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*J Immunol* 2005; 175:4184-4188; doi: 10.4049/jimmunol.175.7.4184

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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

Castle J. Funatake,* Nikki B. Marshall,†‡ Linda B. Steppan, *, Dan V. Mourich,†‡ and Nancy I. Kerkvliet2*,§

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-B6D2F1, 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^{lo}, 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), 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 ($K_d \approx 10^{-10}$–$10^{-9}$ 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 allo-specific 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 F1 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
CD4⁺CD25⁺ cells purified from the spleen of F₃ mice expressed functional characteristics associated with Treg, 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 Treg 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/6 (B6) mice (H-2b, Thy1.2) and B6D2F₁ (F₁) mice (H-2b/d, Thy1.2) were purchased from The Jackson Laboratory. B6.PL-Thy1⁺/CyJ (Thy1.1) mice and B6.129-AhR¹⁰⁻¹²⁻¹⁴⁻/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. F₁ 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 overly 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%. F₁ host mice were injected i.v. with 2 × 10⁶ 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 F₁ hosts (12).

Flow cytometry

Spleen cells were stained with anti-H-2Dd and anti-CD4 Ab to identify the donor CD4⁺ T cells (Fig. 1A) along with Ab to the following markers: CD62L, CD25, CD69, and GITR (BD Pharmingen). Iso- lation kit; Miltenyi Biotec). The purity of the T cells was >90% and viability was >95%. F₁ host mice were injected i.v. with 2 × 10⁶ 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 F₁ hosts (12).

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 expressed 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⁺ T cells were enriched from pooled spleens of six to seven TCDD-treated F₁ mice on day 2 by magnetic bead sorting; the CD4⁺CD25⁻ fraction was further enriched using a MoFlo high-speed cell sorter (DakoCytometry). Pooled spleen cells from three naive B6 mice were sorted into CD4⁺, CD4⁺CD25⁻, and CD4⁺CD25⁺ fractions using a CD4⁺CD25⁺ Treg isolation kit (Miltenyi Biotec). The CD4⁺ T 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.

Results

Loss of donor CD4⁺ T cells in the spleen of TCDD-treated F₁ 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 temporary 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 F₁ mice (Fig. 1D). When the phenotype of donor CD4⁺ cells was examined, treatment with TCDD was associated with a significant increase in the percentage of CD62Llow cells (Fig. 1B). By day 2, 80% of the donor CD4⁺ cells were CD62Llow, and this phenotype was maintained through day 5 (Fig. 1E). At the same time, treatment with TCDD led to a transient increase in the percentage of donor CD4⁺ cells that expressed CD25 (Fig. 1C 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.
B and C, show the expression of CD62L\textsubscript{low} and CD25\textsuperscript{+} on donor CD4\textsuperscript{+} 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\textsubscript{low} was significantly lower on donor CD4\textsuperscript{+} cells from TCDD-treated mice as compared with vehicle. Likewise, after three divisions, the MCF of CD25\textsuperscript{+} on donor CD4\textsuperscript{+} cells from TCDD-treated mice was several-fold higher (Fig. 2C, 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. 2D).

Expression of AhR in the donor T cells is required for induction of the CD25\textsuperscript{+}CD62L\textsubscript{low} phenotype

In the GVH model, both the donor T cells and many types of F\textsubscript{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\textsuperscript{+/+} or AhR\textsuperscript{−/−} donor CD4\textsuperscript{+} cells were examined. Fig. 3A shows the coexpression of CD25 and CD62L on AhR\textsuperscript{+/+} or AhR\textsuperscript{−/−} donor CD4\textsuperscript{+} cells. As expected, when donor T cells expressed AhR, TCDD induced a significant increase in the percentage of donor CD4\textsuperscript{+} cells expressing CD25\textsuperscript{+}CD62L\textsubscript{low} (vehicle = 16.9 ± 1.6; TCDD = 33.0 ± 1.3, p = 0.0001; Fig. 3A, left panel). A concomitant decrease occurred in the CD25\textsuperscript{+}CD62L\textsubscript{high} population, while the CD62L\textsubscript{low} population was unchanged. When the donor T cells did not express AhR, TCDD did not alter the coexpression of CD62L and CD25 (Fig. 3A, right panels).

Donor CD4\textsuperscript{+}CD25\textsuperscript{+} cells from TCDD-treated mice also express high levels of CD28, GITR, and CTLA-4

The CD25\textsuperscript{+}CD62L\textsubscript{low} phenotype has generally been attributed to activated CD4\textsuperscript{+} 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\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} that have a potent ability to suppress allograft responses (21, 22). Therefore, we examined...
additional markers of activated T cells and T<sub>reg</sub> to characterize the donor CD<sup>+</sup> <sup>+</sup> cells. The histograms in Fig. 3, B–D, show the coexpression of CD25 and CD28, GITR, or CTLA-4 on donor CD<sup>+</sup> <sup>+</sup> cells from AhR<sup>+/+</sup> or AhR<sup>−/−</sup> 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 CD<sup>+</sup>CD28<sup>+</sup> cells (Fig. 3B). 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. 3E shows that AhR activation doubled the population of CD<sup>+</sup>CD25<sup>+</sup> 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 CD<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> from unactivated donor CD<sup>+</sup> <sup>+</sup> cells (CD25<sup>-</sup>CD62L<sup>high</sup>CD28<sup>+</sup>GITR−CTLA-4−).

Depletion of CD25<sup>+</sup> cells from the donor T cell inoculum does not influence the TCDD-dependent increase of donor CD<sup>+</sup>CD25<sup>+</sup> cells in F<sub>1</sub> mice

The increase in donor CD<sup>+</sup>CD25<sup>+</sup> cells in TCDD-treated mice could reflect the expansion of natural CD<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> which are present in the donor T cell inoculum at a frequency of ~10%. We used magnetic beads to deplete the CD25<sup>+</sup> cells from the purified donor T cells before injection into F<sub>1</sub> hosts. Fig. 4A shows the TCDD-induced increase in donor CD<sup>+</sup>CD25<sup>+</sup> 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. 4B shows that depletion of CD<sup>+</sup>CD25<sup>+</sup> cells from the donor inoculum did not impair the TCDD-induced increase in CD25<sup>+</sup> cells (vehicle, 10.5 ± 0.6%; TCDD, 33.4 ± 1.3%; p < 0.0001). In addition, depletion of CD25<sup>+</sup> cells did not affect the TCDD-dependent increase in the expression of CD62L<sup>low</sup>, GITR, and CTLA-4 on the donor CD<sup>+</sup>CD25<sup>+</sup> cells (data not shown).

Alloresponsive donor CD<sup>+</sup>CD25<sup>+</sup> cells from TCDD-treated F<sub>1</sub> mice are anergic and suppressive in vitro

T<sub>reg</sub> 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<sub>reg</sub> stimulated with anti-CD3 and accessory cells (23). Donor CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from TCDD-treated F<sub>1</sub> host mice on day 2 after injection failed to proliferate in response to stimulation with anti-CD3 and accessory cells (Fig. 5A). Likewise, CD4<sup>+</sup>CD25<sup>+</sup> cells from naive mice (a natural T<sub>reg</sub> population) failed to proliferate, whereas CD4<sup>+</sup>CD25<sup>+</sup> 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. 5B, donor CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from TCDD-treated F<sub>1</sub> mice inhibited the division of CFSE-labeled CD4<sup>+</sup>CD25<sup>+</sup> cells stimulated with anti-CD3 and accessory cells. The suppressive ability of the CD4<sup>+</sup>CD25<sup>+</sup> cells from TCDD-treated F<sub>1</sub> mice was greater than equivalent numbers of CD4<sup>+</sup>CD25<sup>+</sup> cells from naive mice.

**Discussion**

The initial goal of these studies was to characterize changes in the activation of CD4<sup>+</sup> T cells induced by ligation of the AhR with TCDD that might explain the premature loss of alloresponsive CD4<sup>+</sup> T cells and subsequent suppression of the GVH CTL response (10). Although several AhR-dependent changes in the phenotype of activated CD4<sup>+</sup> cells were observed, further analysis revealed that the changes occurred on a distinct subpopulation of donor CD4<sup>+</sup> cells. This subpopulation of CD4<sup>+</sup> cells expressed high levels of CD25, low levels of CD62L, as well as GITR and CTLA-4. Donor CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from TCDD-treated F<sub>1</sub> 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<sub>reg</sub> (23). If the donor T cells did not express AhR, treatment with TCDD did not induce the CD4<sup>+</sup>CD25<sup>+</sup> population. These results suggest that signaling through the AhR plays a role in the generation of adaptive CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>. Whether this occurs with ligands other than TCDD remains to be determined.

AhR-dependent generation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> early in the immune response is consistent with the potent immunosuppressive effects of TCDD. Previous studies have shown that TCDD suppresses the development of alloreactive CD8<sup>+</sup> CTL.

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**FIGURE 4.** The presence of CD4<sup>+</sup>CD25<sup>+</sup> cells in the donor T cell inoculum is not required for the increase in donor CD4<sup>+</sup>CD25<sup>+</sup> cells in TCDD-treated F<sub>1</sub> mice. Vehicle- or TCDD-treated F<sub>1</sub> mice were injected with undepleted donor T cells (9.4% CD4<sup>+</sup>CD25<sup>+</sup> cells) (A) or CD25-depleted donor T cells (0.1% CD4<sup>+</sup>CD25<sup>+</sup> cells) (B). On day 2 after injection, the expression of CD25 gated on donor CD4<sup>+</sup> cells was determined. Representative data of four to six mice per group are shown.

**FIGURE 5.** Donor CD4<sup>+</sup>CD25<sup>+</sup> cells isolated from TCDD-treated F<sub>1</sub> mice on day 2 are anergic and suppress the proliferation of CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> cells were cultured with anti-CD3, irradiated accessory cells, and increasing numbers of donor or naive CD4<sup>+</sup>CD25<sup>+</sup> cells. Six wells from a 96-well plate were pooled and the percentage of CD4<sup>+</sup>CD25<sup>+</sup> 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+ T cell activity by TCDD was dependent on the presence of AhR+/+ donor CD4+ T cells (10). This CD4-dependent suppression could reflect the development of Treg, since several studies have shown that CD4+CD25+ Treg suppress pathogenic T cell responses in GVH disease (21, 22, 28).

In recent years, several different types of Treg have been described that fall broadly into natural and adaptive categories (11). Natural Treg that derive from the thymus constitutively suppress the activation of alloantigen-specific CD8+ T lymphocytes (29). In addition, the transient nature of the increase in Foxp3 expression in CD4+ T cells, it is likely that activation of the AhR is inducing an adaptive Treg population that may not depend on expression of Foxp3 (11, 29). Furthermore, the level of Foxp3 mRNA was lower in donor T cells isolated from TCDD- versus vehicle-treated F1 mice on day 2, despite the fact that there were twice as many cells expressing the Treg phenotype in the TCDD group (W. R. Vorachek, N. B. Marshall, N. I. Kerkvliet, unpublished observations). Consistent with the low expression of CD25 on the CD4+CD25+ cells, it is likely that activation of the AhR is inducing an adaptive Treg 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 Treg hypothesis, since studies have shown that Treg can down-regulate CD25 while still retaining their suppressive activity (30).

One mechanism by which activation of the AhR could promote the development of Treg is by enhancing expression of the IL-2 gene. The generation and expansion of CD4+ CD25+ Treg 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 and lymphocytes 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 Treg.

Acknowledgments
We thank Julie Oughton for her assistance with data analysis and the Cell and Tissue Analysis Facilities and Service Core of the Environmental Health Sciences Center at Oregon State University for the use of the flow cytometers.

Disclosures
The authors have no financial conflict of interest.

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