The Effects of TCDD on the Activation of Ovalbumin (OVA)-Specific DO11.10 Transgenic CD4+ T cells in Adoptively Transferred Mice

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Exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) suppresses the generation of T cell-dependent immunity, both humoral and cell-mediated. However, the mechanism of TCDD-induced immune suppression remains to be defined. We hypothesized that exposure to TCDD suppresses the activation of naive CD4+ T cells and prevents their expansion and differentiation into effector T-helper cells capable of driving T cell-dependent immune responses. To test this hypothesis, we adoptively-transferred DO11.10 OVA-specific T-cell receptor (TCR) transgenic T cells into syngeneic recipients and used a TCR-specific monoclonal antibody to track the in vivo activation of naive CD4+ T lymphocytes following exposure to OVA. The production of OVA-specific antibodies was suppressed in a dose-dependent manner in adoptively transferred mice that had been exposed to TCDD. Although TCDD exposure had little effect on the expansion or activation of the adoptively transferred, OVA-specific CD4+ T cells, these cells disappeared from the spleen more rapidly in TCDD-treated mice and produced significantly decreased levels of the T cell-derived cytokines IL-2 and IL-10. There was also a trend towards reduced IFN-γ and IL-4 production following in vitro re-stimulation. These data suggest that TCDD may interfere with the survival and/or differentiation of OVA-specific T-helper cells. These results demonstrate for the first time the potential of the DO11.10 adoptive transfer system to directly assess immunotoxic effects of xenobiotics on antigen-specific CD4+ T cells in vivo.

Key Words: immunotoxicity; TCDD; CD4+ T cells; DO11.10; transgenic; mouse; ovalbumin; in vivo.

The activation of naive T lymphocytes is dependent on two distinct signals: ligation of the TCR by specific antigen complexed with major histocompatibility complex (MHC) proteins, and costimulation delivered via interactions of accessory molecules and/or cytokines (Croft, 1994; Mondino et al., 1996; Pape et al., 1997b). After receiving these signals, antigen-specific T cells undergo clonal expansion and modulate their expression of surface adhesion and activation molecules such as CD69 (very-early-activation antigen), CD11a (LFA-1), CD44 (Pgp-1) and CD62L (L-selectin, LECA-M-1) (Croft and Dubey, 1997; Rogers et al., 1997). Ultimately, these lymphocytes will differentiate into cytokine-producing effector cells capable of promoting T-cell-dependent immunity such as antibody production by B cells. Incomplete activation of naive T cells through insufficient TCR engagement or costimulation may lead to antigen-specific T-cell unresponsiveness or anergy (Kearney et al., 1994; Maier et al., 1998; Maier and Greene, 1998). Therefore, proper T-cell activation is essential for the generation of effective humoral and cell-mediated immunity, and disruption of this process may compromise the capacity of an organism to evade pathogenic insult.

The immune system has been identified as a sensitive target of TCDD, and T cell-dependent functions have been shown to be particularly affected in laboratory animals following exposure to TCDD (Kerkvliet, 1995, 1998; Vos and Luster, 1989). Although immune suppression by TCDD has been demonstrated to be dependent on the Ah receptor (Kerkvliet et al., 1990a,b), the subsequent mechanisms of T-cell suppression remain unknown. Previously, TCDD has been shown to inhibit T-cell responsiveness in several experimental models such as the humoral responses to SRBC and OVA, and the cell-mediated CTL response to allogeneic tumor cells (Davis and Safe, 1988; Kerkvliet et al., 1990a; Lundberg et al., 1991; Tomar and Kerkvliet, 1991; Matulka et al., 1997). In these reports, T-cell function was assessed by in vitro restimulation of cells from mice previously exposed to TCDD and antigen. While these assays provide a functional assessment of secondary T-cell responsiveness, they do not directly measure the fate of antigen-specific T cells following a primary exposure to antigen.

Until recently, it has not been possible to directly monitor the activation of antigen-specific CD4+ T cells in vivo, because they are present at frequencies too low to detect by available methods. However, with the development of TCR-transgenic T-cell models such as the DO11.10 adoptive transfer model, we now have the technical means to overcome this obstacle (Pape et al., 1997a). The CD4+ T cells from DO11.10 mice express a transgene that encodes a TCR, specific for the OVA323-339 peptide, in the context of I-A\(^d\) class II MHC. Fol-
allowing the adoptive transfer of small numbers of spleen cells from DO11.10 mice into syngeneic Balb/c recipients, the Ag-specific CD4⁺ T cells are artificially elevated from approximately 1 in 100,000 to a detectable frequency of 1 in 100, or more (Kearney et al., 1994). In addition, this small population of OVA-specific T cells can be followed in the adoptive transfer recipients by flow cytometric methods, using the clonotype-specific monoclonal antibody KJ1-26 (Haskins et al., 1983). In this report, we have utilized the DO11.10 adoptive transfer system to directly monitor 3 phases of antigen-specific CD4⁺ T-cell activation in vivo: (1) clonal expansion, (2) expression of activation markers, and (3) cytokine production. Using this system, we have tested the hypothesis that exposure to TCDD suppresses the activation of naive CD4⁺ T cells, and prevents their expansion and differentiation into effector T-helper cells capable of driving the T cell-dependent antibody response to ovalbumin. The results presented in this report emphasize the potential of the DO11.10 adoptive transfer system to assess possible immunotoxic effects of xenobiotics on CD4⁺ T-helper-cell activation in an antigen-specific transgenic mouse model.

MATERIALS AND METHODS

Mice. Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used as recipients for the adoptive transfer experiments. DO11.10 TCR transgenic mice were generously provided by Dr. Marc Jenkins (University of Minnesota Medical School). DO11.10 mice were bred and maintained in our pathogen-free animal facility in accordance with National Research Council guidelines. DO11.10 mice have been extensively back-crossed (>10 generations) onto the Balb/c background to express H-2d MHC molecules and to serve as syngeneic donors of OVA-specific T cells.

Reagents. 2,3,7,8-Tetrachlorodibenzo-p-dioxin was obtained from Cambridge Isotope Laboratories, Inc. (Woburn, MA). TCDD was dissolved in anisole and diluted in peanut oil. A vehicle solution of anisole in peanut oil was prepared similarly. Complete Freund’s adjuvant was purchased from Difco Laboratories (Detroit, MI). Cyclosporin A was purchased from Sandoz Pharmaceuticals, Corp. (Basel, Switzerland). Chicken ovalbumin was purchased from Sigma (St. Louis, MO). Streptavidin-Red613 from Gibco BRL (Gaithersburg, MD) was used as a second step reagent to visualize staining with the following mAbs from PharMingen (San Diego, CA): FITC-labeled anti-CD11a (2D7), FITC-labeled anti-CD44 (IM7), FITC-labeled anti-CD62L (MEL-14), PE-labeled anti-CD69 (H1.2F3), PE-labeled anti-CD25 (3C7), PE-labeled anti-CD154 (MR1), PE-labeled anti-CD28 (37.51), and Cy-labeled anti-CD4 (GK1.5).

Mouse treatments. Mice were treated with a single dose of TCDD (15 μg/kg) or vehicle by gavage on day –3, relative to OVA/CFA immunization. For the dose response experiment, mice were administered a single dose of 15, 5, 0.5, or 0 μg/kg TCDD on day –3. For the cyclosporin A (CsA) experiment, mice were treated ip with CsA (50 mg/kg) 16 h prior to immunization with CFA-OVA.

Adoptive transfer and immunization. The protocol for the adoptive transfer of DO11.10 T cells was slightly modified from the previously described method (Kearney et al., 1994). Briefly, splenocytes from DO11.10 donor mice were harvested and pooled, and the percentage of CD4⁺/KJ1-26⁺ cells was determined by flow cytometry. Spleens of donor DO11.10 mice contained approximately 18–20% CD4⁺/KJ1-26⁺ cells. An aliquot of cells containing 5 × 10⁵ CD4⁺/KJ1-26⁺ cells per injection was resuspended in HBSS and injected iv in a volume of 0.5 ml into age- and sex-matched Balb/c recipient mice. For our studies, both donor DO11.10 and recipient Balb/c mice were treated with either vehicle or TCDD to provide exposure throughout the entire adoptive transfer procedure. Adoptively transferred mice were rested for 2 days and then immunized with 2 mg OVA emulsified in CFA by ip injection in a volume of 0.25 ml.

Flow cytometric analysis of spleen cells. Spleen-cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides. Erythrocytes were removed by hypotonic lysis. Cells were washed once and resuspended in cold HBSS/5% PBS with 20 mM HEPES, 50 μg/ml gentamicin, and 1.5 mM sodium pyruvate. Non-specific antibody binding was blocked by adding 30 μg of rat IgG to each sample prior to staining with optimal concentrations of fluorochrome-conjugated mAb. Appropriately labeled, isotype-matched Igs were used as controls for non-specific fluorescence. Fifty thousand to 100,000 events were collected by listmode acquisition from freshly prepared cells, using a Coulter XL flow cytometer (Coulter Electronics, Hialeah, FL) and analyzed using WinList (Verity Software House, Topsham, ME). OVA-specific T-helper cells were detected by initially gating on viable spleen cells followed by further gating on the CD4⁺/KJ1-26⁺ population. Subsequent measurement of activation markers and cell size (FALS) was performed by gating on the CD4⁺/KJ1-26⁺ antigen-specific T cells or the CD4⁺/KJ1-26⁻ bystander T-helper-cell population.

ELISAs. For detection of OVA-specific antibodies, enzyme immunoassay plates (Costar, Cambridge, MA) were coated overnight at 4°C with 1 μg/ml of chicken OVA in PBS, blocked with 3% BSA in PBS for 60 min at 37°C, washed, and incubated overnight at 4°C with serial dilutions (1:10 to 1:100,000) of plasma. Plates were then washed and incubated with a 1:5000 dilution of biotinylated anti-mouse IgM, IgG1, IgG2a, or IgG2b (Southern Biotechnology, Birmingham, AL). The secondary biotinylated Abs were complexed with avidin-peroxidase and visualized with 2,2’-azino[3-ethylbenzthiazoline-6-sulfonic acid] as a substrate. Absorbance was read at 405 nm using a Bio-tek model EL309 automated plate reader (Bio-Tek Instruments, Winooski, VT). For detection of cytokines, whole spleen cells (6 × 10⁵) or KJ-depleted spleen cells (6 × 10⁵) were suspended in 1 ml of complete RPMI medium containing 10% FBS with or without OVA (10μM), and incubated for 24 h. KJ-26⁺ cells were depleted by magnetic bead separation, which was performed according to the manufacturers instructions (Miltenyi Biotech, Irvine, CA). Briefly, spleen cells were coated with biotinylated KJ-26 mAb, bound to streptavidin-coated magnetic beads, and separated out by adherence over a Mini-MACS magnetic column. This method of negative selection effectively depleted ~100% of splenic KJ-26⁺ cells as determined by flow cytometry. Cytokine production from individual KJ-26⁻ cells was calculated using the following formula:

\[
\text{[Cytokine WSC]} - \text{[Cytokine KJ-depleted]} \times \left( \frac{\% \text{CD4}^+/\text{KJ1-26}^-}{\% \text{CD4}^+} \right) = \text{[Cytokine KJ-depleted]} \times \left( \frac{6 \times 10^6}{6 \times 10^5} \right) = 100 \times (6 \times 10^6)
\]

where [Cytokine WSC] = cytokine concentration produced by restimulated whole spleen cell cultures; [Cytokine KJ-depleted] = cytokine concentration produced from spleen cells which had been depleted of KJ-26⁺ cells; % CD4⁺/KJ1-26⁻ = the percentage of spleen cells staining positively for CD4 and KJ-26⁻; 6 × 10⁶ = the number of cells restimulated in culture with OVA for 24 h.

Cytokines present in the culture supernatants were measured by sandwich ELISA based on noncompeting pairs of anti-IL-2 (JES6-1A12 and JES6-5H4), anti-IFN-γ (R4-6A2 and XMG1.2), anti-IL-4 (11B11 and BV6-24G2), or anti-IL-10 mAbs (JES5-2A5 and JES5-1E3). Capture and biotinylated detection antibody pairs and their respective standards were purchased from Pharmingen, except for the IL-4 standard, which was obtained from Genzyme (Cambridge, MA). ELISAs were performed according to manufacturer’s directions, with known amounts of recombinant murine cytokines used to generate standard curves for comparison.
Statistical analysis. Results are presented as the mean ± SE of 4–6 mice per group unless indicated otherwise. Analysis of variance modeling was performed using Statview statistical software (Abacus Concepts, Inc., Berkeley, CA). Comparisons between means were made using the least significant difference multiple comparison t test or Dunnet’s t-test for pairwise comparisons. Values of $p \leq 0.05$ were considered statistically significant and were represented with an * (when the comparison was between vehicle and TCDD) or different letters (when the comparison was between several treatment groups).

RESULTS

OVA-specific Antibody Production in Adoptively Transferred Mice is Sensitive to TCDD-Mediated Suppression

The T cell-dependent immune response to OVA results in the production of OVA-specific antibodies. Measurement of these antibodies allows a readout of the effector portion of the immune response and an indirect assessment of T-helper cell function. In order to establish the sensitivity of DO11.10 adoptive transfer model to TCDD, we measured OVA-specific antibody levels in adoptively transferred mice, which had been injected with OVA/CFA. As measured by antigen-specific ELISA, adoptively transferred, vehicle-treated mice produced OVA-specific IgM and IgG antibodies on day 5 post-immunization (Fig. 1A). In contrast, anti-OVA titers in adoptively transferred mice that had been exposed to TCDD were dose-dependently suppressed following immunization with OVA/CFA. Since potent suppression of both IgM and IgG OVA-specific antibody production was observed at 15 μg/kg this dose was used for subsequent investigations.

**FIG. 1.** OVA-specific antibody production in vehicle- and TCDD-treated adoptive transfer recipients. Mice were injected with 2 mg of OVA in CFA and plasma titers of OVA-specific antibodies were determined by isotype-specific ELISAs. (A) The dose-dependent effects of TCDD exposure on plasma levels of OVA-specific IgM and IgG (1:1000 dilution) were determined from vehicle- and TCDD-treated adoptive transfer recipients on day 5 after injection with antigen, as described in Materials and Methods. (B) OVA-specific levels of plasma IgM (1:100), IgG1 (1:1000), and IgG2a (1:100) were measured from vehicle- (open circles) and TCDD-treated (filled circles) adoptive transfer recipients on days 5, 10, and 19 post-immunization. Plasma levels from non-immunized adoptively transferred mice are also shown (open diamonds). Statistically significant differences ($p < 0.05$) between treatment groups are indicated by different letters or asterisks.
A time course of OVA-specific antibody production was conducted to measure the effects of TCDD on plasma levels of IgM, IgG1 and IgG2a (Fig. 1B). By day 5 post-immunization, vehicle-treated mice produced increased levels of OVA-specific IgM antibodies when compared to non-immunized animals. IgM levels increased on day 10 and subsequently decreased by day 19. In contrast, OVA-specific IgG1 and IgG2a antibodies were not significantly produced by day 5. While levels of IgG1 antibodies steadily increased through days 10 and 19, IgG2a was produced in significant amounts by day 10 and remained elevated through day 19 post-immunization. As expected, mice that had been exposed to TCDD produced reduced levels of OVA-specific antibodies. TCDD significantly suppressed levels of IgM on day 5, had no effect on day 10, but showed a trend towards lower IgM levels again by day 19. Although no significant effect of TCDD was observed on IgG1 levels, a trend towards reduced antibody production of this isotype was observed on days 10 and 19 following immunization. On the same hand, levels of OVA-specific IgG2a were significantly suppressed on day 10 and this effect persisted out to day 19 post-immunization.

**The Identification of Antigen-Specific CD4**

A fundamental characteristic of a developing adaptive immune response is the clonal expansion of antigen-specific lymphocytes following encounter with antigen. In our studies, OVA-specific CD4<sup>+</sup> T-helper cells were identified in the spleen by co-staining with anti-CD4 and clonotype-specific KJ1-26 monoclonal antibodies. As shown in Figures 2A and 2B, Balb/c mice do not normally possess detectable numbers of OVA-specific, CD4<sup>+</sup> T-helper cells in their spleens, even following immunization with OVA/CFA. However, a small but defined population of OVA-specific CD4<sup>+</sup> T-helper cells is detectable prior to antigen injection in Balb/c mice that have received adoptively-transferred DO11.10 T cells (Fig. 2C). Furthermore, 3 days after immunizing the adoptively transferred mice with OVA/CFA, the population of antigen-specific T cells has clonally expanded to yield an approximately 8-fold increase in the percentage of CD4<sup>+</sup>/KJ1-26<sup>+</sup> splenic T cells (Fig. 2D).

**The Effects of TCDD Exposure on the Clonal Expansion of DO11.10 T-helper Cells in Adoptively Transferred Mice**

Having established that TCDD suppressed the T cell-dependent antibody response to OVA in adoptively transferred mice, we next tested the hypothesis that TCDD suppresses the activation of naive CD4<sup>+</sup> T cells by inhibiting their clonal expansion. As shown in Figure 3, over a 10 day period, a small population of CD4<sup>+</sup>/KJ1-26<sup>+</sup> T helper cells was detected in the spleens of adoptively transferred mice that were not injected with OVA/CFA. Kearney et al. (1994) have shown that these adoptively transferred cells persist for at least 30 days after transfer in naive animals. Exposure to TCDD did not alter the persistence of these resting T cells in adoptively transferred mice. Following the immunization of vehicle-treated mice with OVA/CFA, the accumulation of CD4<sup>+</sup>/KJ1-26<sup>+</sup> T cells peaked on day 3, followed by a steady decrease in both the percentage and number of these cells through day 10. Surprisingly, TCDD exposure had no effect on the percentage or number of these OVA-specific T-helper cells found in the spleen on day 3 post-immunization. However, TCDD exposure significantly reduced the number of splenic CD4<sup>+</sup>/KJ1-26<sup>+</sup> T-helper cells from adoptively transferred mice on days 5 through 10. It should be noted that a significant decrease was also observed in total splenic cellularity in TCDD-treated mice on days 5–10 (data not shown). These data suggest that TCDD exposure does not interfere with the initial clonal expansion of antigen-specific T cells in vivo, but may directly enhance their deletion following antigen encounter.
The Effects of TCDD on the Expression of Activation Markers on DO11.10 CD4+ T Cells in Adoptively Transferred Mice

Following encounter with antigen, T lymphocytes modulate their surface expression of various activation markers, facilitating their migration in and out of immune tissue and conferring specific effector functions on them. Naive CD4+ T cells express a surface phenotype defined by low levels of CD69, CD44, and CD11a but high levels of CD62L (Swain et al., 1996). This naive phenotype was detected on 2 separate populations of resting CD4+ T-helper cells, the host’s own “bystander” CD4+KJ1-26– T cells, which were not specific for OVA, and the CD4+KJ1-26+ adoptively transferred cells in animals that were not exposed to antigen (data not shown). We monitored the former T-cell population as internal controls for non-antigen-specific effects of our treatments in all subsequent experiments. As shown in Figure 4 and Table 1, no significant modulation of activation markers was detected on the “bystander” CD4+ T cells over the duration of our studies. Conversely, following injection of OVA/CFA, adoptively transferred OVA-specific CD4+KJ1-26+ T cells from vehicle-treated mice upregulated their expression of CD69 within 16 h. By day 3 post-immunization, significantly increased expression of CD44 and CD11a was also detected on the CD4+KJ1-26+ T cells and CD62L expression was down-regulated, as expected to occur on activated T cells (Rogers et al., 1997). Likewise, CD4+KJ1-26+ T cells exhibited an increase in forward light scatter on day 3, which is indicative of increasing cell size and cellular blast transformation. Exposure of adoptively transferred mice to TCDD had no effect on the OVA-induced expression of CD69 and CD44, or the increase in cell size of the OVA-specific DO11.10 T cells. Only minimal
induction of the activation markers CD154 (CD40L), CD28 and CD25 (IL-2Rα) was detected on the CD4⁺/KJ1-26⁺ or "bystander" CD4⁺ KJ1-26⁻ T cells from vehicle- and TCDD-treated mice and then determining percent positive expression and mean channel fluorescence (MCF) on that gated T-cell population. The peak expression of CD69 was detected 16 h after injection with CFA-OVA whereas the expression of CD44, CD62L, CD11a and cell size peaked on day 3 post-immunization. Values are expressed as mean with standard error shown in parentheses. In contrast, TCDD exposure significantly increased the percentage of CD62L in adoptively transferred mice when compared to the vehicle-treated controls. TCDD exposure also significantly suppressed the OVA-induced expression of CD11a on the CD4⁺/KJ1-26⁻ T cells from TCDD-treated mice.

The Effects of TCDD Exposure on Cytokine Production

Another important function of activated T cells is the production of cytokines, which contributes to the development of the antigen-specific response. Initially, IL-2 production by activated T cells drives their clonal expansion. Subsequently, activated T cells polarize their secretion of cytokines to reflect differentiation into Th1 or Th2 effector cells capable of driving cell-mediated or humoral immune responses, respectively (O’Garra and Murphy, 1994). Th1 cells have been characterized by the production of IL-2 and IFN-γ, while Th2 cells primarily produce IL-4, IL-5, and IL-10 (Cherwinski et al., 1987; Mosmann et al., 1986). We measured cytokine production by spleen cells from adoptively transferred mice to characterize the effects of TCDD on T-cell differentiation. To determine the production of cytokines by the KJ1-26⁺ cells, we depleted these cells from our spleen cell preparations, restimulated the remaining cells ex vivo with OVA, and calculated cytokine production as described in Materials and Methods. As shown in Table 2, splenic KJ1-26⁺ cells produced IL-2, IFN-γ, IL-4 and IL-10 when restimulated in vitro with OVA for 24 h. On a per KJ1-26⁺ cell basis, cytokine production was greater on day 1 post-immunization than on day 3, and Th1 cytokines were produced in excess of Th2 cytokines. Interestingly, TCDD exposure significantly suppressed the production of IL-2 on day 1 and IL-10 on day 3. KJ1-26⁻ cells from TCDD-treated mice which had been immunized for one day also showed a trend towards reduced production of IFN-γ and IL-4 following restimulation.

Cyclosporin A Treatment Inhibits the Activation of DO11.10 CD4⁺ T Cells in Adoptively Transferred Mice

The lack of significant effects of TCDD-exposure on the activation of DO11.10 T cells in adoptively transferred mice suggested that CD4⁺/KJ1-26⁺ cells might be refractory to xenobiotic-induced immunosuppression in vivo. To address this possibility, we treated adoptively transferred mice with a known immunosuppressive dose of cyclosporin A (CsA) as a positive control (Neumann et al., 1992). As shown in Table 3, the administration of CsA to adoptively transferred mice significantly inhibited the activation of the CD4⁺/KJ1-26⁺ T cells following immunization with OVA/CFA. CsA suppressed the antigen-induced increase in the percentage and number of DO11.10 T cells on day 3 post-immunization. The frequency of CD4⁺/KJ1-26⁻ T cells in these mice did not significantly increase above the levels detected in mice that received DO11.10 T cells but were not immunized, suggesting that no clonal expansion of OVA-specific T cells had occurred in these mice.
animals. As expected, splenic CD4^+ T cells from CsA-treated mice produced significantly less IL-2 following in vitro restimulation, when compared to controls (Granelli-Piperno, 1990; Shevach, 1985). Interestingly, CD44 expression on CD4^+ T-cells was increased in CsA-treated mice when compared to non-immunized mice but not when compared to immunized control mice. This might reflect that the splenic CD4^+ T cells from CsA-treated mice had received antigenic stimulation yet were unable to clonally expand. Taken together, these results indicate that the adoptively transferred DO11.10 T cells are not uniquely resistant to xenobiotic-induced suppression.

**DISCUSSION**

A fundamental feature of T cell-dependent immune responses is the necessity for a very small population of CD4^+ T cells to undergo clonal expansion and activation following encounter with a specific antigen. While several previous studies have attempted to evaluate the influence of TCDD on CD4^+ T cell function in vivo, due to their low frequency it has not been possible heretofore to directly monitor the effects of TCDD on the activation of antigen-specific CD4^+ T-helper cells. In the studies reported here, we have utilized the DO11.10 adoptive transfer model to directly monitor the effects of TCDD on the in vivo activation of naive, antigen-specific CD4^+ T cells. We have shown in this report that exposure of adoptively transferred mice to TCDD significantly suppressed the production of IgM and IgG OVA-specific antibodies. These results are consistent with a report from Lundberg et al. (1991) in which TCDD suppressed anti-OVA antibody titers in normal Balb/c mice immunized with OVA in CFA. It is interesting to note that in our studies TCDD significantly suppressed the generation of IgM OVA-specific antibodies on day 5 but not on days 10 and 19 post-immunization. Although TCDD exposure produced only a trend towards reduced OVA-specific IgG1 antibody production over time (as represented in Fig. 1), in other experiments, TCDD exposure was found to significantly reduce IgG1 plasma levels in adoptively-transferred mice on day 10 post-immunization (data not shown). In contrast, the production of IgG2a antibodies specific for OVA was profoundly suppressed in TCDD-treated mice on days 10 and 19, an effect that has been consistently observed in all of our experiments. Since IgG antibody production has been demonstrated to be critically dependent on the interaction of activated B cells with effector T-helper cells (Noelle et al., 1992; Parker, 1993), these results are consistent with an effect of TCDD on the function of OVA-specific CD4^+ T cells.

Using the DO11.10 adoptive transfer model, we were able to successfully follow the clonal expansion and activation of OVA-specific CD4^+ T cells. Unexpectedly, TCDD exposure had no effect on the in vivo clonal expansion of DO11.10 T cells in adoptively transferred mice immunized with OVA/ CFA. These results differ from a previous study by Lundberg et al. (1992) in which lymph node cells from mice exposed to a significantly higher dose of TCDD (50 μg/kg) demonstrated diminished proliferation following immunization with OVA. However, this study measured the proliferative response of OVA-specific memory T and B lymphocytes following ex vivo secondary challenge, whereas our studies measured the effects of TCDD on the primary expansion of a clonal population of transgenic T cells specific for OVA. Our results also differ from those reported in a study by Neumann et al. (1993), which measured the effects of TCDD on CD4^+ T-cell proliferation in mice activated with anti-CD3. In these mice, TCDD exposure resulted in a dose-dependent increase in the proliferation of CD4^+ T cells.

The lack of TCDD effects on the clonal expansion of antigen-specific T cells in adoptively-transferred mice could potentially be explained by an accumulation of CD4^+/KJ1-26^+ T cells in the spleen resulting from immigration and not proliferation. However, the increase in relative cell size of the CD4^+/KJ1-26^+ T cells in both the vehicle- and TCDD-treated mice is representative of cellular blast transformation, and

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<th>TABLE 3</th>
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<tr>
<td><strong>The Effects of Cyclosporin A (CsA) Treatment on the Activation of Splenic DO11.10 TCR Transgenic CD4^+ T Cells in Adoptively Transferred Mice</strong></td>
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<table>
<thead>
<tr>
<th></th>
<th>Non-immunized</th>
<th>CFA-OVA</th>
<th>CFA-OVA + CsA</th>
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<tbody>
<tr>
<td>% CD4^+/KJ1-26^+</td>
<td>0.33 (0.05)^a</td>
<td>1.13 (0.05)^b</td>
<td>0.29 (0.03)^a</td>
</tr>
<tr>
<td># CD4^+/KJ1-26^+ (1 x 10^6)</td>
<td>3.8 (0.5)^a</td>
<td>9.3 (0.6)^a</td>
<td>2.6 (0.4)^a</td>
</tr>
<tr>
<td>CD44 MCF</td>
<td>573 (11)^a</td>
<td>1487 (37)^b</td>
<td>1148 (52)^c</td>
</tr>
<tr>
<td>IL-2 per KJ1-26^+ cell (fg/ml)</td>
<td>NA</td>
<td>12.9 (0.7)^a</td>
<td>1.1 (0.6)^a</td>
</tr>
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</table>

Note. Mice were adoptively transferred with DO11.10 spleen cells as described in Materials and Methods. CsA (50 mg/kg) was injected ip 16 h prior to injection with CFA-OVA. Spleen cells were harvested from treated mice 3 days after immunization. The percent and total number of CD4^+/KJ1-26^+ T cells in the spleen was determined as described in Figure 3. The mean channel fluorescence (MCF) of CD44 expression on the CD4^+/KJ1-26^+ T-cell population was determined as described in Table 1. IL-2 levels were determined per KJ-126^+ cell by ELISA from supernatants of splenic cells cultured with OVA (10 μM) for 24 h as described in Table 2. Each value represents the mean (± SEM) from 4 mice. Statistically significant differences (p < 0.01) between treatment groups are indicated by different superscript letters. NA = not applicable.
suggests that these T cells are undergoing proliferation following exposure to OVA. In addition, it was recently demonstrated in a study using mice adoptively transferred with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled DO11.10 T cells that proliferation and not immigration was responsible for the expansion of OVA-specific T cells following immunization with antigen (Gudmundsdottir et al., 1999; our unpublished results). Therefore, although it remains to be definitively demonstrated in adoptively transferred mice that have been exposed to TCDD, it seems likely that the increased numbers of splenic CD4⁺/KJ1-26⁺ cells in our studies was due to proliferation and not an influx of OVA-specific cells.

Alternatively, the lack of effect of TCDD on the numbers of CD4⁺/KJ1-26⁺ T cells in adoptively transferred mice raises the possibility that the transgenic DO11.10 T cells might be insensitive to immune suppression induced by xenobiotic exposure. However, the administration of cyclosporin A to adoptively transferred mice effectively suppressed the clonal expansion of the DO11.10 T cells. Cyclosporin A is a potent immunosuppressive drug that has been used extensively in organ transplantation (Beveridge, 1986). It inhibits T-cell activation by binding to cyclophilin and blocking the function of TCR-mediated signal transducers such as NF-AT (Emmel et al., 1989; Shevach, 1985). The lack of effect of TCDD on the clonal expansion of the adoptively transferred DO11.10 T cells suggests that TCDD does not affect the proximal signaling events associated with TCR ligation.

In contrast to the lack of effects of TCDD on the expansion of OVA-specific T cells, the CD4⁺/KJ1-26⁺ T cells disappeared more rapidly from the spleen in adoptively transferred mice exposed to TCDD. This observation suggests that TCDD may enhance the deletion of the antigen-specific T cells. Although TCDD-induced deletion of thymocytes has been extensively investigated (Silverstone et al., 1994; Staples et al., 1998a,b), only a limited number of reports have implicated an effect of TCDD on peripheral T-cell deletion (Prell et al., 1995; Pryputniewicz et al., 1998). In the study by Prell et al. (1995), the anti-CD3-induced deletion of CD4⁺ but not CD8⁺ T cells was enhanced in TCDD-treated mice when compared to vehicle-treated mice. It was suggested that this deletion was an activation-induced cell death, possibly mediated by increased apoptosis. Likewise, the study by Pryputniewicz et al. (1998) showed increased apoptosis of activated T cells in TCDD-treated but not vehicle-treated mice that had been injected with anti-CD3 in CFA. Studies currently in progress in our laboratory should help delineate the mechanism of enhanced deletion of CD4⁺/KJ1-26⁺ T-helper cells in the spleens of TCDD-treated mice.

The activation of T lymphocytes induces the modulation of specific cell-surface markers. While some of these proteins, such as CD44, CD62L, and CD11a, have been ascribed specific functions, others such as CD69 are less defined (Croft and Dubey, 1997). Our results clearly indicate that OVA-specific DO11.10 T cells in adoptively transferred mice become activated following immunization. Surprisingly, few changes were observed in the expression of activation markers in TCDD-treated mice. In fact, only the induction of CD11a was found to be negatively affected on CD4⁺/KJ1-26⁺ T cells in mice exposed to TCDD. CD11a functions as an adhesion molecule, which enhances the activation of Ag-specific T cells by APC, and disruption of the interaction of CD11a with its receptor CD54 (ICAM-1) leads to profound suppression of T cell activation (Bachmann et al., 1997; Dougherty and Hogg, 1987; Murayama et al., 1997). However, the TCDD-induced decrease in CD11a expression on DO11.10 T cells did not correlate with decreased expansion in adoptively transfused mice following encounter with antigen. Thus, the significance, if any, of the decreased CD11a expression on the CD4⁺/KJ1-26⁺ T cells is unknown.

In contrast to the suppressive effects of TCDD on CD11a expression, the population of DO11.10 T cells that were CD62Llow paradoxically increased in mice exposed to TCDD. Since down-modulation of CD62L is associated with T-cell activation, these results imply that TCDD may increase T-cell activation. This interpretation is supported by recent data using CFSE-labeled cells, which demonstrated that TCDD increased the proliferative capacity of CD4⁺/KJ1-26⁺ T cells in adoptively transfused mice (unpublished observations). Taken together, these results suggest that TCDD may induce hyperstimulation of antigen-specific CD4⁺ T cells and contribute to their activation-induced cell death.

The differentiation of T-helper cells from naive Th0 cells into Th1 and Th2 effector cells is characterized by the production of specific patterns of cytokines (Mosmann et al., 1986). Th1 cells primarily produce IL-2, TNF, and IFN-γ, while Th2 cells secrete IL-4, IL-5, IL-6, and IL-10 (O’Garra and Murphy, 1994). T cell-derived cytokines such as IL-4 and IFN-γ contribute to immunoglobulin (Ig) class switching of antibodies in B cells from the IgM isotype to downstream isotypes such as IgG1 and IgG2a, respectively (Finkelman et al., 1990). Previously, TCDD was shown to suppress T-cell production of IL-2 but not TNF, IFN-γ, or IL-6 in mice injected with anti-CD3 (Prell et al., 1995). In contrast, the production of IL-2, IFN-γ, and TNF by CD8⁺ T cells was suppressed by TCDD following challenge with allogeneic P815 tumor cells, while no effect was observed on the production of IL-4 and IL-6 (Kerkvliet et al., 1996). However, since immunization of mice with P815 cells produces a pronounced Th1 response, it was not clear from these studies if TCDD would affect Th2 development. In the DO11.10 adoptive transfer model, both Th1 and Th2 cytokines were produced by the OVA-specific KJ1-26⁺ spleen cells from adoptively transfused mice, although Th1 cytokines were produced in greater amounts than the Th2 cytokines. Interestingly, TCDD exposure significantly suppressed the production of IL-2 and IL-10 and caused a trend towards decreased production of IFN-γ and IL-4 by KJ1-26⁺ cells. Previously, in a report by Lai et al. (1996), putative dioxin response elements (DREs) were identified in several cytokine
genes including IL-2, IL-10, and IFN-γ. This suggests that it is possible for TCDD to act directly on activated OVA-specific T cells to suppress their production of cytokines, which may ultimately compromise antibody isotype switching by B cells. However, the degree of cytokine suppression produced by TCDD in our studies does not appear to be proportional to the more severe suppression of antibody production observed in adoptively transferred mice exposed to TCDD.

In addition to the role that cytokines play in Ig-class switching by B cells, cognate interactions between B and T cells have also been demonstrated to be important (Noelle et al., 1992; Parker, 1993). Because activated T cells are required to migrate to B cell areas to induce Ig class switching, we speculate that, although OVA-specific T lymphocytes in TCDD-treated mice may undergo activation events such as those described in this paper, they may yet be unable to migrate to specific areas of peripheral immune organs to effectively interact with antigen-specific B cells. In this scenario, TCDD-induced suppression of cognate interactions between OVA-specific CD4+ T and OVA-specific B cells could ultimately result in the suppressed production of IgG OVA-specific antibodies. Alternatively, the suppression of OVA-specific IgG antibodies may result from a direct effect of TCDD on Ig class switching by B cells. Investigations are currently underway in our laboratory to explore these possibilities by following the migration of the DO11.10 T cells into B cell follicles and the formation of germinal centers in adoptively transferred mice exposed to TCDD.

Although modulation of lymphocyte effector functions may be used as a marker of TCDD-induced effects, elucidation of the specific mechanisms underlying TCDD immunosuppression will require identification of the direct cellular target(s) of this xenobiotic. The Ah receptor (AhR) mediates the immunosuppressive effects of TCDD (Kerkvliet, 1998); however, the function of this receptor in specific immune-cell populations remains to be characterized. Although the AhR has been identified in both T and B lymphocytes, these cells constituively expressed only low levels of AhR and required activation to increase their expression (Lawrence et al., 1996; Sulentic et al., 1998). However, in T cells, no binding of the AhR to consensus DRE sequences could be demonstrated, suggesting that T cells are not directly affected by TCDD (Lawrence et al., 1996). This finding is consistent with our results showing that antigen-specific CD4+ T cells appear to be activated in TCDD-treated mice. However, additional studies are necessary to evaluate T-cell effector functions such as CD154 expression or T-cell survival signals in TCDD-treated animals. These events occur downstream from initial activation of T cells such as proliferation, coincide with AhR induction and thus may be more sensitive to the effects of TCDD exposure. At this time, it is unknown if the AhR in B cells is capable of binding to a consensus DRE. However, a study by Sulentic et al. (1998) using transformed B cell lines has demonstrated an AhR-dependent suppression of LPS-induced IgM production, suggesting that B cells can be directly affected by TCDD.

Other potential direct cellular targets of TCDD could be APC populations such as monocytes, macrophages, and dendritic cells. The AhR has been reported to be induced in monocytes following activation, and capable of binding DRE consensus DNA in macrophages (Hayashi et al., 1995; Lawrence et al., 1996). In addition, our laboratory has recently documented constitutive AhR expression in dendritic cells and macrophages and preliminary results suggest that this receptor is functional in these cells (unpublished observations). Precisely, we have demonstrated that TCDD inhibited the expression of costimulatory molecules on APC following activation (Prell and Kerkvliet, 1997). More recently, our laboratory has characterized the suppressive effects of TCDD on the production of IL-12 by splenic APC (Shepherd et al., manuscript submitted). Therefore, it is possible that direct effects of TCDD on APC lead to defective differentiation or survival of antigen-specific lymphocytes and ultimately contributes to compromised adaptive immunity. This hypothesis would be consistent with normal clonal expansion and activation marker expression on antigen-specific CD4+ T lymphocytes followed by enhanced deletion and suppressed effector functions as observed in our studies.

In summary, this report demonstrates for the first time the effects of xenobiotic exposure on the in vivo activation of antigen-specific CD4+ T helper cells by evaluating the effects of TCDD in the DO11.10 adoptive transfer model. Although TCDD exposure of adoptively-transferred mice did not affect the in vivo clonal expansion or activation of OVA-specific CD4+ T cells, DO11.10 cells from TCDD-treated mice disappeared more rapidly from the spleen and produced reduced levels of T cell-derived cytokines following in vitro restimulation. These data suggest that TCDD disrupts the survival and/or differentiation of OVA-specific effector T-helper cells which ultimately contributes to suppression of the humoral immune response to OVA. In addition, these results highlight the potential of the DO11.10 adoptive transfer system to serve as an antigen-specific transgenic mouse model to assess possible immunotoxic effects of xenobiotics on CD4+ T-helper cell activation.

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