Consequences of AhR Activation in Steady-State Dendritic Cells

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Received August 12, 2010; accepted November 11, 2010

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical aryl hydrocarbon receptor (AhR) ligand and a potent immunotoxin. However, the mechanisms underlying TCDD-induced immunomodulation remain to be defined. Dendritic cells are professional antigen-presenting cells that constitutively express the AhR and are sensitive to TCDD-induced AhR activation. We hypothesized that AhR activation alters the differentiation and function of steady-state bone marrow-derived dendritic cells (BMDCs). To test this hypothesis, steady-state BMDCs from C57BL/6 mice were grown in the presence of TCDD or vehicle. TCDD-treated steady-state BMDCs (TCDD-BMDCs) displayed decreased expression of CD11c and CD11a, whereas increasing the frequency of major histocompatibility complex class II, CD86, CD80, and CD54. Similar phenotypic alterations were observed with the AhR ligands 6-formylindolo[3,2-*b*]carbazole and 2-(1H-indole-3'-carbonyl)-thiazole-4-carboxylic acid (ITE). TCDD-BMDCs from AhR^{-/-} mice were refractory to TCDD-induced surface marker alterations, whereas TCDD-BMDCs from AhR^{dbd/dbd} mice displayed similar phenotypic alterations as AhR^{+/+} TCDD-BMDCs. Following lipopolysaccharide (LPS), cytosine-phosphate-guanine (CpG), or Imiquimod stimulation, TCDD-BMDCs secreted less interleukin (IL)-6, tumor necrosis factor- α (TNF- α), IL-10, and IL-12. TCDD also altered NF- κ B family member-binding activity in unstimulated and LPS- or CpG-stimulated steady-state BMDCs. The internalization of the soluble antigens, ovalbumin, and acetylated low-density lipoprotein was decreased, whereas internalization of latex beads was increased in TCDD-BMDCs when compared with vehicle-BMDCs. TCDD-BMDCs displayed increased messenger RNA expression of the regulatory gene IDO2 and following LPS stimulation upregulated IDO1, IDO2, TGF β 1, and TGF β 3 gene expression. Additionally, TCDD-BMDCs increased the generation of CD4⁺ CD25⁺ FoxP3⁺ Tregs *in vitro* in an IDO-dependent fashion. However, TCDD-treated BMDCs did not alter antigen-specific T-cell activation *in vivo*. Overall, TCDD-induced AhR activation alters the differentiation, activation, innate, and immunoregulatory function but not the T cell-activating capacity of steady-state BMDCs.

Key Words: aryl hydrocarbon receptor; steady-state dendritic cells; TCDD; immunotoxicity.

activated AhR translocates into the nucleus where it binds the AhR nuclear translocator (ARNT). Subsequently, in the canonical signaling pathway, the ligand-bound AhR/ARNT heterodimeric complex binds target genes containing dioxin response elements (DREs) and modulates gene expression. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical AhR ligand and a potent environmental toxicant. Low-level TCDD exposure causes immune suppression in a number of animal species and increases susceptibility to infections and cancer (Kerkvliet, 2002). The immunosuppressive effects of TCDD, as well as many other TCDD-like chemicals, are predominantly mediated via the AhR. Within the immune system, TCDD-induced AhR activation affects many cell populations, including antigen-presenting cells (APCs) and CD4⁺ T cells (Funatake *et al.*, 2004; Hauben *et al.*, 2008; Jin *et al.*, 2010; Kerkvliet, 2002; Kerkvliet *et al.*, 2002; Lawrence *et al.*, 2008; Ruby *et al.*, 2005; Vorderstrasse and Kerkvliet, 2001). Recently, the induction of regulatory T cells (Tregs) has been linked to TCDD-induced AhR activation and may underlie the immunosuppressive effects of TCDD (Funatake *et al.*, 2005; Quintana *et al.*, 2008). In contrast, the high-affinity natural AhR ligand 6-formylindolo[3,2-*b*]carbazole (FICZ) has been shown to induce Th17 cells during the course of autoimmune encephalomyelitis in mice (Quintana *et al.*, 2008). However, Kimura *et al.* (2008) demonstrated that in the presence of TGF β both TCDD and FICZ induce FoxP3⁺ Tregs in an AhR-dependent manner. Although TCDD-induced AhR activation has been characterized in T-cell populations, less is known about the role of AhR activation in dendritic cells (DCs).

DCs are professional APCs that function in both the innate and adaptive branches of the immune system. DCs act as sentinels to survey and detect foreign pathogens and can elicit multifarious immune responses. As part of their innate functions, DCs recognize pathogen-associated molecular patterns through pathogen recognition receptors, such as Toll-like receptors (TLRs), and can help clear extracellular pathogens through phagocytosis. Following pathogen recognition, DCs become activated and increase their expression of various accessory molecules, including major

Many environmental pollutants exert their toxic effects via activation of the aryl hydrocarbon receptor (AhR). The ligand-

histocompatibility complex (MHC) class I and class II, CD80, CD86, and CD54. Activated DCs secrete cytokines that tailor the generation of both innate and adaptive immune responses. DCs are considered “professional APCs” based on their constitutive expression of accessory molecules, mobility, and ability to internalize, process, and present antigens to T cells. In unimmunized animals, DCs typically exist as pre-DCs or steady-state DCs (Geissmann *et al.*, 2010; Shortman and Naik, 2007). Pre-DCs do not display a DC phenotype but have the capacity to develop into DCs upon inflammatory or pathogenic encounter (Geissmann *et al.*, 2010; Naik, 2008; Shortman and Naik, 2007). These DCs are referred to as “inflammatory DCs” or “tumor necrosis factor (TNF) and iNOS-producing DCs” (Tip DCs) (Geissmann *et al.*, 2010; Naik, 2008). *In vitro* granulocyte-macrophage colony-stimulating factor (GM-CSF) is used to generate DCs, which model inflammatory/Tip DCs *in vivo* (Kimura *et al.*, 2008; Naik, 2008; Shortman and Naik, 2007; Wu and Liu, 2007; Xu *et al.*, 2007). In contrast, immature steady-state DCs consist of both migratory and lymphoid tissue-resident conventional DC populations (Naik, 2008; Shortman and Naik, 2007). The generation of steady-state DCs *in vitro* is achieved using the growth factor Fms-like tyrosine kinase 3 ligand (Flt3L) and represent DCs residing in peripheral immune tissues (Brasel *et al.*, 2000; Naik *et al.*, 2007; Vremec and Shortman, 2008).

Previously, Vorderstrasse *et al.* reported that splenic CD11c^{high} DC numbers were reduced and their immunophenotypes altered in mice exposed to immunosuppressive doses of TCDD (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2002). Bankoti *et al.* (2010a) further characterized AhR activation in naive DCs, both in the spleen and in the peripheral lymph nodes, and demonstrated that TCDD selectively affected splenic CD11c^{high} CD8 α [−] 33D1⁺ DCs but not the CD11c^{high} CD8 α ⁺ DEC205⁺ DCs. In inflammatory bone marrow-derived dendritic cells (BMDCs) AhR activation induced surface marker alterations, impaired antigen uptake, and enhanced secretion of TNF- α and interleukin (IL)-6, following stimulation with LPS or CpG (Bankoti *et al.*, 2010b). Moreover, TCDD-induced AhR activation in inflammatory DCs caused NF- κ B p65 binding to decrease, whereas upregulating RelB binding (Bankoti *et al.*, 2010b). Surprisingly, TCDD did not alter the ability of inflammatory BMDCs to activate antigen-specific CD4⁺ T cells *in vivo* (Bankoti *et al.*, 2010b).

In this study, we have evaluated the role of AhR activation in steady-state DCs. We hypothesized that similar to inflammatory BMDCs, AhR activation would modulate the phenotype and function of steady-state BMDCs. Phenotypic status, TLR responsiveness, NF- κ B activity, gene expression, antigen uptake, and CD4⁺ T-cell stimulatory capacity were assessed, and steady-state BMDCs from AhR null and AhR DRE-binding-deficient mice were used to mechanistically determine the role of the AhR in steady-state BMDC function. Data from this study advance our understanding of how AhR activation modulates conventional DCs, an effect that undoubtedly

contributes to immune modulation following exposure to AhR ligands.

MATERIALS AND METHODS

Animals. Six- to eight-week-old male and female C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the University of Montana (UM). AhR^{−/−} mice (Schmidt *et al.*, 1996) were a kind gift from Dr Paige Lawrence (University of Rochester Medical Center, Rochester, NY). AhR^{dbd/dbd} mutant mice (Bunger *et al.*, 2008) were generously provided by Dr Chris Bradfield and Dr Ed Glover (University of Wisconsin, Madison, WI). OTII FoxP3^{cGFP} mice were kindly provided by Dr Randolph J. Noelle (Dartmouth Medical School, Lebanon, NH), who originally obtained these mice from Dr Alexander Rudensky (University of Washington School of Medicine, Seattle, WA). All animals were housed and maintained at UM and provided water and chow *ad libitum*. All animal experiments were approved by the UM Institutional Animal Care and Use Committee and adhered to the current National Institutes of Health (NIH) guidelines for animal usage.

Chemicals and reagents. TCDD was purchased from Cambridge Isotope Laboratories (Andover, MA). FICZ was purchased from BIOMOL International (Plymouth Meeting, PA) and ITE was obtained from Tocris Bioscience (Ellisville, MO). All AhR ligands were supplied or suspended in tissue culture grade dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). BMDCs were grown in complete RPMI (cRPMI): 20mM HEPES, 1.5mM sodium pyruvate, 50 μ g/ml gentamicin, and 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific, Waltham, MA). BMDC:T cells were cultured in cRPMI or F10 media: F-10 Nutrient Mixture, 20mM HEPES, 50 μ g/ml gentamicin, and 10% fetal bovine serum (Hyclone). All other media reagents were obtained from Invitrogen (Carlsbad, CA). Whole-chicken ovalbumin (Ova; grade V) and 1-methyl-DL-tryptophan (1-MT) were purchased from Sigma-Aldrich. Ova-peptide, Ova_{323–339}, was obtained from Mimotopes (Clayton, Victoria, Australia). LPS (*Escherichia coli*; 055:B5) was obtained from Sigma-Aldrich, and CpG (ODN 1826; type B) and Imiquimod (R837) were purchased from Invivogen (San Diego, CA).

Generation of steady-state BMDCs. BMDCs were generated using methods as previously described (Brasel *et al.*, 2000). Briefly, bone marrow cells from the tibia and femur were flushed using cRPMI. Progenitor cells were separated from red blood cells and debris by centrifugation using Lympholyte-M (Cedarlane Laboratories, Burlington, NC) and were grown in cRPMI supplemented with 10% conditioned media and 300 ng/ml human Flt3L purchased from PeproTech (Rocky Hill, NJ). The conditioned media was generated by culturing splenocytes from unmanipulated C57Bl/6 mice in cRPMI for 10 days. Levels of IL-6 in the conditioned media were used to standardize each batch and ranged between 2 and 5 ng/ml. On day 5, nonadherent cells were removed, washed, and reseeded in fresh media containing Flt3L, conditioned media, and vehicle or TCDD. On day 10, nonadherent and adherent immature BMDCs were harvested, enumerated, and cell viability assessed using Trypan Blue (Sigma-Aldrich). Unless specified, BMDCs were grown in the presence of 10nM TCDD or vehicle control (0.01% DMSO), treatments that caused no significant effects on BMDC viability (data not shown).

Phenotypic analysis. BMDCs were analyzed by flow cytometry as described previously (Shepherd *et al.*, 2001). Briefly, 1×10^5 to 1×10^6 cells were washed with PBS containing 1% bovine serum albumin and 0.1% NaN₃ and resuspended in immunoglobulin G to block nonspecific binding (Jackson Laboratory). The cells were then stained with fluorochrome-conjugated antibodies for 10 min on ice. Appropriate isotype controls were used in conjunction with the primary antibody staining. The following antibodies were used: CD11c-APC (N418) from Invitrogen; MHC class II-PE (M5/114.15.12), CD80-PE (16-10A1), and CD86-Pacific Blue (GL-1) from Biolegend (San Diego, CA); and CD54-FITC (3E2) and CD11a-PECy7 (2D7)

from BD Pharmingen (San Diego, CA). Samples were analyzed on a FACSAria flow cytometer using BD FACS Diva software, version 4.0 (BD Biosciences, San Jose, CA). Figures were generated using FlowJo, version 8.8.6 (Tree Star Inc., Ashland, OR).

TLR ligand activation and cytokine measurement. Immature BMDCs grown in the presence of TCDD or vehicle were aliquoted into six-well plates (Corning, St Louis, MO), at a density of 1×10^6 cells/ml and stimulated with 1 μ g/ml LPS (Sigma-Aldrich), 0.5 μ M CpG (Invivogen), or 30 μ g/ml Imiquimod (Invivogen) for 24 h. Following stimulation, BMDCs were harvested and analyzed via flow cytometry as described above. Supernatants from individual samples were collected and stored at -20°C until further analysis. TNF- α , IL-6, IL-10, and IL-12p70 cytokine production was measured using ELISAs as per the manufacturer's instructions (BD Biosciences).

NF- κ B activity. Steady-state BMDCs (2×10^6) were seeded into six-well plates and stimulated with LPS (1 μ g/ml) or CpG (0.5 μ M) for 45 min. The cells were then harvested, washed, and nuclear extracts isolated using the Active Motif's Nuclear Extract Kit (Active Motif, Carlsbad, CA). Protein levels from the nuclear extracts were quantified using a bicinchoninic acid protein assay (Pierce, Rockford, IL). The binding activity of NF- κ B family members (p65, p50, p52, and RelB) was measured using the Active Motif TransAM NF- κ B Family kit as per the manufacturer's instructions.

Quantitative real-time reverse transcription-PCR. To determine the levels of gene expression in BMDCs, quantitative real-time reverse transcription (qRT)-PCR studies were conducted as previously described (Bankoti *et al.*, 2010b). BMDCs were harvested on day 10, washed twice in PBS, and resuspended in Trizol (Invitrogen) to isolate RNA. Primers for indoleamine 2,3-dioxygenase 1 (Ido1) and Ido2; transforming growth factor- β 1 (Tgfb1), Tgfb2, and Tgfb3; latent Tgfb-binding protein (Ltbp3); tissue plasminogen activator (Platzer *et al.*, 2009); thrombospondin 1 (Thbs1); aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2); AhR; and Tlr4 were purchased from SA Biosciences (Frederick, MD). Messenger RNA levels were determined using SYBR green in qRT-PCRs (SA Biosciences) on a BIO-RAD IQ 5 Light Cycler (Bio-Rad, Hercules, CA).

Antigen uptake. To assess antigen uptake, Alexa Fluor 488 (AF488)-labeled ova, AF488-labeled acetylated-low-density lipoprotein (LDL) (Invitrogen), or 7–9 μ m FITC-labeled latex beads (Polysciences, Warrington, PA) were added to 1×10^6 BMDCs in six-well plates and incubated at 37°C for 12, 1.5, or 6 h, respectively. Following incubation, the BMDCs were harvested, washed two times, and antigen uptake determined by flow cytometry.

BMDC CD4⁺ T-cell cocultures. CD4⁺ T cells from the spleen and popliteal and brachial lymph nodes of OTII FoxP3^{eGFP} mice were purified to > 75% CD4⁺ using an autoMACS Cell Separator (Miltenyi Biotec Inc., Auburn, CA) and a CD4⁺ T-cell isolation kit according to the manufacturer's instructions (Miltenyi Biotec Inc.). BMDCs were treated with 100 μ M 1-MT on day 9 of culture. On day 10, the BMDCs were harvested, washed, and cultured with ova peptide (2 μ g/ml). After 2.5-h exposure to ova peptide, BMDCs were washed two times and placed with purified CD4⁺ T cells at a 1:5 DC to T cell ratio in cRPMI or F10 media. The cocultures were harvested on day 3, and the frequency of CD4⁺ CD25⁺ FoxP3^{eGFP} T cells was determined by flow cytometry.

T-cell activation. The capacity of BMDCs to activate ova-specific CD4⁺ T cells *in vivo* was assessed using the OTII adoptive transfer model as previously described (Bankoti *et al.*, 2010b). Briefly, CD45.2⁺ BMDCs were generated in the presence of Flt3L and vehicle or TCDD. On day 10, the BMDCs were harvested, washed, and cultured with whole ovalbumin (50 μ g/ml) to load them with antigen. After overnight exposure to ovalbumin, BMDCs were washed two times and then injected (2×10^6) into each of the hind footpads of CD45.1⁺ host mice that had received ova-specific OTII Thy1.1⁺ CD4⁺ T cells (2×10^6) iv 24 h earlier. The popliteal and brachial lymph nodes were harvested 5 days after BMDC injection, and the ova-loaded donor BMDC (CD11c⁺ CD45.2⁺) and OTII T cell (CD4⁺ Thy1.1⁺) populations were analyzed by flow cytometry.

Statistical analysis. Student's *t* tests were used to compare two individual samples and fold changes. One-way ANOVA was used to analyze data with more than two groups, with Bonferroni *post hoc* analysis. *p* Values ≤ 0.05 were considered significant.

RESULTS

AhR Activation Alters Immature Steady-State BMDC Growth and Differentiation

Exogenous Flt3L when added to bone marrow progenitor cultures *in vitro* has been shown to produce immature DCs, which closely resemble steady-state DCs *in vivo* (Brasel *et al.*, 2000; Naik *et al.*, 2007). To assess AhR activation in steady-state DCs, Flt3L-BMDCs were exposed to the prototypical AhR ligand, TCDD. On day 10, the immature (nonadherent) and mature (adherent) BMDC populations were harvested and enumerated. TCDD significantly reduced the number of immature steady-state BMDCs after 10 days in culture (vehicle = $60 \times 10^6 \pm 5.3$; TCDD = $45 \times 10^6 \pm 1.3^*$). However, there was no difference observed in the number of vehicle- and TCDD-treated mature BMDCs (vehicle = $4.8 \times 10^6 \pm 0.5$; TCDD = $3.7 \times 10^6 \pm 0.8$). Steady-state BMDCs were identified using CD11c, the murine DC lineage marker, and were more than 90% CD11c⁺ (Fig. 1). Previous studies have shown that TCDD modulates costimulatory surface marker expression on steady-state splenic DCs in unimmunized mice (Bankoti *et al.*, 2010a; Vorderstrasse *et al.*, 2002). TCDD altered the expression of accessory molecules on steady-state BMDCs, as assessed by flow cytometry (Fig. 1). TCDD decreased the frequency of BMDCs (TCDD-BMDCs) that expressed CD11c and its relative expression when compared with the vehicle-treated controls (vehicle-BMDCs). MHC class II (MHC II) expression was significantly decreased, whereas the percentage of cells expressing MHC II was slightly increased following TCDD exposure (Fig. 1). Furthermore, TCDD decreased the expression and frequency of CD11a (Fig. 1). In contrast, both the expression and frequency of the costimulatory molecules, CD80 and CD86, as well as the adhesion molecule, CD54, were significantly upregulated on TCDD-BMDCs (Fig. 1). To determine if these TCDD-induced effects were concentration dependent, steady-state BMDCs were exposed to 0.1, 1, and 10 nM TCDD. Significant changes in the expression and frequency of CD11c, MHC II, CD86, and CD54 were observed on BMDCs exposed to TCDD concentrations as low as 0.1 nM (Fig. 2). Recently, a number of natural compounds including FIZC and ITE have been shown to bind and activate the AhR with similar affinity as TCDD (Henry *et al.*, 2006; Oberg *et al.*, 2005). To determine if these natural AhR ligands could modulate BMDC differentiation, steady-state BMDCs were exposed to FICZ or ITE. Similar to TCDD, both FICZ and ITE decreased the frequency of steady-state BMDCs expressing CD11c (Fig. 3). Conversely, FICZ and ITE increased the percentage of MHC II, CD86, and CD54 on

FIG. 1. TCDD-induced alteration of steady-state BMDC surface marker expression: steady-state BMDCs were treated with vehicle (solid black line) or 10nM TCDD (dashed line). Isotype staining is represented by gray lines. Immature vehicle- and TCDD-BMDCs were harvested on day 10, and surface marker expression was analyzed by flow cytometry. MHC class II, CD86, CD80, CD54, and CD11a expression was characterized on CD11c⁺ BMDCs. Data are represented as histograms showing surface marker percentage and mean fluorescent intensity (MFI) values with corresponding SEM values and is representative of three separate experiments with $n = 3-6$. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

steady-state CD11c⁺ BMDCs when compared with vehicle-treated controls (Fig. 3).

TCDD-Induced Phenotypic Alterations are AhR Dependent but Not Exclusively DRE Mediated

TCDD-induced phenotypic alterations were previously shown to be AhR dependent in inflammatory BMDCs and steady-state splenic DCs (Bankoti *et al.*, 2010a,b; Lee *et al.*, 2007; Vorderstrasse and Kerkvliet, 2001). As expected, steady-state BMDCs derived from AhR^{-/-} mice were insensitive to the effects of TCDD (Table 1). The frequency of BMDCs expressing CD11c, MHC II, CD86, and CD54 remained unchanged, as did the relative expression of CD11c, MHC II, CD86, and CD54 on AhR^{-/-} TCDD-BMDCs when compared with AhR^{-/-} vehicle-BMDCs (Table 1). On the other hand, BMDCs derived from AhR^{dbd/dbd} mice, which lack the ability to bind DRE sequences in AhR target genes, were not insensitive to TCDD when compared with the AhR^{-/-}

BMDCs (Table 1). AhR^{dbd/dbd} TCDD-BMDCs displayed an increased frequency and relative expression of CD11c (Table 1). Similar to the effects observed in TCDD-BMDCs from AhR^{+/+} mice, the frequency and relative expression of MHC II, CD86, and CD54 on AhR^{dbd/dbd} TCDD-BMDCs were increased when compared with AhR^{dbd/dbd} vehicle-BMDCs.

TCDD Disrupts the TLR Responsiveness of Steady-State BMDCs

Innate immune responses can be initiated via stimulation of TLRs, an event that leads to DC maturation and activation. Subsequently, activated DCs produce inflammatory cytokines that contribute to the development of tailored immune responses. To determine if TCDD-induced AhR activation disrupts steady-state DC activation and/or cytokine production, immature steady-state BMDCs treated with TCDD or vehicle were stimulated for 24 h with three TLR agonists: LPS (TLR4), CPG (TLR9), or Imiquimod (TLR7). As expected, TLR activation of immature BMDCs induced increased expression of CD11c, MHC II, and CD86 when compared with unstimulated BMDCs (Table 2). It should be noted that after growth in cRPMI supplemented with conditioned media and Flt3L, TCDD-BMDCs placed in cRPMI for 24 h without growth factors experienced changes in the expression of CD11c and MHC II when compared with the unstimulated vehicle-treated BMDCs (Table 2). TCDD-BMDCs stimulated with LPS, CpG, or Imiquimod displayed decreased levels of CD11c (Table 2). In contrast to CD11c, TCDD-BMDCs expressed higher levels of MHC II, CD86, and CD54 (Table 2). In addition, TLR-stimulated TCDD-BMDCs displayed increased frequencies of MHC II, CD86, and CD54 when compared with the TLR-stimulated vehicle-BMDCs. Furthermore, the production of IL-6, TNF- α , IL-12, and IL-10 was assessed following TLR stimulation to determine if TCDD disrupts cytokine production by steady-state BMDCs. Small but detectable amounts of IL-12 were produced by unstimulated BMDCs, whereas IL-6, TNF- α , and IL-10 levels were undetectable (Fig. 4). Following LPS or CpG stimulation, steady-state BMDCs secreted IL-6, TNF- α , IL-10, and IL-12 (Fig. 4). Only IL-6 and IL-12 were detectable following stimulation with Imiquimod (Fig. 4). BMDCs treated with 10nM TCDD produced lower levels of IL-6, TNF- α , IL-10, and IL-12 following stimulation with LPS or CpG, whereas no significant changes were seen in response to Imiquimod at 10nM TCDD (Fig. 4). Following LPS stimulation, BMDC production of IL-6, TNF- α , and IL-10 was decreased with concentrations of TCDD as low as 1nM, whereas IL-12 production decreased following exposure to concentrations of TCDD as low as 0.1nM (Fig. 4). Following Imiquimod stimulation, 0.1nM TCDD-exposed BMDCs secreted less IL-12 when compared with Imiquimod-stimulated vehicle-BMDCs. Similar effects were observed following stimulation with CpG (Fig. 4). Activated DCs also produce nitric oxide (NO); however, no differences were observed in the secretion

FIG. 2. TCDD-induced surface marker alterations are concentration dependent: steady-state BMDCs were generated in the presence of vehicle control or varying concentrations of TCDD (0.1, 1.0, and 10nM). CD11c⁺ BMDCs were harvested on day 10 and stained for CD11c, MHC class II, CD86, and CD54 expression as described in the “Materials and Methods” section. (A) Frequency (%) and (B) mean fluorescent intensity (MFI) values with corresponding SEM. Data are representative of three independent experiments with $n = 4$. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

of NO between vehicle-BMDCs and TCDD-BMDCs following stimulation with LPS, CpG, or Imiquimod (data not shown).

NF- κ B Activity Is Altered in TCDD-BMDCs

NF- κ B signaling pathways are initiated following TLR ligation in DCs and subsequently mediate DC activation. Recently, it has been demonstrated that the AhR can directly interact with multiple NF- κ B family members, including RelA,

RelB, and p52 (Ruby *et al.*, 2002; Vogel *et al.*, 2007a,b). Therefore, we measured the effects of NF- κ B activity in steady-state BMDCs. Following stimulation with LPS or CpG, the activity of p65, p52, and p50 was increased, whereas RelB activity remained constant when compared with vehicle-BMDCs (Fig. 5). In unstimulated TCDD-BMDCs, RelB binding was increased, whereas p65 and p52 levels were unchanged when compared with unstimulated vehicle-BMDCs (Fig. 5). In contrast, p50 activity was decreased in unstimulated TCDD-BMDCs. Following LPS stimulation, TCDD-BMDCs

FIG. 3. Natural AhR ligands alter BMDC accessory molecules: BMDCs were treated with vehicle, (A) 10nM FICZ, or (B) 10nM ITE and harvested on day 10. CD11c, MHC II, CD86, and CD54 expression was evaluated on CD11c⁺ BMDCs. (A) Data are representative of two independent experiments with $n = 4$ –6 and (B) represents one experiment with $n = 4$. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

TABLE 1
The Effects of TCDD on Accessory Molecule Expression on BMDCs from AhR^{-/-} and AhR^{dbd/dbd} Mice^a

		AhR ^{-/-}		AhR ^{dbd/dbd}	
		Vehicle-BMDCs	TCDD-BMDCs	Vehicle-BMDCs	TCDD-BMDCs
CD11c	Percentage	95.9 ± 0.3	95.6 ± 0.1	90.1 ± 0.3	92.1 ± 0.3*
	MFI	3059 ± 91	3026 ± 28	6040 ± 123	6354 ± 80*
MHC II	Percentage	94.3 ± 0.2	95.0 ± 0.3	89.7 ± 0.3	91.6 ± 0.4*
	MFI	9994 ± 258	9764 ± 194	23910 ± 552	25660 ± 398*
CD54	Percentage	5.5 ± 0.1	5.4 ± 0.1	14.9 ± 0.3	22.6 ± 0.6*
	MFI	258 ± 1.9	258 ± 4.0	306 ± 1.1	328 ± 3.0*
CD86	Percentage	50.8 ± 0.4	50.2 ± 0.5	48.4 ± 0.5	55.1 ± 0.4*
	MFI	187 ± 0.6	184 ± 0.9	398 ± 1.4	444 ± 1.6*

Note. MFI, mean fluorescent intensity.

^aExpression of accessory molecules on vehicle- and TCDD-treated steady-state BMDCs derived from AhR^{-/-} or AhR^{dbd/dbd} mice. BMDCs were generated in vehicle or 10nM TCDD as described in the “Materials and Methods” section. BMDCs were harvested on day 10 and CD11c, MHC II, CD86, and CD54 expression evaluated by flow cytometry. AhR^{-/-} data are representative of two experiments with $n = 3-4$, and AhR^{dbd/dbd} data represent one experiment with $n = 5$. *Indicates $p \leq 0.05$ when compared with the representative vehicle-treated controls.

displayed increases in RelB and p52 activity and decreases p65 and p50 activity when compared with LPS-stimulated vehicle-BMDCs. RelB binding was unchanged following CpG stimulation in TCDD-BMDCs, whereas p52 binding increased (Fig. 5). Furthermore, the binding levels of both p65 and p50 were reduced in TCDD-BMDCs following stimulation with CpG when compared with CpG-stimulated vehicle-BMDCs.

AhR Activation Increases Regulatory Gene Expression in Steady-State BMDCs

Inflammatory BMDCs exposed to TCDD increase their expression of various immune regulatory genes, such as TGFβ and IDO (Bankoti *et al.*, 2010b; Vogel *et al.*, 2008). Therefore, the expression of key immunoregulatory genes was measured in steady-state BMDCs following TCDD exposure. Although there were no significant fold changes in the expression of Ltbp, Ahr, Thsp1, or TLR4, IDO2 was significantly upregulated in unstimulated TCDD-BMDCs when compared with vehicle-BMDCs (Table 3). In contrast, TCDD downregulated TGFβ2 and Aldh1a2 expression in unstimulated steady-state BMDCs when compared with vehicle-BMDCs. To determine if TCDD altered the expression of regulatory genes in activated BMDCs, gene expression was measured in vehicle-BMDCs and TCDD-BMDCs stimulated with LPS (Table 3). TCDD-BMDCs upregulated IDO1, IDO2, TGFβ1, and TGFβ3 expression following LPS activation, whereas tissue plasminogen activator expression was downregulated (Table 3).

TCDD Modulates Antigen Uptake by Steady-State BMDCs

Following recognition of antigens, such as ovalbumin, LDL, and latex beads, DCs internalize these compounds via macropinocytosis, receptor-mediated endocytosis, or phago-

cytosis, respectively. TCDD disrupts antigen uptake by splenic DCs and inflammatory BMDCs (Bankoti *et al.*, 2010b; Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2002). To assess whether antigen uptake by steady-state BMDCs is affected by TCDD, BMDCs were exposed to Ova, acetylated LDL, or latex beads, and internalization was determined by flow cytometry. On a per cell basis, TCDD-BMDCs internalized less Ova and LDL antigen while increasing the uptake of latex beads when compared with antigen-exposed vehicle-BMDCs (Fig. 6). The overall frequency of Ova-positive and latex bead-positive BMDCs was increased in BMDCs exposed to TCDD (Fig. 6). The frequency of LDL-positive BMDCs was unchanged between vehicle-BMDCs and TCDD-BMDCs (Fig. 6).

TCDD-Treated BMDCs Increase the Frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs In Vitro

AhR activation has recently been linked to the generation of Tregs in both graft versus host and autoimmune disease settings (Funatake *et al.*, 2005; Hauben *et al.*, 2008; Quintana *et al.*, 2008). To determine whether AhR-activated steady-state BMDCs can alter or induce Tregs *in vitro*, antigen-specific CD4⁺ OTII T cells from mice expressing FoxP3^{eGFP} were utilized. Following 3 days in culture, Ova peptide-loaded TCDD-treated BMDCs increased the frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs ~15% when compared with Ova peptide-loaded vehicle-treated BMDCs (Fig. 7A). A 23% increase in CD4⁺ CD25⁺ FoxP3⁺ Tregs with TCDD-treated BMDCs was also observed when BMDC:T cells were cultured in F10 media that contains lower levels of tryptophan (Fig. 7B). To test whether the observed increases in IDO gene expression in TCDD-treated BMDCs correlated with the increased frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs, vehicle- and TCDD-treated BMDCs were exposed to 1-MT,

TABLE 2

Effects of TCDD on the Activation of Steady-State BMDCs via TLR Stimulation^a

	% CD11c		CD11c MFI	
	Vehicle	TCDD	Vehicle	TCDD
Unstimulated	89.8 ± 0.1	89.1 ± 1.0	4213 ± 51.7	3570 ± 146*
LPS	95.9 ± 0.1†	95.0 ± 0.4†*	6416 ± 140†	4268 ± 184*
CpG	91.9 ± 0.3	90.9 ± 0.3*	5210 ± 61.2†	3839 ± 75.3*
Imiquimod	93.6 ± 0.2†	91.3 ± 0.2*	3405 ± 28.8†	3142 ± 84.0*
	% MHC II		MHC II MFI	
	Vehicle	TCDD	Vehicle	TCDD
Unstimulated	94.7 ± 0.6	96.7 ± 0.2*	9157 ± 378	9901 ± 534
LPS	96.6 ± 0.2	97.8 ± 0.1*	23030 ± 1394†	22760 ± 851†
CpG	97.8 ± 0.2†	98.1 ± 0.1†	18610 ± 424†	26070 ± 1444†*
Imiquimod	94.6 ± 0.4	97.1 ± 0.1*	13030 ± 554†	20560 ± 864†*
	% CD86		CD86 MFI	
	Vehicle	TCDD	Vehicle	TCDD
Unstimulated	16.3 ± 0.8	32.1 ± 0.7*	306.3 ± 2.3	314.0 ± 2.2*
LPS	80.2 ± 0.8†	89.6 ± 0.4†*	970.0 ± 17.3†	1538 ± 53.7†*
CpG	61.7 ± 1.2†	80.2 ± 0.6†*	635.0 ± 5.6†	929.8 ± 24.1†*
Imiquimod	43.6 ± 0.7†	70.5 ± 0.8†*	805.8 ± 14.3†	1048 ± 29.8†*
	% CD54		CD54 MFI	
	Vehicle	TCDD	Vehicle	TCDD
Unstimulated	33.1 ± 0.2	46.2 ± 0.6*	260.8 ± 2.3	297.8 ± 2.4*
LPS	88.8 ± 0.4†	89.3 ± 0.1†*	887.8 ± 13.8†	988.0 ± 21.3†*
CpG	72.9 ± 0.9†	83.1 ± 0.4†*	665.3 ± 19.7†	901.3 ± 10.3†*
Imiquimod	29.6 ± 0.8	55.8 ± 0.8†*	370.0 ± 4.1†	524.0 ± 6.2†*

^aBMDCs treated with vehicle or 10nM TCDD were stimulated with LPS (1 µg/ml), CpG (0.5µM), or Imiquimod (30 µg/ml) for 24 h, harvested and CD11c, MHC II, CD86, and CD54 expression determined by flow cytometry. Mean values of frequency (%) and mean fluorescent intensity (MFI) are displayed with corresponding SEM. Data are representative of two independent experiments with $n = 4-6$ per treatment group. *Indicates $p \leq 0.05$ between vehicle- and TCDD-treated samples; † indicates $p \leq 0.05$ between unstimulated and LPS-, CpG-, or Imiquimod-stimulated samples.

a tryptophan analog, and IDO enzyme inhibitor (Mellor and Munn, 2004). 1-MT pretreatment blocked the observed increase in CD4⁺ CD25⁺ FoxP3⁺ Tregs in TCDD-treated BMDC:CD4⁺ T-cell cocultures in both the conventional and tryptophan-low media (Fig. 7).

Steady-State TCDD-BMDCs Initiate Successful

Antigen-Specific CD4⁺ T-Cell Activation In Vivo

To determine if TCDD-induced AhR activation alters the ability of steady-state DCs to activate CD4⁺ T cells *in vivo*, we utilized the OTII adoptive transfer model. CD45.1⁺/Thy1.2⁺ host mice were seeded with CD4⁺/Thy1.1⁺ OTII T cells and injected with Ova-loaded CD45.2⁺ vehicle- or TCDD-

BMDCs. Distal brachial lymph nodes and proximal draining popliteal lymph nodes were harvested 4 days after DC immunization. Donor DCs and OTII T cells were identified from host immune cells based on dual expression of CD11c/CD45.2 and CD4/Thy1.1, respectively. Overall, no significant differences were observed in the percent or number of vehicle- or TCDD-treated donor DCs in the draining lymph nodes (Fig. 8A). TCDD-treated donor DCs displayed an increase in CD86 expression, whereas MHC II expression was unchanged when compared with vehicle-treated donor DCs (data not shown). The percentage and overall number of CD4⁺/Thy1.1⁺ OTII T cells was significantly increased in the popliteal lymph node when compared with the brachial lymph node, demonstrating expansion of Ova-specific T cells in response to the injection of Ova-loaded BMDCs (Fig. 8B). However, no differences in the percent or number of OTII⁺ donor T cells in the draining lymph nodes were detected following the transfer of TCDD-BMDCs when compared with the vehicle-BMDCs. Furthermore, no differences were observed in the activation of donor T cells between mice immunized with vehicle-BMDCs or TCDD-BMDCs as assessed by CD62L and CD44 expression (data not shown).

DISCUSSION

DCs are integral to the function of both the innate and adaptive branches of the immune system. The generation of an immune response, whether stimulatory or tolerogenic, is largely dependent on DC differentiation, activation, and maturation (Banchereau *et al.*, 2000). TCDD-induced AhR activation has been shown to disrupt DC differentiation (Bankoti *et al.*, 2010a,b; Lee *et al.*, 2007; Vorderstrasse and Kerkvliet, 2001). Both naive splenic DCs and inflammatory (GM-CSF-derived) BMDCs display altered surface molecule expression following TCDD-induced AhR activation (Bankoti *et al.*, 2010a,b; Lee *et al.*, 2007; Ruby *et al.*, 2005; Vorderstrasse and Kerkvliet, 2001). Similar to these studies, our data show that AhR activation modulates the differentiation of Flt3L-derived steady-state BMDCs. DCs constitutively express the murine DC lineage marker CD11c and MHC class II, whereas costimulation molecule expression including CD80, CD86, and CD54 is lower but present on immature DCs. However, growth in TCDD altered the differentiation of steady-state BMDCs causing immature BMDCs to decrease their expression of CD11c, MHC class II, and CD11a and upregulate CD80, CD86, and CD54 costimulation molecule expression in the absence of a maturation signal. Overall, TCDD altered steady-state BMDC differentiation and induced a unique phenotype indicative of partially matured DCs. Some of the TCDD-induced changes observed in steady-state BMDCs such as the expression of cell surface molecules and immunoregulatory genes were similar to those in inflammatory BMDCs, whereas

A LPS-Stimulated BMDCs **B CpG-Stimulated BMDCs** **C Imiquimod-Stimulated BMDCs**

FIG. 4. TCDD decreases BMDC cytokine production following TLR stimulation: Vehicle- and TCDD-BMDCs were harvested on day 10 and stimulated for 24 h with (A) LPS (1 μ g/ml) or (B) CpG (0.5 μ M) or (C) Imiquimod (30 μ g/ml) or unstimulated. Cells were harvested, supernatants collected, and IL-6, TNF- α , IL-12p70, and IL-10 levels determined by ELISA. B.D. denotes below detection. Data are representative of two separate experiments with $n = 4-6$. $*p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

FIG. 5. TCDD alters NF- κ B activity: Vehicle- and TCDD-treated BMDCs were stimulated with LPS (1 μ g/ml) or CpG (0.5 μ M) for 45 min. Following stimulation, BMDCs were harvested, nuclear lysates isolated, and RelB-, p65-, p52-, and p50-binding activity determined as described in the “Materials and Methods” section. Data represent mean $\pm$ SEM of four samples per treatment group. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups; # indicates significance between unstimulated and LPS- or CpG-stimulated samples, respectively.

others such as LPS-activated cytokine production differed significantly (Table 4). These effects may highlight the biological functions of both steady-state DCs and inflammatory DCs as mediators of T-cell immunity and inflammatory responses, respectively. Moreover, altered BMDC differentiation caused by AhR activation may be relevant to human exposures as AhR activation by B[a]P has been shown to disrupt human monocyte-derived DC differentiation and maturation *in vitro* (Laupeze *et al.*, 2002). Furthermore, AhR activation by the anti-allergenic compound, VAF347, has recently been shown to upregulate AhR target gene expression in human monocyte-derived DCs and alters the differentiation of human myeloid progenitor cells and Langerhan cells (Lawrence *et al.*, 2008; Platzer *et al.*, 2009).

Two natural AhR ligands, FICZ, and ITE, which have recently been shown to affect immune cell populations and modulate specific immune responses, were evaluated for their ability to alter steady-state BMDC differentiation (Bankoti *et al.*, 2010b; Henry *et al.*, 2006; Kimura *et al.*, 2008; Quintana *et al.*, 2008; Vogel *et al.*, 2008). FIZC and ITE induced similar phenotypic alterations in steady-state BMDCs when compared with TCDD-BMDCs and vehicle controls. This suggests that high-affinity exogenous ligands (TCDD) and high-affinity endogenous ligands (FICZ and ITE) can act similarly to alter the differentiation of steady-state BMDCs.

A number of studies have demonstrated that immunomodulation by TCDD and TCDD-like chemicals is dependent on the AhR and AhR signaling events (Bankoti *et al.*, 2010b; Jin *et al.*,

TABLE 3
Vehicle- and TCDD-BMDC Regulatory Gene Expression^a

	Unstimulated BMDCs		LPS-stimulated BMDCs	
	Fold change	<i>p</i> Value	Fold change	<i>p</i> Value
<i>Ido1</i>	−1.18	0.346	2.37*	0.005
<i>Ido2</i>	6.55*	0.0008	3.62*	0.002
<i>TGFβ1</i>	1.1	0.873	1.65*	0.006
<i>TGFβ2</i>	−2.72*	0.011	−1.25	0.322
<i>TGFβ3</i>	1.51	0.194	2.37*	0.013
<i>Plat</i>	−1.49	0.356	−2.82*	0.007
<i>Ltbp</i>	−1.05	0.863	−1.22	0.639
<i>Ahr</i>	−2.26	0.211	1.03	0.833
<i>Thsp1</i>	−1.12	0.69	1.01	0.981
<i>Aldh1a2</i>	−3.68	0.01	2.69	0.059
<i>Tlr4</i>	−3.95*	0.199	1.49	0.150

^aRegulatory gene expression in unstimulated or LPS-stimulated BMDCs was determined by qRT-PCR. Gene expression values represent the fold change between vehicle and TCDD treatment from two separate experiments with $n = 4$ per treatment group. *Indicates statistically significant ($p \leq 0.05$) fold change between vehicle-BMDCs and TCDD-BMDCs.

FIG. 6. TCDD alters antigen uptake by steady-state BMDCs: AF488-Ova, AF488-LDL, and FITC-latex beads were utilized to assess antigen uptake by BMDCs. Vehicle- and TCDD-BMDCs were incubated with LDL (1.5 h), latex beads (6 h), or Ova (12 h). Antigen uptake was assessed via flow cytometry as described in the “Materials and Methods” section. (A) Histograms displaying isotype staining (gray lines), Vehicle-BMDC antigen uptake (solid black lines) and TCDD-BMDC antigen uptake (dashed black lines) with corresponding mean fluorescence intensity (MFI) values ± SEM. (B) Frequency (%) of antigen uptake. Data are representative of three independent experiments with $n = 3-4$. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

2010; Kerkvliet *et al.*, 2002; Lee *et al.*, 2007; Vorderstrasse and Kerkvliet, 2001). Steady-state BMDCs derived from $AhR^{-/-}$ mice were refractory to TCDD-induced phenotypic alterations. To determine whether canonical AhR signaling was involved, we investigated the role of DRE binding using steady-state BMDCs derived from $AhR^{dbd/dbd}$ mice. $AhR^{dbd/dbd}$ steady-state BMDCs displayed many similar phenotypic changes following exposure to TCDD when compared with $AhR^{+/+}$ steady-state TCDD-BMDCs. These results indicate that canonical signaling

via DREs is not required for TCDD to alter the differentiation of steady-state DCs and suggests that noncanonical AhR signaling may mediate the observed TCDD-induced phenotypic alterations. In inflammatory BMDCs, Bankoti *et al.* (2010b) also demonstrated that the effects of TCDD were similar between $AhR^{dbd/dbd}$ and $AhR^{+/+}$ TCDD-BMDCs, suggesting that non-canonical AhR signaling mediates TCDD-induced defects in both inflammatory and steady-state DCs. Several possible alternatives exist to explain these results, such as the propensity

FIG. 7. TCDD-treated BMDCs increase the frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs in an IDO-dependent manner: Vehicle- or TCDD-BMDCs were treated with 1-MT 24 h preharvest. Both 1-MT treated and untreated BMDCs were loaded with Ova peptide, washed twice, and seeded with purified Ova-specific CD4⁺ T cells from OTII mice expressing FoxP3^{GFP} in 96-well plates at a 1:5 DC to T cell ratio. The cultures were harvested on day 3 and the frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs cultured in (A) cRPMI and (B) F10 media were determined. Data are representative of one experiment with $n = 4$. * $p \leq 0.05$ indicates significant differences between vehicle- and TCDD-treated groups.

FIG. 8. *In vivo* activation of Ova-specific $CD4^+$ OTII T cells by Ova-loaded BMDCs: OTII $^+$ $CD4^+$ $Thy1.1^+$ T cells were adoptively transferred into congenic ($CD45.1^+$) host mice on day 1 relative to immunization. On day 0, adoptively transferred mice were immunized with vehicle- or TCDD-BMDCs loaded with whole ovalbumin. On day 4 after immunization, popliteal and brachial lymph nodes were harvested from host mice and analyzed by flow cytometry. The frequency and numbers of (A) donor DCs ($CD45.2^+/CD11c^+$) from the popliteal lymph nodes and (B) Ova-specific OTII T cells ($CD4^+/Thy1.1^+$) from the popliteal and brachial lymph nodes were determined. Data are representative of one experiment utilizing seven animals per treatment group.

of the activated AhR to physically interact with NF- κ B family members or possibly STAT proteins (Kimura *et al.*, 2008; Ruby *et al.*, 2002; Vogel *et al.*, 2007a). However, the possibility that activated AhR physically interacts with NF- κ B or STAT proteins in steady-state BMDCs remains to be examined.

As a part of the innate immune system, DCs become activated following pathogenic encounter increasing their expression of various surface molecules and secreting multiple cytokines (Banchereau *et al.*, 2000). Studies have demonstrated that TCDD-induced AhR activation can alter the expression of CD11c, MHC class II, and CD86 and cytokine production following TLR stimulation in inflammatory BMDCs (Bankoti *et al.*, 2010b; Lee *et al.*, 2007). Following stimulation with

LPS (TLR4) or CpG (TLR9), $CD11c^+$ inflammatory TCDD-BMDCs increased their expression of MHC class II and secreted more IL-6 and TNF- α , without affecting their production of IL-10 or IL-12 (Bankoti *et al.*, 2010b). Furthermore, Lee *et al.* (2007) demonstrated that inflammatory BMDCs derived in the presence of GM-CSF, IL-4, and TCDD downregulated CD11c expression and upregulated MHC class II and CD86 expression following LPS stimulation. Similar to these studies, following stimulation with LPS or CpG, TCDD-treated steady-state BMDCs displayed altered surface marker expression. Moreover, TCDD-treated steady-state BMDCs produced less IL-6, TNF- α , IL-10, and IL-12 following stimulation. This is in contrast to TCDD-treated

TABLE 4
Comparison of TCDD-Treated Steady-State and Inflammatory Murine BMDCs^a

	Steady-state BMDCs	Inflammatory BMDCs
Surface molecule expression ^b	↓CD11c, ↓MHC II, ↑CD86, ↑CD54, ↓CD11a	↓CD11c, ↑MHC II, ↑CD86, ↑CD54, ↓CD11a
Cytokine production ^c	↓TNF- α , ↓IL-6, ↓IL-12, ↓IL-10	↑TNF- α , ↑IL-6, n.s. IL-12, n.s. IL-10
NF- κ B activity	↓p65, ↑RelB	↓p65, n.s. RelB
Antigen uptake	↑Ova, n.s. LDL, ↑particulate	↓Ova, ↓LDL, ↑particulate
Immunoregulatory gene expression	n.s. IDO1, ↑IDO2, n.s. TGF β 3	↑IDO1, ↑IDO2, ↑TGF β 3
Treg generation capacity (<i>in vitro</i>)	↑CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Tregs	↑CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Treg ^d
Antigen-specific T-cell activation (<i>in vivo</i>)	No effects on OTII CD4 ⁺ T cells	No effects on OTII CD4 ⁺ T cells

Note. ↑, increase; ↓, decrease; n.s., not significant.

^aComparison of TCDD-induced alterations in steady-state and inflammatory murine BMDCs.

^bSurface molecule expression from unstimulated BMDCs as reported by Bankoti *et al.* (2010b) and Lee *et al.* (2007).

^cBMDCs stimulated with LPS for 24 h (cytokine production) or 30–45 min (NF- κ B activity).

^dUnpublished results.

inflammatory BMDCs that secreted more IL-6 and TNF- α following LPS or CpG stimulation (Bankoti *et al.*, 2010b). The differences in cytokine production between AhR-activated inflammatory and steady-state BMDCs is likely due to the fact that they model different DC populations *in vivo*. It is interesting that the levels of each cytokine measured, IL-6, TNF- α , IL-12, and IL-10, were reduced by stimulated TCDD-treated steady-state BMDCs, suggesting that AhR activation may disrupt DC maturation. However, the observed increased expression of MHC class II, CD86, and CD54 following stimulation suggests that AhR activation may differentially affect steady-state DC maturation by increasing costimulatory molecule expression, whereas decreasing the production of key cytokines. The molecular underpinnings and biological significance of such effects remain to be determined.

The altered expression of costimulatory molecules and decreased cytokine production in TCDD-treated steady-state BMDCs following innate recognition of pathogenic components may be due to noncanonical AhR signaling. Recently, a number of noncanonical AhR signaling pathways have been described that include interactions between activated AhR and the NF- κ B family member RelB (Kimura *et al.*, 2008; Vogel *et al.*, 2007a). Vogel *et al.* (2007b) showed that production of the chemokines, BAFF, BLC, and CCL1, from TCDD-treated U937 macrophages was dependent on the expression of both the AhR and RelB and controlled by AhR/RelB complexes binding to promoter elements upstream of the BAFF, BLC, and CCL1 genes. Ruby *et al.* (2002) demonstrated that stimulation of TCDD-exposed DC2.4 cells with TNF- α or anti-CD40 induced a physical association between the AhR and p65, effectively blocking p65 activity. To characterize AhR/NF- κ B interactions in nontransformed DCs, Lee *et al.* (2007) used inflammatory BMDCs and demonstrated that following LPS stimulation TCDD-treated BMDCs reduce RelB gene expression. However, Bankoti *et al.* (2010b) showed that there was an increasing trend of RelB activity in TCDD-treated inflammatory BMDCs following LPS or CpG stimulation, whereas p65

activity was significantly reduced). In unstimulated and stimulated steady-state BMDCs treated with TCDD, NF- κ B RelB, p65, p50, and p52 activity was altered. These data suggest that in steady-state BMDCs activated AhR may interact with RelB or p52 to increase DNA binding or alternatively interact with p65 or p50 to block target gene binding. It is currently unknown if activated AhR physically interacts with NF- κ B family members to alter target gene binding in steady-state BMDCs or conventional DCs *in vivo*.

DCs contribute to innate immune responses by clearing pathogens via phagocytosis. Two previous studies have evaluated the ability of DCs to internalize antigens following AhR activation (Bankoti *et al.*, 2010b; Vorderstrasse *et al.*, 2002). In the first study, splenic DCs isolated following TCDD exposure showed no difference in their ability to internalize FITC-labeled latex beads (Bankoti *et al.*, 2010b; Vorderstrasse *et al.*, 2002). In the second study, inflammatory BMDCs exposed to TCDD displayed decreases in AF488-labeled Ova and LDL antigen uptake, whereas increasing FITC-labeled latex bead uptake (Bankoti *et al.*, 2010b; Vorderstrasse *et al.*, 2002). In this study, TCDD altered the ability of steady-state BMDCs to internalize both soluble and particulate antigens. The inconsistencies in these results may be due to differences in the type of DCs (i.e., *ex vivo* conventional DCs vs. *in vitro*-derived inflammatory or steady-state BMDCs). Nonetheless, defects in antigen uptake following AhR activation in DCs could affect their innate clearance of pathogens and potentially alter antigen processing and presentation and subsequent T cell-mediated immune responses.

Altering DC differentiation and antigen uptake following AhR activation may affect the functional capacity of DCs to induce appropriate antigen-specific T-cell responses (Lee *et al.*, 2007; Vorderstrasse *et al.*, 2002). T-cell activation not only requires TCR stimulation through antigen recognition in the context of MHC molecules but also costimulation through interactions between CD86/CD80 and CD28. AhR activation in steady-state BMDCs decreases MHC class II expression,

whereas increasing CD86 and CD80 expression, which could affect T-cell activation. Recently, increases in CD86 on inflammatory BMDCs have been attributed to increased T-cell activation in mixed lymphocyte reactions (Lee *et al.*, 2007). However, interactions between CD86/CD80 expressed on the DC and CTLA4 on T cells has been shown to inhibit T-cell activation and may induce regulatory T cells (Funatake *et al.*, 2005; Lane, 1997). Furthermore, decreased expression of the adhesion molecule CD11a following AhR activation could affect interactions between T cells and antigen-bearing DCs, leading to suboptimal T-cell stimulation. In contrast to inflammatory BMDCs, which display decreased levels of both CD11a and CD54 following TCDD-induced AhR activation, the increased expression of CD54 on steady-state TCDD-BMDCs could enhance T-cell adhesion (Bankoti *et al.*, 2010b). However, the TCDD-induced alterations of surface molecules on steady-state BMDCs were modest, and thus, it remains to be determined if these changes are biologically significant.

The induction of tolerance or regulation of immune responses by DCs is dependent on a variety of mediators including TGF β , IDO, as well as other essential amino acid-metabolizing enzymes and retinoic acid (Cobbold *et al.*, 2010; Sharma *et al.*, 2007; Yamazaki and Steinman, 2009). Steady-state BMDCs treated with TCDD upregulated the expression of IDO2 approximately sevenfold when compared with vehicle-treated steady-state BMDCs. Following LPS stimulation, steady-state BMDCs treated with TCDD upregulated expression of IDO1, IDO2, TGF β 1, and TGF β 3. The TCDD-induced induction of IDO2 suggests that TCDD induces a regulatory phenotype in steady-state BMDCs. However, TCDD-treated inflammatory BMDCs displayed greater than 10-fold increases in IDO1, IDO2, and TGF β 3 (Bankoti *et al.*, 2010b). The differences in regulatory gene expression between these two DC populations may be because these BMDCs represent separate DC populations *in vivo* (Geissmann *et al.*, 2010; Naik, 2008; Shortman and Naik, 2007). Inflammatory DCs are hyperresponsive immune cells and play an integral role in the regulation of inflammation. On the other hand, steady-state BMDCs represent conventional DC populations that are more likely to be refractory to rapid environmental changes, which may account for their overall lower regulatory gene expression when compared with TCDD-treated inflammatory BMDCs.

The regulatory capacity of TCDD-treated steady-state BMDCs is illustrated by their ability to increase the frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs *in vitro*. Furthermore, the increased generation of CD4⁺ CD25⁺ FoxP3⁺ Tregs was IDO-dependent, as 1-MT-treated AhR-activated BMDCs did not generate an increased frequency of CD4⁺ CD25⁺ FoxP3⁺ Tregs. These data demonstrate the functional capacity of IDO, which is induced following AhR activation in steady-state BMDCs, to contribute to the generation of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells. It has yet to be determined if AhR-activated steady-state BMDCs under suboptimal T-cell stim-

ulatory conditions induce CD4⁺ CD25⁺ FoxP3⁺ Tregs or other types of regulatory T cell, such as Tr1 cells, *in vivo*.

In contrast to our *in vitro* results, TCDD-treated steady-state BMDCs failed to suppress antigen-specific CD4⁺ T-cell responses *in vivo*. This lack of an effect may be due to several plausible outcomes. First, the *in vivo* response may have been oversaturated with antigen (via the ova-loaded BMDCs), essentially “washing out” any TCDD-induced immunoregulatory effects of the BMDCs. Second, the population of BMDCs that are more significantly affected by TCDD may have defects in their ability to migrate to the draining popliteal lymph node and thus never reach that immune tissue to modify the subsequent T-cell response. Third, TCDD-treated BMDCs may have to undergo some form of maturation via stimulatory signals such as LPS before they acquire functional immunosuppressive capabilities *in vivo* as was previously observed with VAF347-treated BMDCs (Hauben *et al.*, 2008). Fourth, OTII adoptively transferred mice may need to receive TCDD-treated BMDCs via a different route of administration (i.e., iv and not via the footpad) to realize an immunosuppressive effect. Finally, it could simply be that TCDD-treated steady-state BMDCs possess only a phenotypic, and not a functional, immunoregulatory capacity.

However, as shown in Figure 7, it would appear that TCDD-treated steady-state BMDCs do indeed possess an immunoregulatory capacity. In these experiments, TCDD-exposed BMDCs were loaded with ovalbumin at concentrations somewhat lower than had been used for the *in vivo* experiments. These BMDCs were then cultured with OTII/Foxp3^{egfp} T cells, leading to a significant increase in the generation of regulatory T cells *in vitro*. These experiments are consistent with the possibility that in our *in vivo* experiments: (1) The immunosuppressive BMDCs may not have been migrating properly to the draining lymph nodes as previously described for lung DCs (Jin *et al.*, 2010); (2) injecting them iv may be a more effective “therapeutic” approach (Hauben *et al.*, 2008); and/or (3) the concentration of ovalbumin used to load BMDCs for the *in vivo* experiments may have been inappropriate. On the other hand, our *in vitro* results indicate that TCDD-treated BMDCs are (1) functionally immunoregulatory and (2) do not need to be LPS stimulated to obtain this capability. Consequently, additional studies are necessary to define the optimal conditions that are required to translate the effects of TCDD-treated steady-state BMDCs *in vivo*. These experiments are currently being addressed in our laboratory and will attempt to determine the therapeutic potential of AhR-activated BMDCs in several animal models of immune-mediated disease.

In summary, we report here that AhR activation disrupts the differentiation, TLR responsiveness, NF- κ B signaling, uptake of antigen, and induction of immunoregulatory gene expression in steady-state BMDCs. Moreover, TCDD-treated steady-state BMDCs can generate CD4⁺ CD25⁺ FoxP3⁺ Tregs *in vitro* in an IDO-dependent fashion. However,

somewhat unexpectedly, TCDD-treated steady-state BMDCs successfully activated CD4⁺ antigen-specific T cells *in vivo*. Overall, our study offers mechanistic data to further explain the immunomodulatory effects of AhR activation. Moreover, this study defines several specific biomarkers of immunotoxicity following exposure to toxicants that can activate the AhR and identifies selective AhR modulators that may possess therapeutic value.

FUNDING

National Institute of Environmental Health Sciences (R01 ES013784); National Center for Research Resources (P20RR17670).

ACKNOWLEDGMENTS

We acknowledge the assistance of the Center for Environmental Health Sciences Fluorescence Cytometry Core and the Molecular Biology Core at the University of Montana. The authors would like to thank Dr Jerry Smith for his expert editorial assistance.

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