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CUTTING EDGE

Cutting Edge: Activation of the Aryl Hydrocarbon Receptor by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Generates a Population of $CD4^+CD25^+$ Cells with Characteristics of Regulatory T Cells[1]

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***Activation of the aryl hydrocarbon receptor (AhR) by its most potent ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), leads to immune suppression in mice. Although the underlying mechanisms responsible for AhR-mediated immune suppression are not known, previous studies have shown that activation of the AhR must occur within the first 3 days of an immune response and that $CD4^+$ T cells are primary targets. Using the B6-into-B6D2F_1 model of an acute graft-vs-host response, we show that activation of AhR in donor T cells leads to the generation of a subpopulation of $CD4^+$ T cells that expresses high levels of CD25, along with $CD62L^{low}$, CTLA-4, and glucocorticoid-induced TNFR. These donor-derived $CD4^+CD25^+$ cells also display functional characteristics of regulatory T cells in vitro. These findings suggest a novel role for AhR in the induction of regulatory T cells and provide a new perspective on the mechanisms that underlie the profound immune suppression induced by exposure to TCDD. The Journal of Immunology,* 2005, 175: 4184–4188.**

The aryl hydrocarbon receptor (AhR),[3] along with its nuclear binding partner, ARNT, is a ligand-activated transcription factor that belongs to the basic-helix-loop-helix PER-ARNT-SIM (bHLH-PAS) family of transcriptional regulators (1). PAS domain-containing proteins play a role in sensing and responding to changes induced by environmental stimuli, such as oxygen partial pressure, redox potential, light intensity, and xenobiotic chemicals (2). Ligands for AhR are diverse and include products of cellular metabolism such as tryptophan derivatives and arachidonic acid metabolites, as well as dietary components such as indole-3-carbinol found in cruciferous vegetables and quercetin found in green and black teas (3, 4). However, the functional effects of AhR activation have been elucidated using ligands of toxicological concern, such as the polycyclic aromatic hydrocarbon benzo[*a*]pyrene, found in cigarette smoke and broiled meats, and a variety of halogenated aromatic hydrocarbons, noted for their widespread contamination of the environment. Of the latter group of chemicals, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent AhR agonist due to its high binding affinity (K_d 10^{-10}–10^{-9} M) and resistance to metabolism (5).

Extensive studies in laboratory rodents have shown that a single treatment with TCDD in the low microgram per kilogram range induces profound suppression of Ab- and cell-mediated immune responses and alters host resistance to many diseases (6). Human exposure to TCDD and other dioxin-like compounds has also been linked to altered immune function, particularly when exposure occurs during fetal/neonatal development (7, 8). Although most cells of the immune system express AhR, adult $AhR^{-/-}$ mice have no reported defects in immune development or immune responsiveness (9). In contrast, $AhR^{-/-}$ mice are highly resistant to the immune suppression associated with exposure to TCDD.

The underlying cellular mechanisms that drive AhR-dependent immune suppression have not been elucidated. Recent studies from our laboratory have shown that expression of AhR in both $CD4^+$ and $CD8^+$ T cells is required for TCDD to fully suppress an allospecific CTL response generated in an acute graft-vs-host (GVH) model (10). In this study, we used the same model to determine whether TCDD alters the activation of donor $CD4^+$ cells and to assess the dependence of the effects observed on the presence of AhR in the donor T cells. Treatment of F_1 hosts with TCDD resulted in a significant increase in the percentage of donor $CD4^+$ cells that expressed high levels of CD25, low levels of CD62L as well as glucocorticoid-induced TNFR (GITR) and CTLA-4, a phenotype associated with some types of regulatory T cells (T_{reg}) (11). Donor

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[3] Abbreviations used in this paper: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; GVH, graft-vs-host; GITR, glucocorticoid-induced TNFR; T_{reg}, regulatory T cell; MCF, mean channel fluorescence.

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$CD4^+CD25^+$ cells purified from the spleen of F_1 mice expressed functional characteristics associated with T_{reg}, namely, unresponsiveness to stimulation with anti-CD3 unless exogenous IL-2 was also provided and a potent ability to suppress the proliferation of $CD4^+CD25^-$ cells. The development of the $CD4^+CD25^+$ population was dependent on the presence of AhR in the donor T cells, but not on the presence of preexisting $CD4^+CD25^+$ cells. Taken together, these results suggest a novel role for AhR in the generation of T_{reg} and provide a new perspective on the mechanisms that underlie the profound immune suppression induced by exposure to TCDD.

Materials and Methods

Mice and treatment with TCDD

C57BL/6J (B6) mice (H-2^b, Thy1.2) and B6D2F_1/J (F_1) mice (H-$2^{b/d}$, Thy1.2) were purchased from The Jackson Laboratory. B6.PL-Thy1^a/CyJ (Thy1.1) mice and B6.129-AhR^{tm1Bra}/J (AhR$^{-/-}$) mice (originally purchased from The Jackson Laboratory) were bred and maintained in our specific pathogen-free animal facility at Oregon State University. B6 mice purchased from The Jackson Laboratory were used as wild-type controls for AhR$^{-/-}$ mice. All animal procedures were approved by the Institutional Animal Care and Use Committee. F_1 mice were dosed orally with vehicle or 15 μg TCDD/kg body weight 1 day before the injection of donor B6 T cells as previously described (10). This dose of TCDD is not overtly toxic but effectively suppresses the GVH CTL response.

Preparation of donor T cells

T cells were purified from pooled B6 spleens by magnetic bead sorting (Pan T isolation kit; Miltenyi Biotec). The purity of the T cells was ≥90% and viability was >95%. F_1 host mice were injected i.v. with 2×10^7 donor T cells. In some experiments, cell division was assessed by labeling the donor T cells with 5 μM CFSE (Molecular Probes) before injection into F_1 hosts (12).

Flow cytometry

Spleen cells were stained with anti-H-$2D^d$ and anti-CD4 Ab to identify the donor $CD4^+$ T cells (Fig. 1*A*) along with Ab to the following markers: CD62L, CD25, CD28, (BD Pharmingen), and GITR (R&D Systems). Following surface staining, the cells were fixed and permeabilized (Cytofix/Cytoperm Plus kit; BD Pharmingen) and stained with anti-CTLA-4 (BD Pharmingen). Isotype-matched fluorochrome-conjugated Ab were used as controls for nonspecific fluorescence. After gating on live spleen cells, listmode data on 5,000–10,000 donor $CD4^+$ T cells were collected using either a Coulter XL or FC500 flow cytometer (Beckman Coulter). All data analyses, including software compensation, were performed using WinList software (Verity Software House).

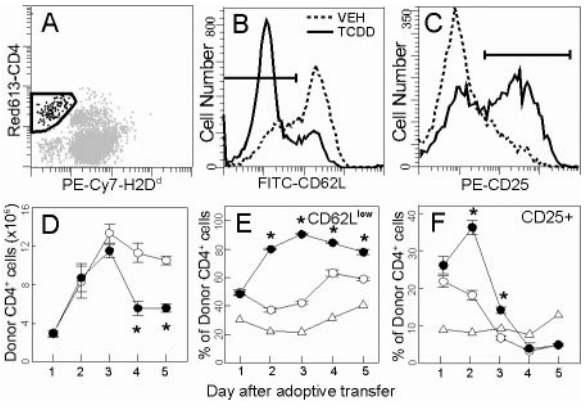

FIGURE 1. The premature loss of donor $CD4^+$ cells from the spleen of TCDD-treated mice is preceded by increased expression of an activated phenotype. F_1 mice were dosed with vehicle (○) or TCDD (●) 1 day before the i.v. injection of B6 donor T cells. As a syngeneic control (△), B6 donor T cells were injected into B6-Thy1.1 congenic mice. *A*, After gating on live spleen cells, the donor $CD4^+$ T cells were identified as H-$2D^{d-}CD4^+$. The region identifying $CD62L^{low}$ (*B*) or $CD25^+$ (*C*) cells is indicated by the black bar, as determined by syngeneic controls and isotype staining, respectively. The number of donor $CD4^+$ T cells in the spleen (*D*) and the percentage of $CD62L^{low}$ (*E*) and $CD25^+$ (*F*) donor $CD4^+$ cells was determined on days 1–5. The data are combined from two time course experiments (days 1–3 and days 3–5, $n = 4$–5 mice/group per day) except for CD25 on days 1 and 2, where $n = 7$ and 20/group, respectively. *, $p \leq 0.001$, compared with vehicle.

In vitro suppression and anergy assays

The ability of donor-derived or naive $CD4^+CD25^+$ cells to proliferate and to suppress the proliferation of CFSE-labeled naive $CD4^+CD25^-$ cells was assessed as described previously (13). Donor Thy1.1$^+$ cells were enriched from pooled spleens of six to seven TCDD-treated F_1 mice on day 2 by magnetic bead sorting; the $CD4^+CD25^+$ fraction was further enriched using a MoFlo high-speed cell sorter (DakoCytomation). Pooled spleen cells from three naive B6 mice were sorted into $CD4^-$, $CD4^+CD25^-$, and $CD4^+CD25^+$ fractions using a $CD4^+CD25^+$ T_{reg} isolation kit (Miltenyi Biotec). The $CD4^-$ cells were irradiated (3000 rad) and used as accessory cells. The $CD4^+CD25^-$ cells were labeled with 2 μM CFSE before culturing with anti-CD3 (BD Pharmingen) and accessory cells; donor-derived or naive $CD4^+CD25^+$ cells were added to some wells. After 72 h, CFSE dilution was measured by flow cytometry.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute). Comparisons between means were made using the least-significance difference multiple comparison *t* test, with $p < 0.05$ considered to be statistically significant.

Results

Loss of donor $CD4^+$ T cells in the spleen of TCDD-treated F_1 hosts is preceded by an increase in their activation phenotype

Previous studies have shown that treatment with TCDD does not alter the initial expansion of activated $CD4^+$ T cells in the spleen of Ag-challenged mice but promotes a premature decline in their number before effector cell development (12, 14, 15). TCDD produced a similar effect on the expansion and contraction of donor $CD4^+$ cells following their transfer into F_1 mice (Fig. 1*D*). When the phenotype of donor $CD4^+$ cells was examined, treatment with TCDD was associated with a significant increase in the percentage of $CD62L^{low}$ cells (Fig. 1*B*). By day 2, 80% of the donor $CD4^+$ cells were $CD62L^{low}$, and this phenotype was maintained through day 5 (Fig. 1*E*). At the same time, treatment with TCDD led to a transient increase in the percentage of donor $CD4^+$ cells that expressed CD25 (Fig. 1, *C* and *F*). On day 2, the percentage of donor $CD4^+CD25^+$ cells in TCDD-treated mice was consistently 2-fold greater than the percentage in vehicle-treated mice. The percentage of $CD4^+CD25^+$ cells declined on day 3 and thereafter in both treatment groups.

Division-dependent changes in expression of CD62L and CD25 are enhanced by TCDD

The expression levels of CD62L and CD25 on newly activated $CD4^+$ T cells decreases and increases, respectively, with progressive rounds of cell division (16, 17). We injected CFSE-labeled donor T cells to determine whether treatment with TCDD altered this relationship. As previously observed, on day 2, TCDD induced a significant increase in the percentage of donor $CD4^+$ cells that were $CD62L^{low}$ (vehicle, 32.3 ± 1.3%; TCDD, 56.5 ± 1.6%; $p < 0.0001$) and $CD25^+$ (vehicle, 16.6 ± 0.9%; TCDD, 45.7 ± 1.1%; $p < 0.0001$). Based on dilution of CFSE, no division of the donor $CD4^+$ cells was apparent 1 day after transfer into vehicle- or TCDD-treated F_1 mice (data not shown). On day 2, >80% of the donor $CD4^+$ cells from both treatment groups had undergone one to four cell divisions, with no observable effect of TCDD (Fig. 2*A*). Fig. 2,

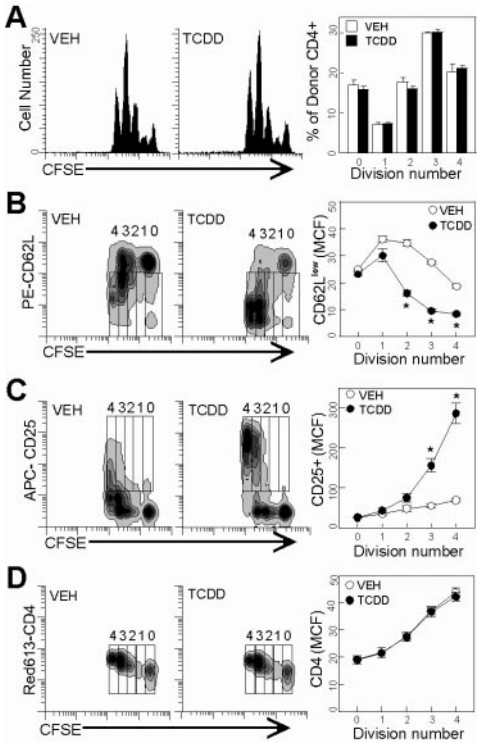

FIGURE 2. TCDD alters the intensity of CD25 and CD62L expression on donor $CD4^+$ cells but preserves the relationship to cell division. B6 donor T cells were labeled with CFSE before injection into F_1 mice. On day 2, spleen cells were collected, and the H-$2D^{d-}CD4^+$ cells were analyzed for cell division (*A*) and expression of $CD62L^{low}$ (*B*), $CD25^+$ (*C*), and CD4 (*D*). Data are representative of five independent experiments, with $n = 4–6$ mice/group. *B–D,* The boxes correspond to each cell division and indicate the regions from which the MCF data for $CD62L^{low}$, $CD25^+$, and CD4 are derived. *, $p \leq 0.01$, compared with vehicle.

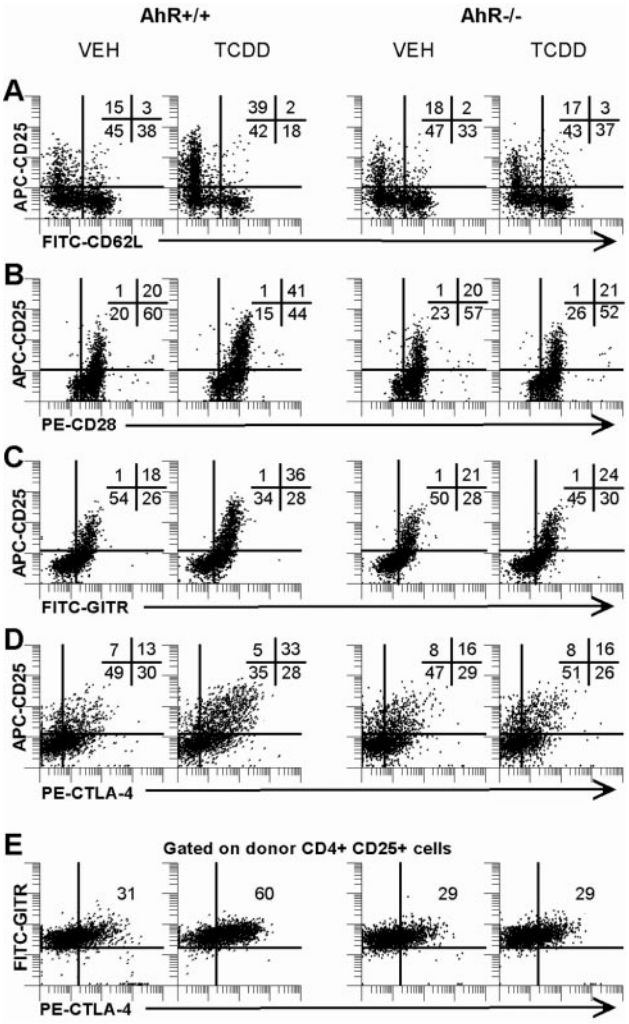

FIGURE 3. Activation of AhR increases the subpopulation of donor $CD4^+$ cells that coexpresses CD25 with $CD62L^{low}$, CD28, GITR, and CTLA-4. $AhR^{+/+}$ or $AhR^{-/-}$ donor T cells were injected into vehicle- or TCDD-treated F_1 mice. On day 2, spleen cells were collected and stained for H-$2D^d$, CD4, and CD25 along with CD62L (*A*), CD28 (*B*), GITR (*C*), or CTLA-4 (*D*). *E,* The coexpression of GITR and CTLA-4 was examined on the H-$2D^{d-}CD4^+CD25^+$ cells from $AhR^{+/+}$ or $AhR^{-/-}$ mice. Data show the percentage of cells within each quadrant for a single animal representative of 8–10 mice from two independent experiments. For quadrant means above 5, the SEs averaged 12% of the mean. Quadrants for CD62L were set based on syngeneic controls; all others were set based on isotype.

B and *C*, show the expression of $CD62L^{low}$ and $CD25^+$ on donor $CD4^+$ cells in relation to the number of cell divisions. For both treatment groups, at least two rounds of cell division were required before changes in expression level of CD62L or CD25 were observed. TCDD did not influence the overall kinetics, but augmented the degree of down-regulation of CD62L and up-regulation of CD25 within each cell division. As early as the second cell division, the mean channel fluorescence (MCF) of $CD62L^{low}$ was significantly lower on donor $CD4^+$ cells from TCDD-treated mice as compared with vehicle-treated mice (Fig. 2*B*, *right panel*). Likewise, after three divisions, the MCF of $CD25^+$ on donor $CD4^+$ cells from TCDD-treated mice was severalfold higher (Fig. 2*C*, *right panel*). Similar to the findings of Maury et al. (18), the expression of CD4 increased on donor T cells with progressive rounds of cell division; however, this increase was unaffected by TCDD (Fig. 2*D*).

Expression of AhR in the donor T cells is required for induction of the $CD25^+CD62L^{low}$ phenotype

In the GVH model, both the donor T cells and many types of F_1 host cells express AhR. To determine whether AhR expression in the donor T cells was required for the altered phenotype induced by TCDD, T cells from $AhR^{-/-}$ mice were examined. Fig. 3*A* shows the coexpression of CD25 and CD62L on $AhR^{+/+}$ or $AhR^{-/-}$ donor $CD4^+$ cells. As expected, when donor T cells expressed AhR, TCDD induced a significant increase in the percentage of donor $CD4^+$ cells expressing $CD25^+CD62L^{low}$ (vehicle = 16.9 ± 1.6; TCDD = 33.0 ± 1.3, $p = 0.0001$; Fig. 3*A*, *left panels*). A concomitant decrease occurred in the $CD25^-CD62L^{high}$ population, while the $CD25^-CD62L^{low}$ population was unchanged. When the donor T cells did not express AhR, TCDD did not alter the coexpression of CD62L and CD25 (Fig. 3*A*, *right panels*).

Donor $CD4^+CD25^+$ cells from TCDD-treated mice also express high levels of CD28, GITR, and CTLA-4

The $CD25^+CD62L^{low}$ phenotype has generally been attributed to activated $CD4^+$ T cells, suggesting that ligation of AhR by TCDD could be promoting CD25-mediated activation-induced cell death (19, 20). However, the same phenotype defines some $CD4^+CD25^+$ T_{reg} that have a potent ability to suppress allograft responses (21, 22). Therefore, we examined

additional markers of activated T cells and T_{reg} to characterize the donor $CD4^+$ cells. The histograms in Fig. 3, *B–D*, show the coexpression of CD25 and CD28, GITR, or CTLA-4 on donor $CD4^+$ cells from $AhR^{+/+}$ or $AhR^{-/-}$ mice. Treatment with TCDD led to a 2-fold increase in the percentage of double-positive cells for all three markers. For CD28, a concomitant decrease occurred in the $CD25^-CD28^+$ cells (Fig. 3*B*). For GITR and CTLA-4, a concomitant decrease occurred in the double-negative population (Fig. 3, *C* and *D*). These changes in donor T cell phenotype occurred only if the donor T cells expressed AhR. Fig. 3*E* shows that AhR activation doubled the population of $CD4^+CD25^+$ cells that expressed GITR and CTLA-4 from 31.6 ± 1.7% in vehicle-treated mice to 58.0 ± 2.0% in TCDD-treated mice ($p < 0.0001$). This doubling was dependent on AhR in the donor T cells since no increase was observed following treatment with TCDD if they did not express AhR. Taken together, these results suggest that activation of AhR in T cells may be promoting the development of $CD4^+CD25^+$ T_{reg} from unactivated donor $CD4^+$ cells ($CD25^-CD62L^{high}CD28^+GITR^-CTLA\text{-}4^-$).

Depletion of $CD25^+$ cells from the donor T cell inoculum does not influence the TCDD-dependent increase of donor $CD4^+$ $CD25^+$ cells in F_1 mice

The increase in donor $CD4^+CD25^+$ cells in TCDD-treated mice could reflect the expansion of natural $CD4^+CD25^+$ T_{reg}, which are present in the donor T cell inoculum at a frequency of ~10%. We used magnetic beads to deplete the $CD25^+$ cells from the purified donor T cells before injection into F_1 hosts. Fig. 4*A* shows the TCDD-induced increase in donor $CD4^+CD25^+$ cells on day 2 when undepleted donor T cells were injected (vehicle, 10.7 ± 0.6%; TCDD, 36.8 ± 1.2%; $p < 0.0001$). Fig. 4*B* shows that depletion of $CD4^+CD25^+$ cells from the donor inoculum did not impair the TCDD-induced increase in $CD25^+$ cells (vehicle, 10.5 ± 0.6%; TCDD, 33.4 ± 1.3%; $p < 0.0001$). In addition, depletion of $CD25^+$ cells did not affect the TCDD-dependent increase in the expression of $CD62L^{low}$, GITR, and CTLA-4 on the donor $CD4^+CD25^+$ cells (data not shown).

Alloresponsive donor $CD4^+$ $CD25^+$ cells from TCDD-treated F_1 mice are anergic and suppressive in vitro

T_{reg} are characterized by two functional attributes in in vitro assays: 1) anergy to stimulation with anti-CD3 and accessory cells that can be overcome by addition of exogenous IL-2 and 2) the ability to suppress the proliferative response of non-T_{reg} stimulated with anti-CD3 and accessory cells (23). Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F_1 host mice on day 2 after injection failed to proliferate in response to stimulation with anti-CD3 and accessory cells (Fig. 5*A*). Likewise, $CD4^+CD25^+$ cells from naive mice (a natural T_{reg} population) failed to proliferate, whereas $CD4^+CD25^-$ cells from the same naive mice proliferated extensively. Addition of IL-2 led to increased proliferation in all of the cultures. As shown in Fig. 5*B*, donor $CD4^+CD25^+$ cells isolated from TCDD-treated F_1 mice inhibited the division of CFSE-labeled $CD4^+CD25^-$ cells stimulated with anti-CD3 and accessory cells. The suppressive ability of the $CD4^+CD25^+$ cells from TCDD-treated F_1 mice was greater than equivalent numbers of $CD4^+CD25^+$ cells from naive mice.

Discussion

The initial goal of these studies was to characterize changes in the activation of $CD4^+$ T cells induced by ligation of the AhR with TCDD that might explain the premature loss of alloresponsive $CD4^+$ T cells and subsequent suppression of the GVH CTL response (10). Although several AhR-dependent changes in the phenotype of activated $CD4^+$ cells were observed, further analysis revealed that the changes occurred on a distinct subpopulation of donor $CD4^+$ cells. This subpopulation of $CD4^+$ cells expressed high levels of CD25, low levels of CD62L, as well as GITR and CTLA-4. Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F_1 mice were anergic and suppressed the proliferative response of naive T cells in vitro, demonstrating that these cells possessed what is currently the best functional definition of T_{reg} (23). If the donor T cells did not express AhR, treatment with TCDD did not induce the $CD4^+CD25^+$ population. These results suggest that signaling through the AhR plays a role in the generation of adaptive $CD4^+CD25^+$ T_{reg}. Whether this occurs with ligands other than TCDD remains to be determined.

AhR-dependent generation of $CD4^+CD25^+$ T_{reg} early in the immune response is consistent with the potent immunosuppressive effects of TCDD. Previous studies have shown that TCDD suppresses the development of allospecific $CD8^+$ CTL

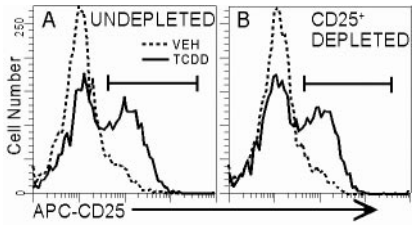

FIGURE 4. The presence of $CD4^+CD25^+$ cells in the donor T cell inoculum is not required for the increase in donor $CD4^+CD25^+$ cells in TCDD-treated F_1 mice. Vehicle- or TCDD-treated F_1 mice were injected with undepleted donor T cells (9.4% $CD4^+CD25^+$ cells) (*A*) or CD25-depleted donor T cells (0.1% $CD4^+CD25^+$ cells) (*B*). On day 2 after injection, the expression of CD25 gated on donor $CD4^+$ cells was determined. Representative data of four to six mice per group are shown.

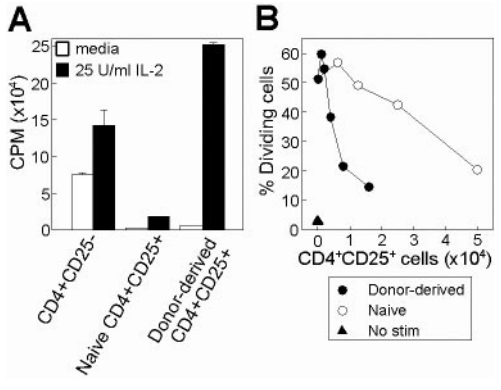

FIGURE 5. Donor $CD4^+CD25^+$ cells isolated from TCDD-treated F_1 mice on day 2 are anergic and suppress the proliferation of $CD4^+CD25^-$ cells in vitro. *A,* The indicated subsets of cells were cultured in triplicate with anti-CD3 and irradiated accessory cells with or without IL-2, and the incorporation of $[^3H]$TdR was measured on day 3. *B,* CFSE-labeled $CD4^+CD25^-$ cells were cultured with anti-CD3, irradiated accessory cells, and increasing numbers of donor or naive $CD4^+CD25^+$ cells. Six wells from a 96-well plate were pooled and the percentage of $CD4^+CD25^-$ cells that had divided was determined on day 3 by flow cytometry. Data are representative of two independent experiments.

activity to P815 tumor cells by reducing the number of CTL precursors that are activated early in the response (24, 25). This effect on CTL activation was lost if treatment with TCDD was delayed >3 days after the injection of P815 cells (26) and required the presence of $CD4^+$ T cells (27). Furthermore, in an acute GVH response, suppression of allospecific $CD8^+$ CTL activity by TCDD was dependent on the presence of $AhR^{+/+}$ donor $CD4^+$ T cells (10). This CD4-dependent suppression could reflect the development of T_{reg}, since several studies have shown that $CD4^+CD25^+$ T_{reg} suppress pathogenic T cell responses in GVH disease (21, 22, 28).

In recent years, several different types of T_{reg} have been described that fall broadly into natural and adaptive categories (11). Natural T_{reg} that derive from the thymus constitutively express CD25, CTLA-4, GITR, and CD62L, as well as the transcription factor Foxp3. In our model, depletion of the $CD25^+$ cells from the donor inoculum before injection into TCDD-treated F_1 hosts did not alter the development of the $CD4^+CD25^+$ subpopulation, suggesting that ligation of the AhR is not simply expanding the population of natural T_{reg}. Furthermore, the level of Foxp3 mRNA was lower in donor T cells isolated from TCDD- vs vehicle-treated F_1 mice on day 2, despite the fact that there were twice as many cells expressing the T_{reg} phenotype in the TCDD group (W. R. Vorachek, N. B. Marshall, N. I. Kerkvliet, unpublished observations). Consistent with the low expression of CD62L on the $CD4^+CD25^+$ cells, it is likely that activation of the AhR is inducing an adaptive T_{reg} population that may not depend on expression of Foxp3 (11, 29). In addition, the transient nature of the increase in expression of CD25 is not contradictory with a T_{reg} hypothesis, since studies have shown that T_{reg} can down-regulate CD25 while still retaining their suppressive activity (30).

One mechanism by which activation of the AhR could promote the development of T_{reg} is by enhancing expression of the *IL-2* gene. The generation and expansion of $CD4^+CD25^+$ T_{reg} have been shown to depend on IL-2 (31, 32). Interestingly, Jeon and Esser (33) reported that the mouse *IL-2* promoter contains three AhR/ARNT response elements that bind the ligand-activated AhR and induce reporter gene expression. Increased expression of the *IL-2* gene was also observed in thymocytes after in vivo exposure to TCDD and in mitogen-activated spleen cells. Studies are underway to delineate the potential role of AhR-induced production of IL-2 in the development of T_{reg}.

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Disclosures

The authors have no financial conflict of interest.

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