig. 2. Solid gray line (vehicle), solid black line (TCDD), dotted line (isotype control). Values represent mean MCF \pm SEM for vehicle (V)- and TCDD (T)-treated groups ($n = 3$ /group). Data are representative of three to five independent experiments. *Different from vehicle ($p \leq 0.05$).

TCDD Does Not Decrease Recovery of DC Nor Affect Accessory Molecule Expression in AhR^{-/-} Mice

Many, if not all, of the immunotoxic effects of TCDD result from activation of the AhR (Vecchi *et al.*, 1983; Kerkvliet *et al.*, 1990). To address whether TCDD-induced changes in DC were AhR-dependent, we examined DC from AhR^{-/-} mice exposed to 0 or 15 μ g/kg TCDD 7 days previously. As shown in Table 3, the absence of the AhR in the null mice did not appear to affect the overall development of splenic DC, since vehicle-treated C57Bl/6 and AhR^{-/-} mice had equivalent numbers of splenic DC that expressed similar levels of accessory molecules. Furthermore, TCDD treatment of the AhR^{-/-} mice did not alter the number of DC recovered from the spleen nor the expression of any markers examined, including ICAM-1, CD24, CD40, B7-2, or LFA-1. In contrast, DC from TCDD-treated C57Bl/6 mice, included in the study as positive controls, showed alterations in marker expression and number similar to previous experiments.

TABLE 1
Number of Splenic DC in C57Bl/6 Mice on Days 7 and 14 after TCDD Exposure^a

	CD11c ^{HI} ($\times 10^5$)
Vehicle	7.8 \pm 0.2
TCDD, day 7	4.4 \pm 0.1*
TCDD, day 14	4.6 \pm 0.2*

^a Data represent the average number \pm SEM of DC defined as CD11c^{HI} cells in the low-density spleen cell fraction ($n = 3$ /group). Data are representative of three independent experiments.

* Different from vehicle ($p \leq 0.05$).

TABLE 2

Number and Phenotype of Splenic DC in Balb/c Mice on Days 3 and 7 after TCDD Exposure^a

		Vehicle	TCDD, day 3	TCDD, day 7
DC	Number ($\times 10^5$)	7.1 \pm 0.8	4.4 \pm 0.5*	3.7 \pm 0.5*
ICAM-1	%	91.8 \pm 0.4	95.3 \pm 0.3*	95.1 \pm 0.4*
	MCF	80.3 \pm 0.7	92.7 \pm 0.7*	97.0 \pm 0.6**
CD24	%	89.9 \pm 0.4	93.5 \pm 0.2*	96.3 \pm 0.2**
	MCF	102.3 \pm 2.6	124.0 \pm 1.0*	131.3 \pm 0.9**
CD40	%	68.4 \pm 2.6	78.1 \pm 1.8*	82.2 \pm 1.3*
	MCF	69.0 \pm 1.2	76.3 \pm 1.5*	81.0 \pm 1.5*
B7-2	%	66.3 \pm 0.8	72.9 \pm 1.7*	76.6 \pm 1.3*
	MCF	78.7 \pm 0.9	83.3 \pm 1.2*	85.0 \pm 1.0*
LFA-1	%	74.0 \pm 1.0	63.7 \pm 0.9*	62.3 \pm 2.2*
	MCF	88.3 \pm 0.7	83.3 \pm 0.3*	82.3 \pm 2.2*

^a Data represent the mean \pm SEM of three animals per group. Data were analyzed by ANOVA and comparisons between group means were performed using LSD.

* Different from vehicle ($p \leq 0.05$).

** Different from vehicle and TCDD, day 3 ($p \leq 0.05$).

Effect of TCDD Exposure on the CD8 α + DC Population

Current evidence suggests that two populations of DC are present in the spleen, one derived from a lymphoid precursor and one of myeloid origin (Vremec and Shortman, 1997; Shortman and Caux, 1997). The expression of CD8 α has been used to identify the lymphoid-derived cells, which have also been identified by their coexpression of high levels of CD24 (Crowley *et al.*, 1990; Vremec *et al.*, 1992). Since CD24 expression was increased following TCDD exposure, it was possible that TCDD was selectively increasing the CD8 α + lymphoid DC population in the spleen. Therefore, CD8 α expression was evaluated on splenic DC from C57Bl/6, Balb/c,

and AhR^{-/-} mice at various times after treatment with 0 or 15 μ g/kg TCDD. As shown in Fig. 5, TCDD exposure increased the percentage of DC expressing CD8 α in C57Bl/6 and Balb/c mice but not in AhR^{-/-} mice. This increase in the percentage of CD8 α + DC was seen on days 7 and 14, but not on day 3, in C57Bl/6 mice. In Balb/c animals, TCDD had similar effects on both day 3 and day 7. Paradoxically, although the percentage of CD8 α + DC increased after TCDD treatment, the intensity of CD8 α staining was decreased in both C57Bl/6 and Balb/c mice. The significance of this observation is not currently known.

Effect of in Vivo TCDD Exposure on DC Function ex Vivo

The functional status of DC is often evaluated by their ability to activate allogeneic T cells in an MLR (Moser *et al.*, 1995; Girolomoni *et al.*, 1992; Metlay *et al.*, 1989). To determine if TCDD alters DC function, DC were enriched from the spleens of vehicle- or TCDD-treated C57Bl/6 mice 3 days after exposure and cultured with splenic T cells from untreated DBA animals for various periods of time. As shown in Fig. 6, TCDD exposure tended to enhance the ability of DC to activate T cells. Specifically, cultures containing DC from TCDD-treated mice demonstrated enhanced T cell proliferation (Fig. 6A) and IFN- γ production (Fig. 6B). TCDD exposure also resulted in a slight increase in IL-2 production (representative experiment shown in Fig. 6C). Although not statistically significant in all experiments, the increased IL-2 in 28-h cultures was significant when analyzed across four experiments ($p < 0.01$). Similar results were obtained in a single experiment in which proliferation and cytokine production were measured in cultures containing DC from animals treated with TCDD 7 days previously (data not shown).

As shown in Fig. 7, TCDD exposure was also associated

TABLE 3

Number and Phenotype of Splenic DC in AhR^{-/-} vs C57Bl/6 Mice on Day 7 after TCDD Exposure^a

		C57Bl/6		AhR ^{-/-}	
		Vehicle	TCDD	Vehicle	TCDD
DC	Number ($\times 10^5$)	9.6 \pm 0.6	5.5 \pm 0.6*	11.0 \pm 2.0	12.0 \pm 4.0
ICAM-1	%	91.1 \pm 1.4	94.8 \pm 0.9	94.9 \pm 1.5	89.0 \pm 4.0
	MCF	91.0 \pm 3.9	150.4 \pm 3.6*	105.7 \pm 5.7	92.2 \pm 11.6
CD24	%	90.3 \pm 1.0	96.4 \pm 0.8*	90.7 \pm 2.0	92.8 \pm 1.4
	MCF	41.7 \pm 4.7	59.8 \pm 9.1	45.1 \pm 17.7	46.2 \pm 2.5
CD40	%	65.2 \pm 1.3	85.4 \pm 0.6*	71.9 \pm 2.0	64.7 \pm 0.4
	MCF	14.5 \pm 0.4	28.1 \pm 0.9*	18.2 \pm 1.0	15.5 \pm 0.5
B7-2	%	65.0 \pm 0.7	77.8 \pm 0.7*	69.5 \pm 1.8	69.5 \pm 1.8
	MCF	41.7 \pm 1.3	59.0 \pm 1.4*	49.0 \pm 5.3	44.6 \pm 2.4
LFA-1	%	88.7 \pm 0.6	73.0 \pm 1.0*	94.6 \pm 1.3	94.2 \pm 0.8
	MCF	47.0 \pm 1.0	39.3 \pm 0.8*	54.3 \pm 10.6	51.4 \pm 0.9

^a Data represent the mean \pm SEM of two or three animals per group.

* Different from vehicle ($p \leq 0.05$).

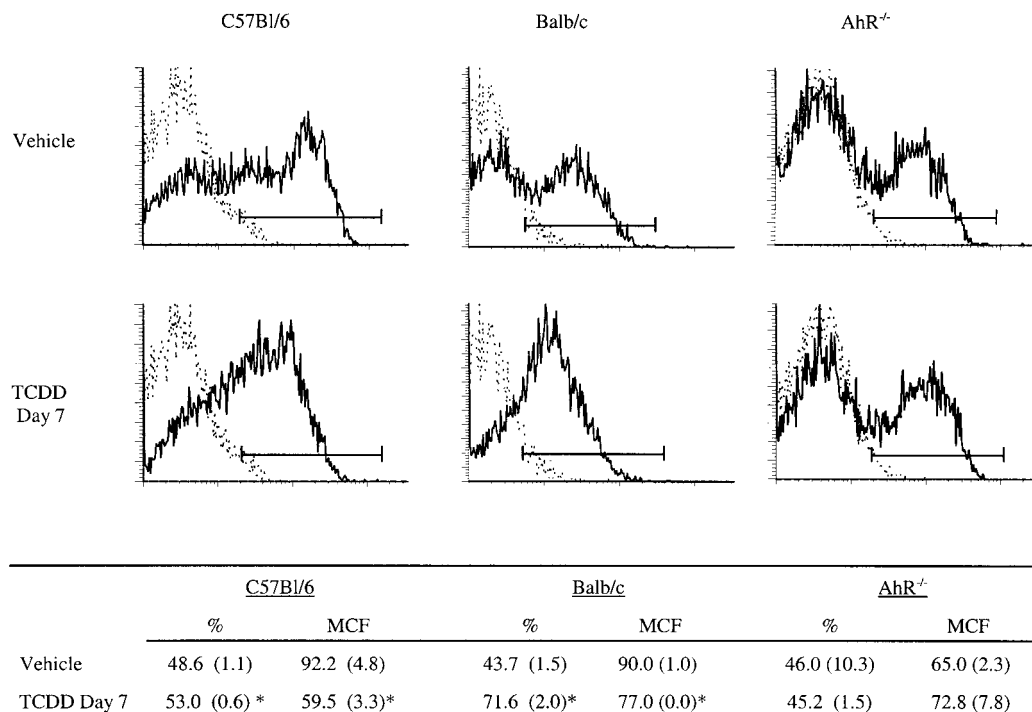


FIG. 5. Effect of TCDD on CD8 α expression on DC 7 days after exposure. Mice were treated with vehicle control or 15 $\mu\text{g}/\text{kg}$ TCDD. Seven days later, spleens were digested with collagenase, enriched for DC by density gradient centrifugation, and analyzed by flow cytometry. Dotted lines show staining of isotype control antibody. Region delineates CD8 α + cells as defined by staining above isotype control antibody. MCF was calculated on cells within this region. Data are representative of three experiments for C57Bl/6 mice and a single experiment for Balb/c and AhR^{-/-} mice. *Different from vehicle ($p \leq 0.05$).

with increased production of IL-12, an APC-derived cytokine that induces IFN- γ production by T cells and thus promotes a Th1-type immune response. When DC were cultured alone, we observed low, but detectable production of IL-12, which was augmented by TCDD treatment. Likewise, when T cell-enriched allogeneic splenocytes were added to the DC cultures, IL-12 production was higher in wells containing DC from TCDD-treated mice. In the wells containing both cell populations, IL-12 production was significantly enhanced relative to wells containing DC only, indicating cross-talk between the cell populations. This type of cell-cell communication likely occurs via interaction of cell surface molecules such as CD40-CD40L, or possibly as a response to production of IFN- γ , which has been shown to induce or enhance the production of IL-12 by macrophages (Ma *et al.*, 1996; Yoshida *et al.*, 1994). To address the possibility that augmented IL-12 production resulted from contaminating macrophages in the T cell preparation, we performed a separate experiment in which T cell preparations were depleted of adherent cells prior to addition to DC cultures. This depletion did not affect IL-12 production, indicating that the DC were the source of the IL-12 (data not shown).

DISCUSSION

The effects of TCDD exposure on DC phenotype and function have not been previously reported. Due to the immuno-

suppressive properties of TCDD, we hypothesized that TCDD exposure would suppress the expression of key accessory molecules on DC and/or disrupt their ability to activate T cells. Contrary to expectation, we instead found that DC from TCDD-treated mice expressed higher levels of many important accessory molecules, produced more IL-12, and enhanced the *in vitro* activation of T cells. The changes induced by TCDD were dose-dependent and persisted for at least 14 days. Similar effects on DC were seen in two AhR-responsive mouse strains (C57Bl/6 and Balb/c), but not in AhR^{-/-} animals, indicating that changes in DC were mediated by the AhR. The changes in DC phenotype were induced by TCDD in the absence of antigenic stimulation and suggest that TCDD may inappropriately activate DC.

Although these results appear to be difficult to reconcile with TCDD's immunosuppressive effects, there are several biologically plausible hypotheses that are compatible with current understanding of DC biology. For example, DC lose their ability to take up particulate antigens when activated (Kitajima *et al.*, 1997; Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1995). Thus, inappropriate activation of DC by TCDD may result in a diminished ability to internalize antigen for processing, a scenario that was previously observed following LPS-induced activation of DC (DeSmedt *et al.*, 1996). In our studies, antigen presentation by DC was assessed in an allogeneic MLR assay in which T cells recognize the alloMHC on the surface of the

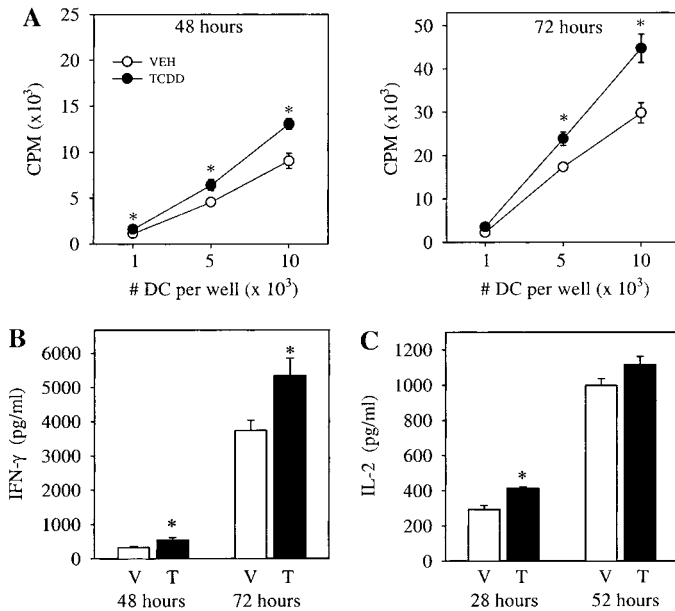


FIG. 6. Effect of TCDD exposure on ability of DC to stimulate T cell proliferation and cytokine production in a mixed leukocyte reaction. Spleens were removed from C57BL/6 mice 3 days after treatment with vehicle (open circles/bars) or 15 $\mu\text{g}/\text{kg}$ TCDD (solid circles/bars). Spleens from three mice were pooled, and DC were enriched by collagenase digestion, density gradient centrifugation, and transient adherence to plastic. Indicated numbers of DC were cultured with 3×10^5 T cells enriched from spleens of DBA/2 mice. Proliferation (A) was measured at 48 and 72 h by ^3H -TdR incorporation. Background activity was below 1000 cpm in wells containing T cells alone and below 400 cpm in wells containing DC alone. For IFN- γ (B) and IL-2 (C) analysis, culture supernatants were harvested at indicated times from wells containing T cells and 10^4 DC. Cytokine concentrations were determined by ELISA. Wells containing only DC or T cells were below the limit of detection. Data points represent the means \pm SEM ($n = 4/\text{group}$). Data are representative of three (IFN- γ) or four (proliferation, IL-2) experiments. *Different from vehicle ($p \leq 0.05$).

DC, independent of antigen processing. Studies are in progress to evaluate the ability of DC from TCDD-treated mice to internalize and present exogenous antigen to antigen-specific T cells.

Another potential link between TCDD-induced DC activation and immune suppression relates to the significant decrease in numbers of DC in the spleen of TCDD-treated mice. In a normal immune response, once DC are activated, they are committed to undergo apoptosis (DeSmedt *et al.*, 1996, 1998; Winzler *et al.*, 1997). This is likely an important mechanism by which the immune response is downregulated following antigen clearance. It is possible that the inappropriate activation of DC by TCDD leads to their premature deletion. This may result in an immune response that never fully develops or one that is prematurely terminated. This possibility is supported by recent studies that suggest both the number and life span of DC *in vivo* influence the strength of the subsequent immune response (Josien *et al.*, 2000).

One of the most significant changes in DC induced by TCDD was an increase in CD24 expression. Since high levels

of CD24 are coexpressed with CD8 α on lymphoid DC (Vreemec *et al.*, 1992), it suggests that TCDD treatment might be selectively increasing this subset of DC. This possibility is intriguing because current evidence suggests that lymphoid DC may play a role in downregulation of an immune response, including the induction of tolerance (Suss and Shortman, 1996; Kronin *et al.*, 1996). Interestingly, when CD8 α expression on DC from TCDD-treated mice was examined, the percentage of DC staining positive for CD8 α increased, but the intensity of the staining decreased. Although this staining pattern confounded a straightforward interpretation of the data, it was noteworthy that the increased percentage of CD8 α + DC occurred on the same days that DC numbers decreased in the spleen. Thus, taken together, these results suggest that TCDD may selectively reduce the myeloid DC population rather than increase the CD8 α + lymphoid DC. Additional markers defining the lymphoid and myeloid subpopulations of DC will need to be examined to verify subtype-specific TCDD effects.

In contrast to the increased expression of other accessory molecules, TCDD caused a marked reduction in LFA-1 expression on DC. This reduced expression is unlikely to reflect activation of DC since LFA-1 is upregulated on activated macrophages and T cells (Kurtzinger *et al.*, 1981; Strassmann *et al.*, 1985, 1986). Furthermore, LFA-1 expression was not reduced on splenic DC subjected to overnight culturing (Inaba *et al.*, 1994, and our unpublished observations), a stimulus that causes a substantial increase in expression of activation markers such as B7 and CD40. LFA-1 is a β -2 integrin protein that is important in cell-cell adhesion and plays a role in leukocyte trafficking and extravasation into tissue (Andrew *et al.*, 1998; Ma *et al.*, 1994). For example, studies by Ma *et al.* (1994) showed that blocking LFA-1 *in vivo* reduced the antigen-

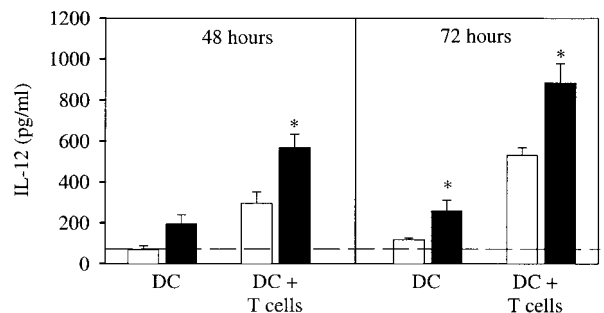


FIG. 7. Effect of TCDD exposure on IL-12 production by DC in a mixed leukocyte reaction. Spleens were removed from C57BL/6 mice 3 days after treatment with vehicle (open bars) or 15 $\mu\text{g}/\text{kg}$ TCDD (solid bars). Spleens were pooled from three animals and dendritic cells were isolated by collagenase digestion, density gradient centrifugation, and transient adherence to plastic. 1×10^4 DC were cultured alone or with 3×10^5 allogeneic T cells enriched from spleens of untreated DBA/2 mice. Culture supernatants were harvested at indicated times, and cytokine concentration was determined by ELISA. Data points represent the means \pm SEM ($n = 4/\text{group}$). Data are representative of three experiments. Dashed line represents limit of detection for ELISA. Wells containing only T cells were below the limit of detection. *Different from vehicle ($p \leq 0.05$).

induced migration of DC to the lymph nodes and the subsequent development of a contact hypersensitivity response. Therefore, it is possible that the reduced LFA-1 expression induced by TCDD may interfere with antigen-specific activation of T cells by inhibiting the ability of DC to carry antigen to the T cell areas of the lymphoid organs. In addition, if LFA-1 plays a role in general trafficking of DC, a decrease in expression of this molecule could affect normal repopulation of splenic DC lost to cell turnover or death and thus could account for the loss of splenic DC we observed in TCDD-treated mice. Further studies are warranted to address the functional significance of decreased LFA-1 expression.

Finally, the increased production of IL-12 by DC from TCDD-treated mice observed in the absence of exogenous antigen, although consistent with DC activation, was unexpected given the results of other studies in our laboratory. Specifically, exposure to TCDD during the development of an immune response to allogeneic tumor cells or to ovalbumin suppressed the *in vitro* production of IL-12 by antigen-restimulated spleen cells (Shepherd *et al.*, submitted for publication, and unpublished observations). It is possible that these contrasting results illuminate differences in TCDD's effects on DC in the presence and absence of antigen. Alternatively, since macrophages and other inflammatory cells also make IL-12, it is possible that the decreased IL-12 production reflects a suppressive effect of TCDD on other IL-12-producing cells present in the spleen.

In conclusion, our studies have shown that splenic DC from TCDD-treated mice demonstrate enhanced expression of many accessory molecules required for activation of T cells, produce more IL-12, and are relatively more efficient at stimulating T cells *in vitro* compared to DC from vehicle-treated mice. Although these findings were contrary to our original hypothesis, there are several potential mechanisms that could link DC activation to suppression of the immune response. In future studies, we are particularly interested in the relationship between DC survival and immune suppression as well as the effects of TCDD on different DC subpopulations.

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