Inhibition of TC-1 Cytokine Production, Effector Cytotoxic T Lymphocyte Development and Alloantibody Production by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin[1]

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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a widespread environmental contaminant and prototypic ligand for the aryl hydrocarbon receptor, is a potent immunotoxicant. To understand the underlying mechanisms of TCDD immunotoxicity, we have characterized the time course of changes in CTL, alloantibody, and cytokine responses to the P815 tumor allograft in C57Bl/6 mice treated with 0 or 15 μg TCDD/kg. Suppression of CTL activity by TCDD directly correlated with reduced numbers of splenic CTL effector cells identified by their $CD8^+CD44^{high}CD45RB^{low}$ phenotype, while suppression of the alloantibody response correlated with a lack of expansion of the $B220^+$ splenocyte population. Cytokine production was differentially modulated following TCDD treatment. Although type 1 cytokine production (IFN-γ, IL-2, and TNF) was initially induced in TCDD-treated mice, production failed to increase normally after day 5. In contrast, the production of IL-1β, IL-4, and IL-6 was mostly unaffected by TCDD exposure. This differential effect of TCDD on cytokine production was reflected in the degree of suppression of specific alloantibody isotypes. TCDD abrogated the production of IgG2a (promoted by IFN-γ), but had much less effect on the level of IgG1 (promoted by IL-4). IgM Ab titers were also highly suppressed. $CD8^+$ cells were the exclusive producers of IFN-γ and IL-2 when spleen cells from P815-injected mice were cultured in vitro on days 4 to 7 after P815 injection. However, $CD4^+$ cells were shown to play a crucial role in the generation of both CTL and alloantibody responses, since their depletion in vivo abolished both responses. Based on similar temporal effects produced by TCDD and anti-CD4 Ab on alloimmune responses, we postulate that TCDD interferes with the initial activation of $CD4^+$ T cells, which leads to downstream inhibition of the activation and/or differentiation of $CD8^+$ T cells and B cells. In addition, since delayed treatment with either anti-CD4 Ab or TCDD suppressed the alloantibody but not the CTL response, TCDD may also affect later $CD4^+$ T helper-B cell interactions. *The Journal of Immunology,* **1996, 157: 2310–2319.**

The immune system has been identified as one of the most sensitive targets for the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD),[4] the prototype and most toxic member of a large group of structurally similar chlorinated aromatic compounds that are widely dispersed in the environment. These chemicals represent a unique group of environmental contaminants, in that much of their toxicity is initiated by binding to an intracellular receptor, the aryl hydrocarbon receptor (AhR). The AhR functions as a ligand-activated transcription factor to induce a battery of genes coding for cytochrome P4501A1 and other enzymes important in xenobiotic metabolism (1). Transcriptional activation or repression of other, as yet unidentified, genes is hypothesized to underlie the specific toxic effects of TCDD (2, 3). Accumulating evidence suggests that alterations in the production of several different growth factors and cytokines may be involved in TCDD-induced toxicity in different tissues including the skin (4–6), liver (7, 8), and palate (9). The possible role that cytokines play in TCDD immunotoxicity, however, has not been determined.

In mice, TCDD-induced immune dysfunction is characterized by suppression of acquired immunity, with both Ab and cell-mediated responses susceptible to suppression under different exposure paradigms (reviewed in Refs. 10 and 11). In contrast, nonspecific immunity mediated by macrophages and NK cells is more resistant to suppression by TCDD (12), and inflammatory responses are sometimes enhanced following TCDD exposure (13, 14). However, investigations into the mechanisms by which TCDD exerts its immunotoxicity have been hampered by the difficulty in demonstrating consistent direct immunotoxic effects of TCDD in vitro (15–21). Hence, the cellular targets and biochemical mechanisms that mediate the immunotoxicity of TCDD remain poorly understood.

We (21, 22) as well as others (23) have previously shown that treatment of C57Bl/6 mice with TCDD results in a dose-dependent suppression of the in vivo CTL response to allogeneic P815 mastocytoma cells. Structure-activity studies using polychlorinated biphenyl congeners that differ in their affinity for the AhR (22, 23), and the use of AhR-congenic mice (22) indicates that the suppression of the CTL response is mediated through the AhR. However, when TCDD or polychlorinated biphenyl is added directly to

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[4] Abbreviations used in this paper: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; CTL_E, effector cytotoxic T lymphocyte; CTL_P, precursor cytotoxic T lymphocyte.

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mixed lymphocyte-tumor cell cultures, neither the proliferative response nor the development of CTL activity is altered (21, 24, 25). Thus, the basis for the suppression of CTL responses in vivo by AhR ligands is unknown.

In this report, we have attempted to provide insight into the mechanisms of suppression of the CTL response by characterizing the effects of TCDD on the phenotypes of the cells responding to P815 tumor challenge and their ability to produce a panel of cytokines important in the development of CTL activity. We also evaluated the cytotoxic alloantibody response to P815 tumor cells to directly compare the sensitivity of humoral and cell-mediated responses to suppression by TCDD. The results of these studies indicate that TCDD targets early activation events that may involve $CD4^+$ cells, which lead to premature termination of cytokine production by $CD8^+$ TC-1 cells, suppression of CTLp activation, and suppression of B cell activation. Additional effects of TCDD on $CD4^+$-B cell interactions appear to contribute to the suppression of the alloantibody response.

Materials and Methods

Animals

Male C57Bl/6 and female DBA/2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DBA/2 mice were housed six per cage and were used for weekly in vivo propagation of the P815 mastocytoma cells. C57Bl/6 mice were housed singly and used in experimental studies at 7 to 9 wk of age.

Antibodies and cytokine standards

Anti-CD4 (GK1.5) and anti-CD8 (53–6.72) mAb used for T cell depletion experiments were purified by HPLC from ascites and generously provided by Dr. Randy Noelle, Dartmouth Medical School (Lebanon, NH). Rat IgG used as a control Ab for T cell depletion experiments was purchased from Cappel (Organon Teknika, West Chester, PA). The following were used as primary Abs for flow cytometric analysis: phycoerythrin-labeled anti-CD8a (53–6.72), FITC-labeled anti-B220 (RA3–6B2), FITC-labeled anti-CD45RB (16A), and FITC-labeled anti-CD62L (MEL-14) from PharMingen (San Diego, CA); RED670-labeled anti-CD44 (IM7.8.1) and FITC-labeled anti-CD4 (H129.19) from Life Technologies (Grand Island, NY); and FITC-labeled anti-CD8a (53–6.7) from Becton Dickinson (Mountain View, CA). For cytokine ELISA assays, capture and biotinylated second Ab pairs and cytokine standards were obtained from PharMingen, except for IL-4, which was obtained from Genzyme (Cambridge, MA). TNF-α standard was also obtained from Genzyme. An ELISA kit for measuring IL-1β was purchased from Perseptive Diagnostics (Cambridge, MA).

Xenobiotic exposure

TCDD (Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1.5 μg/ml. A vehicle control solution was prepared in a similar manner. Mice were given a single dose of 15 μg of TCDD/kg body weight by gavage, a dose known to produce significant suppression of the CTL response in P815-injected mice (21).

Animal treatments

Mice were treated with TCDD 1 day before the i.p. injection of 1×10^7 viable P815 tumor cells on day 0. In one study, TCDD was administered on days −1, +1, +2, +3, +4, or +5 relative to the injection of P815 cells. Animals were killed by CO_2 overdose at various times after P815 injection. Spleens were removed aseptically. Blood was collected by heart puncture into heparinized syringes. Plasma was separated and stored at −70°C.

Preparation of spleen cells

Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Spleen cells were suspended in cold HBSS supplemented with 2.5% FCS (Rehautin, Intergen, Purchase, NY; 0.065 ng endotoxin/ml), 20 mM HEPES, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate. Erythrocytes were removed by hypotonic lysis. Nucleated spleen cells were enumerated using a Coulter counter (Coulter Electronics, Hialeah, FL).

Flow cytometric analysis of spleen cells

B cells and T cell subsets in the spleen were defined by B220, CD4, or CD8 expression. CTL_E cells were also identified by the expression of CD8, CD44, and CD45RB as described by Mobley and Dailey (26). Cells were incubated with predetermined optimal concentrations of fluorochrome-conjugated mAb in 96-well microtiter plates in the presence of 10 μg of rat IgG to block nonspecific binding. Appropriately labeled, isotype-matched Igs were used as controls for nonspecific fluorescence. Data were collected from 20,000 to 100,000 freshly stained cells by listmode acquisition using an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using Cyclops software (Cytomation, Inc., Fort Collins, CO). A live cell gate was established on the basis of forward angle light scatter and propidium iodide to exclude clumps and dead cells.

CTL assay

The cytolytic activity of spleen cells to P815 tumor cells was measured in a standard 4-h ^{51}Cr release assay as previously described (21). E:T ratios from 200:1 to 6.25:1 were tested in duplicate. The percent cytotoxicity was calculated for each E:T ratio as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{naive release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

where experimental release was obtained with splenocytes from P815-injected mice, naive release was obtained with spleen cells from uninjected mice, maximum release was obtained by incubating tumor cells in SDS, and spontaneous release was obtained by incubating tumor cells in medium. The spontaneous release of ^{51}Cr never exceeded 15%.

Cytotoxic antibody assay

Cytotoxic alloantibody titers were determined using a complement-dependent ^{51}Cr release assay. Plasma samples were heat inactivated at 56°C for 30 min before assay. Serial twofold dilutions (1/10 to 1/2560) of plasma were incubated in wells of 96-well round-bottom plates with 1×10^4 ^{51}Cr-labeled P815 cells for 20 min at 37°C in 5% CO_2. After one wash, 100 μl of Low-Tox-M rabbit complement (1/12, Cedarlane Laboratories, Hornby, Ontario, Canada) was added for 45 min at 37°C. The amount of ^{51}Cr released into the supernatant was measured by gamma scintillation counting. Specific cytotoxicity was calculated by subtracting the background release of ^{51}Cr due to complement only from the experimental release and dividing by the maximum releasable activity using SDS. All samples were tested in duplicate on separate plates. The Ab titer was defined as the highest dilution of plasma at which a minimum of 20% specific cytotoxicity was measured. The titer was transformed to $\log_2$ dilution for statistical analysis.

Alloantibody isotype characterization by flow cytometry

The isotype of Ab produced in response to P815 tumor challenge was characterized by flow cytometry as previously described by Bishop et al. (27). Plasma obtained from mice on different days after P815 injection was used as the primary Ab to stain P815 tumor cells, followed by second step staining with FITC-conjugated goat $F(ab')_2$ anti-mouse IgM, IgG1, or IgG2a (Southern Biotechnology, Birmingham, AL). The mean channel of the fluorescence was determined for each sample and used as a measure of the isotype-specific titer.

Cytokine analysis

Spleen cells were suspended in serum-free Ultraculture medium (BioWhittaker, Walkersville, MD) supplemented with 1.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM L-glutamine, 20 mM HEPES, and 5×10^{-5} M 2-ME. Spleen cells (1×10^7) were incubated for 6 h with 1×10^6 P815 tumor cells in 2-ml cultures in 24-well plates (Corning) at 37°C and 5% CO_2. Cultures of naive spleen cells with P815 tumor cells and P815 tumor cells alone were included as controls in each experiment.

IL-1, IL-2, IL-4, IL-6, and IFN-γ were measured in culture supernatants using Ab sandwich ELISA techniques. The secondary biotinylated Abs 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 plate reader (Bio-Tek Instruments, Wincoski, VT). Supernatant levels of TNF were measured by standard bioassay (28) using a L929 fibroblast cell line (kindly provided by Dr. Mark Blazha, National Institute of Environmental Health Sciences, Research Triangle Park, NC).

RNA preparation and reverse transcription-PCR

Spleen cells were restimulated in vitro with P815 cells for 1 h before RNA extraction, a step required to induce detectable levels of IL-2 and IFN-γ mRNA. Total RNA was obtained by lysis of cells in 4 M guanidine-thiocyanate as previously described by Noelle et al. (29). cDNA was synthesized using RNA extracted from 1×10^6 spleen cells using oligo(dt) primers (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). PCR amplification of cDNA was performed using Taq polymerase (Boehringer Mannheim, Indianapolis, IN), specific cytokine gene oligonucleotide primers (see below), and deoxynucleotides for 30 cycles using an Ericomp thermocycler (San Diego, CA). DNA products were separated by agarose gel electrophoresis and visualized using ethidium bromide. All cDNA samples were initially analyzed for β_2m to determine relative amounts and integrity of mRNA/cDNA. Samples displaying low levels of β_2m gene expression or degraded DNA amplification products were discarded.

TNF-α primers were obtained from Clontech (Palo Alto, CA). Primers for IL-2, IFN-γ, and β_2m were designed using RightPrimer (BioDisk Software, San Francisco, CA) to span introns of the respective cytokine genes to allow for discrimination between amplified DNA and genomic or cDNA. The primer sequences used were: β_2m, 5′-ATGGCTCGCTCGGTGACC CT and 3′-TCATGATGCTTGATCACATG; IL-2, 5′-ATGTACAGGATG CAAATAATGTCTT and 3′-GTTAGTGTTGAGATGATGCTTTGAC; and IFN-γ, 5′-ATGAAATATACAAGTTATATCTTGGCTTTT and 3′-GATGCTCTTCGACCTCGAAACAGCATCTGA.

Depletion of T cell subpopulations in vitro

Spleen cell suspensions were selectively depleted of $CD4^+$ or $CD8^+$ T cells by panning (30). Spleen cells (1×10^7) were incubated for 30 min at 4°C with 10 μg of rat anti-CD4 Ab, rat anti-CD8 Ab, or a control rat IgG. Ab-coated cells were then depleted from the suspension by incubation on plastic petri dishes coated with goat anti-rat IgG (Cappel) for 40 min at 4°C. Nonadherent cells were panned twice. Flow cytometric analysis demonstrated >90% depletion of $CD4^+$ cells and 100% depletion of $CD8^+$ cells.

In vivo depletion of $CD4^+$ and $CD8^+$ cells

To deplete $CD4^+$ cells, mice were injected i.p. with 500 μg of anti-CD4 Ab or a control rat IgG on days −3, −2 and +3 relative to P815 injection on day 0. To deplete $CD8^+$ cells, mice were injected i.p. with 250 μg of anti-CD8 Ab or a control rat IgG on days −1, +1, and +5 relative to P815 challenge. Single doses of these Abs were shown in preliminary studies to achieve >90% depletion of $CD8^+$ cells and >99% depletion of $CD4^+$ cells bearing a naive ($CD44^{low}CD45RB^{high}$) phenotype. The preferential depletion of resting naive $CD4^+$ cells following in vivo treatment with anti-CD4 Ab has been recently reported (31). In a separate study, mice received one i.p. injection of 250 μg of anti-CD4 Ab on days −1, +1, +2, +3, +4, or +5 relative to P815 injection on day 0.

Statistical analysis

Results are presented as the mean ± SEM of six mice per group unless indicated otherwise. Most experiments were repeated at least once. Analysis of variance modeling was performed using SAS statistical software (version 6.03, SAS Institute, Inc., Cary, NC) with a significance level of $\alpha = 0.05$. Comparisons between means were made using the least significant difference multiple comparison t test or Dunnett's t test for pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant.

Results

TCDD exposure suppresses CTL and cytotoxic antibody responses to P815 tumor cells

Previous studies have shown that a single oral dose of TCDD over a dose range of 2.5 to 40 μg/kg suppresses the splenic CTL response when measured 10 days after the injection of P815 tumor cells in C57Bl/6 mice (21). In this experiment, the effect of 15 μg of TCDD/kg on CTL activity and cytotoxic Ab responses were examined in a time-course study. Mice were treated with vehicle or TCDD 1 day before P815 injection, and immune responses were measured from days 4 to 10. As shown in Figure 1, at an E:T ratio of 200:1, CTL activity was first detected in the spleens of both vehicle- and TCDD-treated mice on day 5. In vehicle-treated mice, the CTL response increased to a maximum of 65% specific lysis on day 10, whereas the response in TCDD-treated mice did not exceed 10% lysis on any day tested. In the sera of the same mice, cytotoxic alloantibodies were first detected on day 6 (Fig. 1). Titers increased steadily through day 10 in vehicle-treated mice, while titers did not increase in TCDD-treated mice after day 8.

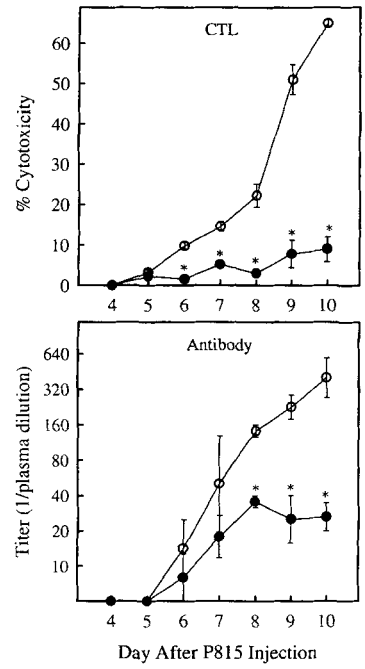

FIGURE 1. Time course of CTL and cytotoxic alloantibody responses in C57Bl/6 mice given a single oral dose of either the vehicle control (○) or 15 μg/kg TCDD (●) 1 day before the i.p. injection of 1×10^7 P815 cells. On days 4 to 10 after P815 injection, splenic CTL activity and plasma titers of cytotoxic Ab were determined as described in *Materials and Methods*. Data points represent the mean ± SEM for six mice per treatment per day. Cytotoxicity data are shown for the 200:1 E:T ratio. * indicates $p \leq 0.05$.

Spleen cell phenotypes reflect immune responsiveness to P815 tumor

Spleen cells were analyzed by flow cytometry on days 5 to 10 following P815 injection to determine whether P815 injection altered the percentage and/or total number of B and T lymphocyte subpopulations. As shown in Figure 2, the number of $CD8^+$ T cells increased dramatically on days 9 and 10 in vehicle-treated mice in parallel with the increase in CTL activity on these days. $B220^+$ B cell numbers were already increased on day 5 following P815 injection and remained elevated through day 9. In contrast, the percentage and total number of $CD4^+$ T cells decreased in P815-injected mice on all days compared with those in naive animals. When TCDD-treated mice were compared with vehicle-treated mice, the most distinctive difference was the absence of an increase in $CD8^+$ cells on days 9 and 10. Also notable was the steady decline in the number of $B220^+$ cells on days 6 to 8. $CD4^+$ cell numbers were less affected by TCDD, with a small decrease in number on day 8 and a larger decrease on day 10. Similar TCDD-induced changes in these cell populations have been documented in two other time-course studies.

Suppression of CTL activity correlates with a reduced percentage of $CD8^+$ T cells expressing a CTL_E phenotype

In a mouse skin allograft model, Mobley and Dailey (26) reported that functions associated with CTL_E, including cytotoxic activity

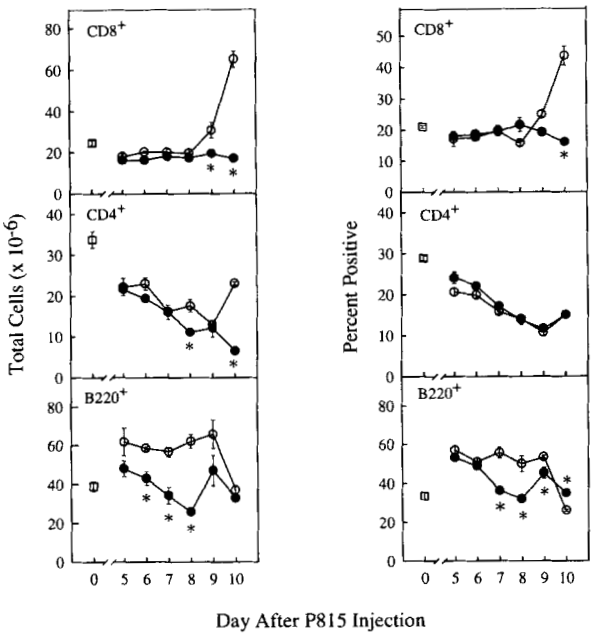

FIGURE 2. Splenic phenotypes in vehicle- and TCDD-exposed mice during the P815 allograft response. Mice were treated as described in Figure 1, with treatment groups represented as naive (□), vehicle control (○), and TCDD (●). Spleen cells were immunophenotyped by flow cytometric analysis for CD8, CD4, and B220 expression. Data points represent the mean ± SEM for six mice per treatment per day. The data for naive mice were pooled from 12 mice tested in groups of two on each day. * indicates $p \leq 0.05$.

in a ^{51}Cr release assay, IFN-γ production, and granzyme activity, were restricted to a subset of $CD8^+$ lymph node cells that expressed high levels of CD44 and low levels of MEL-14 and CD45RB. We used CD44 and CD45RB to identify $CD8^+$ CTL_E in the spleen following P815 injection. As shown in the representative histograms in Figure 3, cells expressing the CTL_E phenotype ($CD44^{high}$ $CD45RB^{low}$) represented the majority of $CD8^+$ cells in mice that had been injected with P815 tumor cells 10 days previously. In contrast, only a small percentage of cells in nonimmune mice expressed this phenotype. To determine whether TCDD treatment inhibited the development of CTL_E cells, mice were treated with different doses of TCDD to induce varying degrees of suppression of CTL activity as previously described (21). The cytotoxic activity of the spleen cells in a ^{51}Cr release assay was then correlated on an individual animal basis with the percentage of $CD8^+$ cells that expressed the CTL_E phenotype. As shown in Figure 4, the cytotoxic activity of splenocytes was highly correlated with the percentage of CTL_E in the splenocyte population. These results indicate that the suppression of cytotoxic activity by TCDD reflects a quantitative reduction in the number of CTL_E.

Cytokine production by spleen cells responding to P815 tumor cells is altered by TCDD exposure

Several different cytokines have been reported to play a role in the development of CTL responses in vitro, including IL-1 (32, 33), IL-2 (33–36), IL-4 (36, 37), IL-6 (32, 36, 38), IFN-γ (35, 39, 40), and TNF (41, 42). These and perhaps other cytokines act together during the process of CTL_p activation and differentiation to promote the acquisition of cytolytic and other effector capabilities as well as to drive clonal expansion. We investigated the effects of TCDD on cytokine production by spleen cells isolated from mice on days 4 to 10 following P815 injection and restimulated in vitro

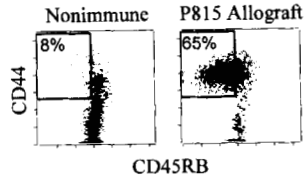

FIGURE 3. Representative histogram showing the correlated expression of CD44 and CD45RB on $CD8^+$ cells obtained from the spleens of nonimmune mice or mice injected with P815 tumor cells 10 days previously. Cells were stained and analyzed by three-color flow cytometry as described in *Materials and Methods*. The region identified represents the population of $CD8^+$ cells that expresses the CTL_E phenotype ($CD44^{high}CD45RB^{low}$) as described by Mobley and Dailey (26).

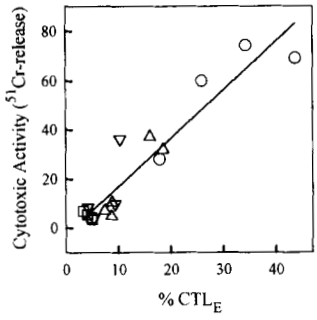

FIGURE 4. Correlation between cytotoxic activity in the ^{51}Cr release assay and the frequency of $CD8^+$ cells expressing the CTL_E phenotype, defined in Figure 3. C57Bl/6 mice were treated with 0 (○), 3.75 (△), 7.5 (▽), or 15 (□) μg TCDD/kg body weight 1 day before the injection of 1×10^7 P815 tumor cells. Mice were killed 9 days after tumor injection for assessment of CTL activity and flow cytometric analysis as described in *Materials and Methods*. The data shown represent the cytotoxic activity at a 100:1 E:T ratio. The two parameters were highly correlated ($r = 0.95$; $p < 0.0001$). Similar correlations were seen with other E:T ratios.

with P815 cells for 6 h. Supernatants were harvested and analyzed for IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF. As summarized in Figure 5, IL-4, TNF, and IL-6 were already elevated in vehicle-treated mice by day 4 following the injection of P815 cells, followed by the appearance of IL-1β and IFN-γ on day 5 and IL-2 on day 6. Peak cytokine production occurred on day 7 for IL-6; on day 8 for IL-4; on day 9 for IL-2, IFNγ, and TNF; and on day 10 for IL-1β. Interestingly, on day 10, IL-2 levels declined to levels found in naive mice despite the ongoing CTL response, whereas levels of TNF and IFN-γ remained significantly elevated.

This cytokine profile was quite different in mice exposed to TCDD. The early increases in TNF (on days 4 and 5) and IFN-γ (on day 5) production were normal, but levels failed to increase thereafter. IL-2 production was significantly elevated above control values on day 5 and then failed to increase further. The small but significant early increase in IL-2 production in TCDD-treated mice has since been corroborated in two additional studies. In contrast, IL-1β, IL-4, and IL-6 production was not significantly affected by TCDD exposure, except for small differences in IL-6 production on day 4 (lower than controls) and day 10 (higher than controls).

TCDD reduces mRNA for IFN-γ and IL-2, but not TNF-α

To corroborate the changes in cytokine production seen at the protein level and to exclude effects that might be due to altered patterns of cytokine consumption, mRNA levels for IL-2, IFN-γ, and TNF-α were evaluated by reverse transcription-PCR in the same

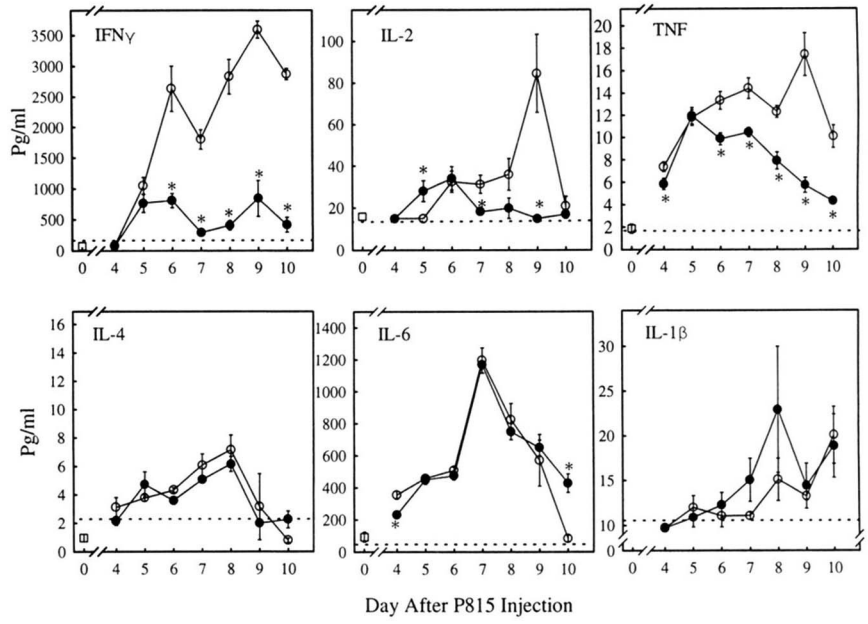

FIGURE 5. Time course of *ex vivo* cytokine production by spleen cells during the allogeneic response to P815 tumor cells in vehicle- and TCDD-treated mice. Mice were treated as described in Figure 1. Spleen cells from naive (□), vehicle-treated (○), or TCDD-treated (●) mice were cultured for 6 h with P815 cells as described in *Materials and Methods.* Culture supernatants from six mice per treatment per day were assayed for IFNγ, IL-2, IL-4, IL-6, and IL-1β by cytokine-specific ELISAs and for TNF using the L929 bioassay. The TNF activity detected in the bioassay was TNF-α based on the ability of Ab to TNF-α to neutralize all activity in randomly selected samples of day 7 or day 9 spleen cell culture supernatants. The dashed lines indicate the lowest dilution of standard tested. Data points represent the mean ± SEM. * indicates $p \leq 0.05$. P815 cells cultured alone for 6 h produced barely detectable levels of TNF and IL-6 and none of the other cytokines.

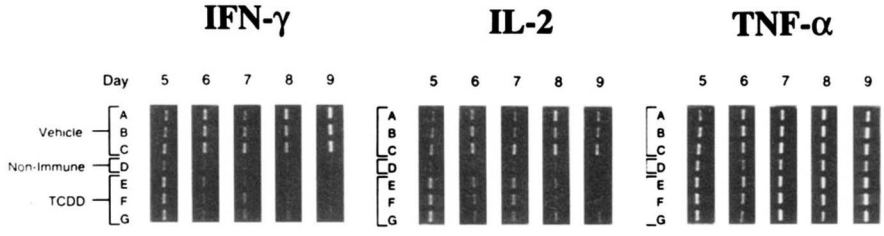

FIGURE 6. Influence of TCDD on splenic mRNA levels of IFN-γ, IL-2, and TNF-α during the allogeneic response to P815 tumor cells. The same spleen cell cultures used for measuring cytokine production in Figure 5 were used to measure cytokine mRNA levels, except that they were harvested after 1 h instead of 6 h of incubation, as described in *Materials and Methods.* The same number of P815 tumor cells alone had no detectable mRNA for IL-2, IFN-γ, or TNF-α.

time-course study as that reported in Figure 5. As shown in Figure 6, mRNA for IL-2 and IFN-γ showed a pattern of expression that directly paralleled the pattern of secreted protein. When compared with vehicle-treated mice, TCDD-treated mice showed higher levels of IL-2 mRNA on day 5, but lower levels than controls on all days thereafter. IFN-γ mRNA levels were reduced on all days except day 5 compared with those in vehicle-treated mice. IL-2 and IFN-γ mRNA were also detected in naive spleen cells after 1 h of culture with P815 cells, but at levels much lower than were present in in vivo activated cells from vehicle-treated mice. TNF-α mRNA levels, on the other hand, did not reflect the TNF activity measured by bioassay, and there were no detectable differences among naive, vehicle-treated, or TCDD-treated mice. These results were not an artifact of overamplification, since dilution of cDNA samples 5- and 25-fold yielded proportionately lower, but equivalent, levels of TNF-α from all three treatment groups (data not shown). These results suggest that TNF protein levels may be significantly modulated post-transcriptionally.

IL-2 and IFN-γ are produced exclusively by CD8⁺ spleen cells on days 4 to 7 following P815 injection

To determine whether the suppression of IL-2 and IFN-γ production by spleen cells from TCDD-treated mice represented a selective effect on $CD4^+$ or $CD8^+$ T cells, cytokine production by spleen cells depleted of either T cell subset was examined. As shown in Table I, depletion of $CD4^+$ cells did not affect the amount of IL-2 or IFN-γ produced on days 4 to 7 after P815 injection. However, when spleen cells were depleted of $CD8^+$ cells, the production of both cytokines was reduced to levels below the limit of detection. These results indicate that $CD8^+$ cells are the major and perhaps exclusive producers of these cytokines in the spleen during the time frame examined. In agreement with this conclusion, the suppressive effects of TCDD on IL-2 and IFN-γ production were still evident in spleen cell cultures depleted of

Table I. *Effect of in vitro depletion of $CD4^+$ or $CD8^+$ cells on IL-2 and IFN-γ production by spleen cells from vehicle or TCDD-treated allograted mice*[a]

		IL-2 (pg/ml)			IFN-γ (pg/ml)		
Day Post P815	TCDD (in vivo)	Control	CD4 depleted	CD8 depleted	Control	CD4 depleted	CD8 depleted
Day 4	−	<7.5	<7.5	<7.5	<200	<200	<200
	+	13	15	<7.5	<200	<200	<200
Day 5	−	39	44	<7.5	2131	2122	<200
	+	74	73	<7.5	1536	1817	<200
Day 6	−	41	52	<7.5	3463	4405	<200
	+	64	53	<7.5	2717	2730	<200
Day 7	−	47	58	<7.5	3758	4412	<200
	+	28	27	<7.5	1009	1416	<200

[a] Spleen cells from vehicle- or TCDD-treated mice were pooled from three to five animals per group on different days after the injection of P815 cells, incubated with either a rat IgG control, anti-CD4 or anti-CD8, and depleted by panning as described in *Materials and Methods.* Depleted spleen cell cultures were restimulated with P815 cells for 6 h and the supernatants analyzed for IL-2 and IFN-γ by ELISA. The lowest point on the standard curve was 7.5 pg/ml for IL-2 and 200 pg/ml for IFN-γ.

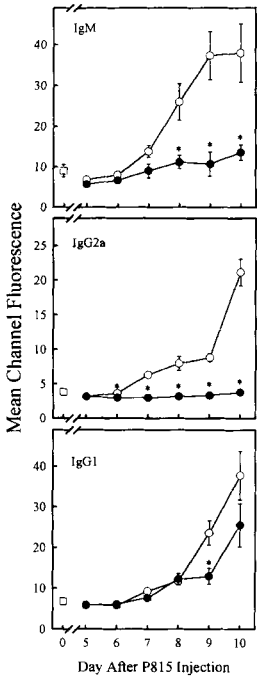

FIGURE 7. Isotype of anti-P815/H-2^d alloantibodies produced in vehicle-treated (○) or TCDD-treated (●) mice compared with that in naive mice (□) treated as described in Figure 1. Plasma was obtained on the days indicated following P815 tumor injection. The isotypes of the alloantibodies were assessed using P815 target cells, isotype-specific secondary Abs, and flow cytometric analysis as described in *Materials and Methods*. Data, reported as mean channel fluorescence, represent the mean ± SEM for five or six mice per treatment per day. * indicates $p \leq 0.05$.

$CD4^+$ cells, while no cytokine activity was detected in CD8-depleted cultures from TCDD-treated mice. These results indirectly demonstrate that TCDD suppresses IL-2 and IFN-γ production by $CD8^+$ cells. Furthermore, the increased IL-2 production observed on days 4 and 5 with whole spleen cells from TCDD-treated mice was evident in cultures that were depleted of $CD4^+$ cells, suggesting that this early increase in IL-2 is derived from $CD8^+$ cells as well.

The effect of depleting $CD4^+$ or $CD8^+$ cells on the production of TNF and IL-6 was also examined in a limited number of samples. TNF production on day 7 was not suppressed by anti-CD4 treatment and was reduced by only 22% following anti-CD8 treatment, suggesting that most of the TNF was produced by a non-T cell source. The amount of IL-6 produced on day 9 was not altered by CD4 or CD8 depletion, suggesting a non-T cell source for this cytokine as well.

TCDD induces differential suppression of alloantibody isotypes

It is generally accepted that IFN-γ, a product of Th1 and TC-1 type cells, promotes IgG2a production, while IL-4, a product of Th2 type cells, promotes IgG1 production (43). Given the profound suppression of IFN-γ compared with IL-4 in TCDD-treated mice, we examined the relative amounts of IgG2a, IgG1, as well as IgM in vehicle- and TCDD-treated mice on different days after P815 injection. As shown in Figure 7, vehicle-treated mice developed significant titers of all three isotypes. On day 9, the relative ratio of IgM to IgG2a to IgG1 alloantibodies was approximately 4:1:3, changing to 2:1:2 on day 10. In TCDD-treated mice, no IgG2a or IgM was detected above background, while a significant titer of IgG1 was detected on days 8 to 10, albeit at a lower level than in vehicle controls. These results support a selective effect of TCDD on the production of $CD8^+$ TC-1-derived cytokines in this model.

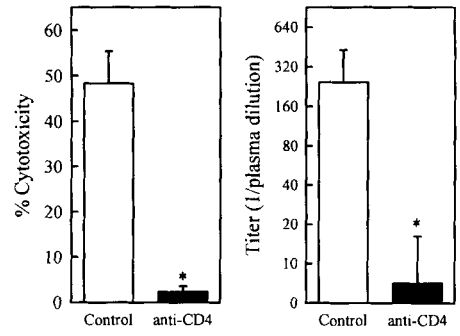

FIGURE 8. Effect of in vivo depletion of $CD4^+$ T cells on CTL and cytotoxic alloantibody responses to the P815 tumor allograft. Mice were treated with anti-CD4 Ab or a control rat IgG as described in *Materials and Methods* and killed on day 10 for immunoassays. Bars represent the mean ± SEM for four to six mice per treatment. CTL data represent an E:T ratio of 50:1. * indicates $p \leq 0.05$.

In vivo depletion of $CD4^+$ cells suppresses allogeneic responses to P815 tumor cells

The apparent lack of cytokine production by P815-primed $CD4^+$ cells in vitro (Table I) coupled with the lack of expansion of the $CD4^+$ population following P815 injection (Fig. 2) led us to wonder whether $CD4^+$ cells played any role in the P815 tumor allograft response. To directly evaluate their importance in the development of CTL and Ab responses to P815 tumor cells in vivo, we depleted mice of $CD4^+$ cells by treatment with anti-CD4 Ab before and following P815 injection. As shown in Figure 8, both CTL and alloantibody responses were nearly abolished in anti-CD4-treated mice, indicating that $CD4^+$ cells play a critical role in the generation of both CTL and alloantibody responses to P815 tumor cells. The results of further study of this phenomenon indicated that $CD4^+$ cells had to be depleted within the first 3 days of the response to impact the development of CTL activity (Fig. 9). In contrast, significant suppression of the alloantibody response was observed even when anti-CD4 Ab treatment was delayed until day 5. These results suggest that there may be early functions of $CD4^+$ cells that are important for the initiation of both the CTL and alloantibody responses as well as later functions relevant only to the generation of the alloantibody response.

In vivo depletion of $CD8^+$ cells suppresses allogeneic responses to P815 tumor cells

Although $CD8^+$ cells have previously been shown to mediate the CTL response to P815 tumor cells (44, 45), we wanted to know whether there were non-$CD8^+$ cells that contribute to the cytotoxic activity in the ^{51}Cr release assay as well as evaluate the role of $CD8^+$ cells in the alloantibody response. Therefore, mice were treated with anti-CD8 Ab as previously described, and the CTL and alloantibody responses were measured on day 9. As shown in Figure 10, treatment of mice with anti-CD8 Ab abolished detectable CTL activity. Treatment with anti-CD8 Ab also partially suppressed the alloantibody response, suggesting that $CD8^+$ cells or the cytokines they produce are necessary for optimum Ab production.

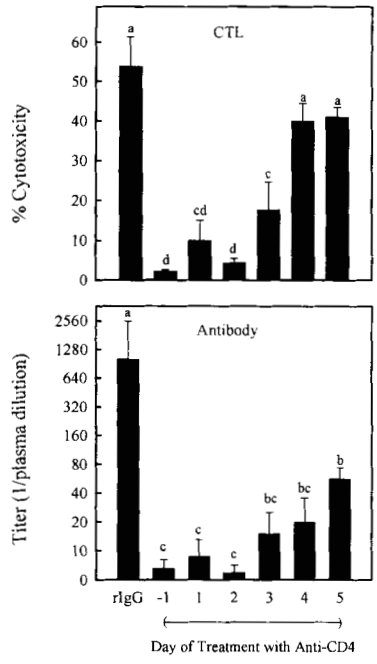

FIGURE 9. Influence of timing of anti-CD4 Ab treatment on CTL and alloantibody responses to the P815 tumor allograft. Mice were injected with 250 μg of anti-CD4 (GK1.5 mAb) on the indicated day relative to the injection of P815 tumor cells on day 0. Controls were treated with 250 μg of rat IgG on day −1. Splenic CTL activity and cytotoxic alloantibody titers were determined on day 10. Data represent the mean ± SEM for five mice per treatment per day, except on day −1, when three mice were tested. CTL activity represents an E:T ratio of 33:1. Multiple comparisons were made using a least significant difference *t* test. Statistically significant differences ($p \leq 0.05$) between treatment groups are indicated by different letters. If a letter is shared by two treatments, there is no significant difference between the treatments.

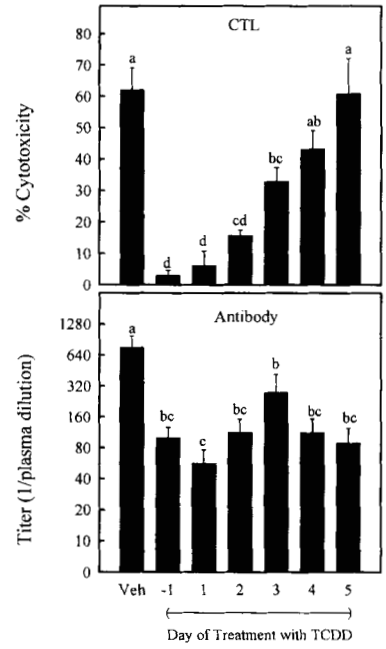

FIGURE 11. Effect of timing of TCDD exposure on the suppression of CTL and alloantibody responses. Mice were treated with vehicle or 15 μg TCDD/kg on different days relative to the i.p. injection of 1×10^7 P815 cells on day 0. Splenic CTL activity and cytotoxic Ab levels were determined 10 days after tumor injection. Bars represent the mean ± SEM for three to eight mice per treatment per day. CTL activity is shown for the 50:1 E:T ratio. Multiple comparisons were made using a least significant difference *t* test. Statistically significant differences ($p \leq 0.05$) between treatment groups are indicated by different letters. If a letter is shared by two treatments, there is no significant difference between the treatments.

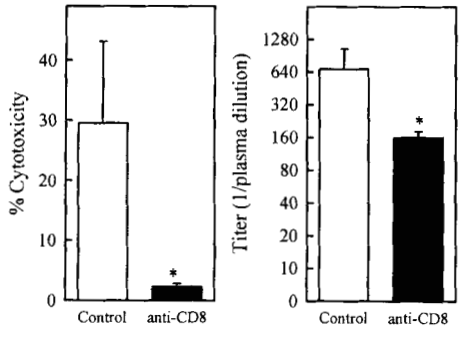

FIGURE 10. Effect of in vivo depletion of $CD8^+$ T cells on CTL and cytotoxic alloantibody responses to the P815 tumor allograft. Mice were treated with anti-CD8 Ab or a control rat IgG as described in *Materials and Methods* and killed on day 9 for immunoassays. Bars represent the mean ± SEM for four to six mice per treatment. CTL data represent an E:T ratio of 100:1. * indicates $p \leq 0.05$.

There is a TCDD-sensitive window within the early days of the allogeneic response that leads to suppression of CTL activity and Ab production

In all previous studies, mice were treated with TCDD 1 day before P815 injection. Since the half-life of TCDD in C57Bl/6 mice has been estimated to be approximately 9 days (46), a single dose of TCDD given before Ag challenge could influence any of the subsequent immunologic processes involved in the development of alloimmunity. Therefore, to provide insight into the potential targets of TCDD in this model, mice were treated with TCDD on different days after P815 injection, and the degree of suppression of the CTL and alloantibody responses was measured on day 10. As shown in Figure 11, the ability of TCDD to suppress the CTL response declined as the time of treatment was delayed after P815 sensitization. The greatest suppression occurred when TCDD was given within the first 2 days of allosensitization. When TCDD treatment was delayed until day 4 or 5, no significant suppression of CTL activity on day 10 was observed. In contrast, the cytotoxic alloantibody response was significantly inhibited when TCDD was given on any of days 1 to 5, with somewhat diminished effectiveness when administered on day 3. This time-dependent pattern of suppression by TCDD is similar to the pattern of suppression observed with anti-CD4 Ab, suggesting that altered reactivity of $CD4^+$ cells may represent one mechanism of immunotoxicity by TCDD.

Discussion

Previous studies have shown that TCDD induces an AhR-dependent dose-responsive suppression of the CTL response in C57Bl/6 mice injected with allogeneic P815 mastocytoma cells (21–23, 25). The objective of the present studies was to compare the time course of the cellular and cytokine responses that occur following the injection of P815 cells in control and TCDD-treated mice in an attempt to understand the basis of TCDD-induced suppression of the CTL response. We also evaluated the concurrent effects of

TCDD exposure on the alloantibody response to directly compare the sensitivity and mechanisms of suppression of cell-mediated and humoral immune responses to the same antigenic stimulation. In the course of these studies, we characterized the previously undescribed profile of cytokines produced in vivo in response to the P815 tumor allograft and identified an essential role for $CD4^+$ cells in the generation of both CTL and alloantibody responses in this model.

The initial time-course studies showed that CTL activity and cytotoxic alloantibody titers in TCDD-treated mice were suppressed concomitantly from the time the responses were first detected on day 6 through their peak on day 10. At the 15 μg/kg dose of TCDD used in these studies, only very low levels of CTL activity (<10% lysis) were detectable at any time, while alloantibody titers showed an initial increase and then failed to increase further after day 8. In a dose-response experiment, the degree of suppression of CTL activity on day 10 directly correlated with the number of $CD8^+$ cells that expressed a CTL_E phenotype, indicating that TCDD decreased the generation of mature effector CTL. Likewise, suppression of the alloantibody response correlated with a reduced expansion of the $B220^+$ cell population, suggesting that TCDD inhibits B cell activation and/or proliferation.

Because cytokines are critical to the activation and clonal expansion of immune effector cells, we evaluated ex vivo cytokine production by spleen cells obtained from vehicle- and TCDD-treated mice on different days following P815 injection. In vehicle-treated mice, spleen cell supernatants contained readily detected levels of IFN-γ, IL-2, TNF-α, and IL-6 and low levels of IL-1 and IL-4. Under the same culture conditions, no IL-10 was detected (0.14 U/ml, limit of detection). All of the detected cytokines have been previously reported to play a role in the development of CTL activity in vitro (39–49). Their increased production in parallel with the increases in CTL activity and alloantibody titers suggests that they also play important roles in effector cell development in vivo. Based on the effects of selective depletion of $CD4^+$ or $CD8^+$ spleen cells before culture, all of the IL-2 and IFN-γ detected on days 4 to 7 of the allograft response was produced by $CD8^+$ T cells, while TNF and IL-6 were produced by non-T cells as well.

Treatment of mice with TCDD resulted in a premature termination of the production of type 1 cytokines, leading to a profound decrease in IFN-γ levels as well as significant reductions in IL-2 and TNF. In contrast, the production of Th2-type cytokines was hardly affected by TCDD. The termination of Th1-type cytokine production by spleen cells from TCDD-treated mice occurred in parallel with suppression of the development of CTL activity and alloantibody production. These results suggest that $CD8^+$ T cells secreting Th1 type cytokines (recently designated TC-1 cells by Sad et al. (47)) are selectively inhibited following TCDD exposure. These TC-1 cells may represent a distinct population of $CD8^+$ cells that produce cytokines for utilization by CTL_P or they may be a population of CTL_P undergoing autocrine-driven differentiation. Nonetheless, since suppressed production of IFN-γ and IL-2 has previously been associated with a state of nonresponsiveness in several transplant models, and administration of either cytokine can prevent anergy (48–50), these results suggested that CTL activity may be suppressed due to the lack of cytokine production. However, preliminary studies have shown that the injection of TCDD-treated mice with IL-2 during the time when IL-2 production was suppressed by TCDD (days 7, 8, and 9) was incapable of altering TCDD-induced suppression of the CTL response (data not shown). Thus, a simple lack of the T cell growth factor for clonal expansion does not appear to represent the underlying defect in TCDD-treated mice. Additional studies are currently in progress to examine the ability of IL-2 and IFN-γ, when given alone or in combination at early and later times following P815 injection, to reconstitute the CTL response in TCDD-treated mice.

In contrast to the suppression of type 1 cytokines, type 2 cytokines normally associated with Ab production (IL-4 and IL-6) were hardly altered by TCDD. Although TCDD may suppress the production of other type 2 cytokines (e.g., IL-5) that were not measured in our studies, it is possible that the suppressed levels of TC-1 cytokines (e.g., IFN-γ and IL-2) influenced alloantibody formation. In this regard, Chan et al. (51) recently reported that in vivo depletion of $CD8^+$ cells suppressed the alloantibody response in a murine heart allograft model. In addition, they found that mice depleted of $CD8^+$ cells produced significantly less IgM and IgG2a and more IgG1 Ab compared with unmodified mice. Interestingly, when we measured the relative amounts of alloantibody isotypes in control and TCDD-treated mice, the TCDD-treated mice resembled $CD8^+$-depleted mice, with IgM and IgG2a reduced to background levels, while IgG1 levels were only slightly suppressed. These results indicate that the suppression of cytokine production by TC-1 cells in TCDD-treated mice may affect the alloantibody response to P815 tumor cells. Paradoxically, however, when mice were totally depleted of TC-1 cells by treatment with anti-CD8 Ab in the absence of TCDD, only a modest suppressive effect was observed on the overall cytotoxic alloantibody response (Fig. 10). These results suggest that suppression of the alloantibody response by TCDD cannot be fully explained by suppression of cytokine production by TC-1 cells and point to additional alterations in other cells, such as $CD4^+$ T helper cells or B cells, that may contribute to the suppression. In other models, both T helper cells and B cells have been reported to be altered following TCDD exposure (11).

Although previous studies have implicated T helper cells in the suppression of Ab responses by TCDD (11), the lack of cytokine production by P815-primed $CD4^+$ cells in vitro (Table I) coupled with the lack of expansion of the $CD4^+$ population following P815 injection (Fig. 2) led us to question whether $CD4^+$ cells were involved in the response to P815 tumor cells. Since P815 cells are MHC class II^- (52), such $CD4^+$ cell involvement would have to occur via host-derived APC. Also, Sprent and Schaefer (53) reported that $CD8^+$ cells can be directly activated to kill P815 cells in the absence of $CD4^+$ cells in the Winn assay, and Gajewski et al. (38) recently reported that anti-P815 alloCTL effector cells could be generated in vitro independent of $CD4^+$ cells. Therefore, we were surprised to find that in vivo depletion of $CD4^+$ cells resulted in complete abrogation of the alloantibody response as well as >90% suppression of the CTL response to P815 tumor cells. Further study of this phenomenon suggested that $CD4^+$ cells were required only within the first 3 days of the CTL response, whereas the Ab response was dependent on $CD4^+$ cells during the first 5 days of the response, and perhaps longer. The actual role(s) played by the $CD4^+$ cell in each response is not known, but probably involves the provision of costimulatory signals to naive $CD8^+$ cells and B cells either through cognate receptor-ligand binding interactions or through the release of costimulatory cytokines (54, 55), or both. Although the cytokine responses measured on days 4 to 7 did not appear to involve $CD4^+$ cells, this does not preclude their provision of early and perhaps locally secreted cytokines to $CD8^+$ cells or B cells.

We conducted similar timing studies with TCDD and found an interesting parallel between the effects of TCDD and the effects of CD4 depletion on anti-P815 responses. Thus, mice had to be treated with TCDD within the first 3 days following P815 injection for the CTL response to be suppressed. In contrast, suppression of the Ab response occurred even if TCDD treatment was delayed

until day 5, a pattern also observed with anti-CD4 treatment. Together these results provide circumstantial evidence that the pathways of activation of $CD8^+$ cells and B cells involving $CD4^+$ cells may be altered by TCDD exposure. However, the results do not provide insight into what the alteration is or what cell is directly targeted by TCDD. In fact, results of recent studies in our laboratory on the distribution and behavior of the AhR in T cells would argue against a direct effect of TCDD on $CD4^+$ T cells themselves. Although T cells express detectable levels of AhR, the ability of the activated AhR to bind to a consensus dioxin response element appears to be defective in T cells (56). Furthermore, the addition of TCDD directly to cultures of TH1 and TH2 clones did not influence the production of several cytokines, including IFN-γ and IL-2 (56). Likewise, TCDD has little effect on IL-2 or IFN-γ production by spleen cells in response to anti-CD3 stimulation (57). Taken together, these results are consistent with an indirect effect of TCDD on T cell activation.

In the absence of direct effects of TCDD on T cells, one hypothesis that is currently being tested in our laboratory is that TCDD alters T cell activation indirectly via effects on APCs. In this regard, recent studies have shown that TCDD exposure reduces the expression of the important costimulatory molecules B7–1 and B7–2 on B cells and, to a lesser extent, on macrophages following P815 tumor challenge.[5] Furthermore, if mice are injected with B7-transfected P815 tumor cells, the CTL response, but not the alloantibody response, is restored in TCDD-treated mice.[5] These results are compatible with a model in which the B7-transfected P815 cells are able to provide both Ag-specific and costimulatory signals to $CD8^+$ TC1/CTLp, while being unable to substitute for the cognate interaction between $CD4^+$ cells and B cells. Since TCDD is most effective as an immunosuppressant when given very early in the allograft response, the interaction between dendritic cells and naive $CD4^+$ cells may represent the earliest and most critical target of TCDD, leading to suppression of both CTL and alloantibody responses.

Finally, one potentially important, yet unexplained, effect of TCDD in these studies was the small, but significantly elevated, production of IL-2 by TC-1 cells that was seen on days 4 to 5 following allosensitization (Fig. 5 and Table I). Based on a parallel increase in the level of IL-2 mRNA on day 5 (Fig. 6), this appeared to reflect a true increase in production rather than a decreased utilization of IL-2. A comparable elevation of IL-2 production also preceded the suppression of IL-2 and IFN-γ production and CTL activity in mice treated with another immunosuppressive AhR ligand, 3,4,5,3′,4′,5′-hexachlorobiphenyl (G. K. DeKrey and N. I. Kerkvliet, unpublished observations). Since excess IL-2 has been shown to inhibit IFN-γ production in a graft-vs-host model (58) and to inhibit the alloreactivity of cloned T cells (59), this early IL-2 response in TCDD-treated mice could potentially play a role in the premature termination of cytokine production. However, the mechanism that underlies this paradoxical enhanced response in TC-1 cells remains to be determined.

Acknowledgments

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