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

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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


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 CD62Llow, 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 (Kd 10⁻¹⁰–10⁻¹⁵ 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 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, and glucocorticoid-induced TNFR (GITR) and CTLA-4, a phenotype associated with some types of regulatory T cells (Treg) (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; Treg, regulatory T cell; MCF, mean channel fluorescence.
CD4<sup>+</sup>CD25<sup>+</sup> cells purified from the spleen of F<sub>1</sub> mice expressed functional characteristics associated with T<sub>reg</sub>, namely, unresponsiveness to stimulation with anti-CD3 unless exogenous IL-2 was also provided and a potent ability to suppress the proliferation of CD4<sup>+</sup>CD25<sup>+</sup> cells. The development of the CD4<sup>+</sup>CD25<sup>+</sup> population was dependent on the presence of AHR in the donor T cells, but not on the presence of preexisting CD4<sup>+</sup>CD25<sup>+</sup> cells. Taken together, these results suggest a novel role for AHR in the generation of T<sub>reg</sub> 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<sup>b</sup>, Thy1.2) and B6D2F1/J (F<sub>1</sub>) mice (H-2<sup>b/d</sup>, Thy1.2) were purchased from The Jackson Laboratory. B6.PL-Thy1<sup>+</sup>CyJ (Thy1.1) mice and B6.129-AhR<sup>tm1Bra/J</sup> (AhR<sup>−/−</sup>) 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<sup>−/−</sup> mice. All animal procedures were approved by the Institutional Animal Care and Use Committee. F<sub>1</sub> 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 >99%. F<sub>1</sub> host mice were injected with 2 × 10<sup>6</sup> 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<sub>1</sub> hosts (12).

**Flow cytometry**

Spleen cells were stained with anti-H-2<sup>d</sup> and anti-CD4 Ab to identify the donor CD4<sup>+</sup> T cells (Fig. 1A) 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/Cytoperme Plus kit; BD Pharmingen) and stained with anti-CTLA-4 (BD Pharmingen). Iso-type-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<sup>+</sup> T cells were collected using either a Coulter XL or FACSII flow cytometer (Beckman Coulter). All data analyses, including software compensation, were performed using WinList software (Verity Software House).

**In vitro suppression and anergy assays**

The ability of donor-derived or naive CD4<sup>+</sup>CD25<sup>+</sup> cells to proliferate and to suppress the proliferation of CFSE-labeled naive CD4<sup>+</sup>CD25<sup>+</sup> cells was assessed as described previously (13). Donor Thy1.1<sup>+</sup> T cells were enriched from pooled spleens of six to seven TCDD-treated F<sub>1</sub> mice on day 2 by magnetic bead sorting; the CD4<sup>+</sup> CD25<sup>+</sup> fraction was further enriched using a MoFlo high-speed cell sorter (DakoCytomation). Pooled spleen cells from three naive B6 mice were sorted into CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, and CD4<sup>+</sup>CD25<sup>−</sup> fractions using a CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> isolation kit (Miltenyi Biotec). The CD<sup>+</sup>CD25<sup>−</sup> cells were irradiated (3000 rad) and used as accessory cells. The CD4<sup>+</sup>CD25<sup>+</sup> cells were labeled with 2 μM CFSE before culturing with anti-CD3 (BD Pharmingen) and accessory cells; donor-derived or naive CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup> T cells in the spleen of TCDD-treated F<sub>1</sub> 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<sup>+</sup> 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<sup>+</sup> T cells following their transfer into F<sub>1</sub> mice (Fig. 1D). When the phenotype of donor CD4<sup>+</sup> cells was examined, treatment with TCDD was associated with a significant increase in the percentage of CD62L<sup>lo</sup> cells (Fig. 1B). By day 2, 80% of the donor CD4<sup>+</sup> cells were CD62L<sup>lo</sup>, 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<sup>+</sup> cells that expressed CD25 (Fig. 1, C and F). On day 2, the percentage of donor CD4<sup>+</sup>CD25<sup>+</sup> cells in TCDD-treated mice was consistently 2-fold greater than the percentage in vehicle-treated mice. The percentage of CD4<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells that were CD62L<sup>lo</sup> (vehicle, 32.3 ± 1.3%; TCDD, 56.5 ± 1.6%; p < 0.0001) and CD25<sup>+</sup> (vehicle, 16.6 ± 0.9%; TCDD, 45.7 ± 1.1%; p < 0.0001). Based on dilution of CFSE, no division of the donor CD4<sup>+</sup> cells was apparent 1 day after transfer into vehicle- or TCDD-treated F<sub>1</sub> mice (data not shown). On day 2, >80% of the donor CD4<sup>+</sup> cells from both treatment groups had undergone one to four cell divisions, with no observable effect of TCDD (Fig. 2A). Fig. 2,
nor T cells expressed AhR, TCDD induced a significant increase in the percentage of donor CD4+ cells expressing CD25+CD62Llow (vehicle = 16.9 ± 1.6; TCDD = 33.0 ± 1.3, p = 0.0001; Fig. 3A, left panel). A concomitant decrease occurred in the CD25+CD62Lhigh population, while the CD25+CD62Llow 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 panel).

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

The CD25+CD62Llow 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+ Treg that have a potent ability to suppress allograft responses (21, 22). Therefore, we examined
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.
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 Treg, since several studies have shown that CD4+CD25+Treg suppress pathogenic T cell responses in GVH disease (21, 22, 28).

In recent years, different studies of Treg have been described that fall broadly into natural and adaptive categories (11). Natural Treg 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 CD4+CD25+ Treg suppressed pathogenic T cell responses in GVH disease (21, 22, 28).

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 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.

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References