Effects of TCDD on the Fate of Naive Dendritic Cells

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The environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), causes immune suppression via activation of the aryl hydrocarbon receptor. Dendritic cells (DCs), the professional antigen-presenting cells in the immune system, are adversely affected by TCDD. We hypothesized that TCDD alters DC homeostasis, resulting in a loss of DCs in naive mice. To test this hypothesis, C57Bl/6 mice were gavaged with either vehicle or an immunosuppressive dose of TCDD (15 μg/kg). TCDD exposure decreased the frequency and number of splenic CD11c-high DCs on day 7 when compared with vehicle-treated controls. TCDD increased the expression of CD86 and CD54, while decreasing the frequency of splenic CD11c-high DCs expressing CD11a and major histocompatibility complex (MHC) class II. Moreover, TCDD selectively decreased the CD11clow/CD8α-33D1+ splenic DCs specialized at activating CD4+ T cells but did not affect the regulatory CD11c-high/CD8α+DEC205+ splenic DCs. TCDD did not alter the number or frequency of CD11c-low splenic DCs but decreased their MHC class II and CD11a expression. Loss of splenic CD11c-high DCs was independent of Fas-mediated apoptosis and was not due to alterations in the numbers of common DC precursors in the bone marrow or their ability to generate steady-state DCs in vitro. Instead, increased CCR7 expression on CD11c-high DCs suggested involvement of a migratory event. Popliteal and brachial lymph node DC11c+ cells showed elevated levels of MHC class II and CD40 following TCDD exposure. Collectively, this study shows the presence of a TCDD-sensitive splenic DC subpopulation in naive mice, suggesting that TCDD may induce suppression of T-cell-mediated immunity by disrupting DC homeostasis.

Key Words: aryl hydrocarbon receptor; dendritic cells; immunotoxicity; TCDD; apoptosis; CCR7.

The immune system has been identified as a sensitive target of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Luster et al., 1989). Exposure of laboratory rodents to very low doses of TCDD suppresses the generation of adaptive immunity and increases the susceptibility to infectious diseases (Kerkvliet and Burleson, 1994). TCDD primarily exerts its toxic effects by binding and activating the aryl hydrocarbon receptor (AhR; Fernandez-Salgueiro et al., 1996; Perdew, 2008).

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and the primary immune cells responsible for the activation of naive T cells. DCs populate most tissues in mice and can be identified by the expression of the integrin CD11c. DCs progress through multiple differentiation stages based on their activation status. Immature DCs are characterized by expression of low levels of CD11c and of costimulatory molecules such as major histocompatibility complex (MHC) class II, CD86, and CD54, but are very efficient at the phagocytosis of antigens. Following activation, immature DCs migrate to the draining lymph nodes (LNs), undergo maturation, and activate naive T cells leading to the generation of antigen-specific, T-cell-mediated immune responses. Mature DCs are CD11c-high but exhibit a decreased capacity to internalize antigens. The activation, maturation, and migration of DCs to T-cell-rich areas such as LNs can be triggered by phagocytic uptake of antigens or exposure to pathogen-associated molecular patterns such as lipopolysaccharide (Banchereau et al., 2001; Winzler et al., 1997a,b). Migration of DCs to LNs is mediated by chemokine receptors such as CCR7 (Gunn, 2003; Martin-Fontecha et al., 2003; Randolph et al., 2005). Deficiency of CCR7, or its primary ligands CCL19 or CCL21, results in abnormal LN development and dysfunctional leukocyte trafficking (Forster et al., 1999; Gunn et al., 1999; Mori et al., 2001; Martin-Fontecha et al., 2003).

DCs exist in a variety of subsets specialized in mediating distinct effector functions. In the murine spleen, two distinct subpopulations of DCs have been identified phenotypically by the expression of CD11c: CD11c-high and CD11c-low cells. CD11c-low DCs have been characterized to be immature DCs, whereas the CD11c-high DCs are considered mature. Recently, two functionally distinct CD11c-high DC subsets in the spleen were identified based on their expression of CD11c-high/CD8α+DEC205+ and CD11c-high/CD8α-33D1+ (Dudziak et al., 2007). CD8α-DEC205+ DCs are specialized at cross-presentation of antigens to CD8+ T cells and at inducing Foxp3+ regulatory T cells (Dudziak et al., 2007; Yamazaki et al., 2008). In contrast, CD8α-33D1+ DCs are more effective at the uptake of exogenous antigens and subsequent activation of CD4+ T cells.

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DCs are sensitive to TCDD; however, limited information exists on the effects of AhR activation in these cells. Because DCs play a key role in the generation of successful T-cell-mediated immunity, it is important to define the potential effects of persistent environmental pollutants such as TCDD on their fate and function. Studies conducted by Vorderstrasse and coworkers provided an initial characterization of the effects of TCDD on naive murine DCs. In these studies, TCDD induced a decrease in CD11c+ DCs and also affected the expression of key surface costimulatory molecules in an AhR-mediated fashion (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse et al., 2003). In a separate study, TCDD was reported to enhance the tumor necrosis factor (TNF)-α-induced maturation of bone marrow (BM)–derived DCs (BMDCs) rendering them more sensitive to CD95-mediated apoptosis (Ruby et al., 2005).

However, the effects of TCDD on specific murine DC subsets remain to be defined and the mechanisms underlying the loss of naive splenic DCs remain to be elucidated. In the present study, we have characterized the fate of distinct DC subsets in the spleen and LNs of TCDD-treated naive mice. In addition, we have explored potential mechanisms responsible for TCDD-induced disruption of DC homeostasis in unimmunized mice.

MATERIALS AND METHODS

Animals. Six- to 8-week-old C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of B6.Ahr<sup>tm1Smn</sup> (AhR<sup>−/−</sup>) mice were obtained from Dr. Paige Lawrence (University of Rochester Medical College, Rochester, NY) and subsequently bred as previously described (Schmidt and Bradfield, 1996). C57Bl/6<sup>Flt3<sup>−/−</sup></sup> (B6.Smn.C3-Fasl<sup>−/−</sup>) mice that possess a natural mutation in the Fas ligand (FasL) gene were originally purchased from Jackson Laboratories and generously provided by Dr. Andrij Holian (University of Montana). C57Bl/6<sup>Lin<sup>−/−</sup></sup> (B6.MRL-Fas<sup>−/−</sup>) mice possessing a natural mutation in the Fas gene were obtained from Jackson Laboratories. All animal experiments were approved by the University of Montana Institutional Animal Care and Use Committee and adhered to the current National Institutes of Health guidelines for animal usage. Animals were provided rodent chow and tap water ad libitum. For all experiments, animals were euthanized by CO2 overdose followed by cervical dislocation.

TCDD exposure. TCDD was obtained from Cambridge Isotope laboratories, Inc. (Woburn, MA), dissolved in anisole, and diluted in peanut oil. Control mice received vehicle consisting of comparable doses of anisole and peanut oil. For all experiments, mice were gavaged once with vehicle or an immunosuppressive dose of TCDD (15 µg/kg; Kerkvliet et al., 1996). Animals were sacrificed either on day 5 or 7 after treatment and immune tissues harvested for subsequent analyses.

Preparation of splenocytes. Spleens were harvested and processed as previously described (Shepherd et al., 2001). Briefly, spleens were processed between the frosted ends of slides, erythrocytes were depleted by hypotonic lysis, and cells were washed and resuspended in cold HBSS, 5% fetal bovine serum (FBS), supplemented with 20 mM Hepes, 50 µg/ml gentamicin, and 1.5 mM sodium pyruvate (referred to as cHBSS).

Preparation of splenocytes harvested 5 days after vehicle or TCDD treatment. LN tissue was processed using the end of a 1-ml syringe and cell strainer in 5 ml of cHBSS. The cell suspension was centrifuged at room temperature at 290 × g for 10 min. The supernatant was discarded and cells resuspended in 1 ml of cHBSS.

Preparation of BM cells and generation of steady-state BM DCs. C57Bl/6 mice (10 weeks old) were gavaged once with vehicle or TCDD (15 µg/kg). On day 7, mice were sacrificed and their BM harvested. BM cells were collected as previously described (Grauer et al., 2002) with modifications. Briefly, to collect BM cells, the femur and tibia of mice were flushed using a 27-gauge needle with complete media (cRPMI) that comprised RPMI (GibcoBRL, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), 50 µM mercaptoethanol, 20 mM Hepes, 10 mM sodium pyruvate, and 50 µg/ml gentamicin (GibcoBRL). Cells were subjected to a density gradient using Lympholyte-M reagent (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) to enrich hematopoietic cells and then washed with cRPMI. A fraction of the cells recovered were then used for detection of common DC precursors (CDPs) by flow cytometry. The remaining BM precursor cells were placed in culture to generate steady-state BMDCs.

Flt3 ligand–derived, steady-state BMDCs were prepared as previously described (Brasel et al., 2000; Brawand et al., 2002) with modifications. Briefly, the BM cells were grown in 1.0 ml conditioned media and 300 ng/ml Flt3 ligand (PeproTech, Inc., Rocky Hill, NJ) to a final concentration of 1 × 10<sup>6</sup> cells/ml in six-well plates. Conditioned media containing interleukin-6 was obtained by culturing red blood cell–depleted splenocytes for 10 days in cRPMI, collecting the supernatant, and storing it at −20°C for future use. Conditioned media and growth factor were removed and replenished on day 5. Nonadherent cells, representative of immature DCs, were harvested after 10 days and evaluated for viability and expression of CD11c. Cells were ≥95% viable as determined by trypan blue exclusion and were ≥90% CD11c<sup>+</sup> as determined by flow cytometry.

Flow cytometry. Immune cells were stained for flow cytometric analysis as previously described (Shepherd et al., 2001). Rat, hamster, or mouse immunoglobulin G (IgG) block was added to aliquots of prepared leukocytes for 10 min on ice to prevent nonspecific binding. Splenocytes, LN cells, or BM cells were stained for 10 min on ice with monoclonal antibodies (mAbs) from BD Pharmingen (San Diego, CA): CD11c (HL3), CD11b (M1/70), B20 (RA3-6B2), Ly6C (AL-21), CD11a (2D7), MHC II (2G9), CD86 (GL1), and CD40 (3/23). CD3e (145-2C11), Ly6G/Ly6C (RB6-8C5), and the erythroid marker (TER-119); from Miltenyi Biotec (Auburn, CA): DEC205 (NLDC-145); from BioLegend (San Diego, CA): CD11c (N418), CCR7 (4B12), CD8 (53-6.7), CD4 (3/23), CD54 (Y1/17.4), CD117 (2B8), NK-1.1 (PK136), and CD19 (6D5); and from eBioscience (San Diego, CA): 33D1, CD11d (AFS98), and CD135 (A2F10). All mAbs used were titrated for optimal concentration, and appropriately labeled isotype-matched mAbs were used as controls. Acquisition of 100,000–300,000 events from freshly prepared cells was carried out using a FACS Aria flow cytometer (BD Biosciences, San Jose, CA) and analyzed by BD FACSDiva software (version 4.0).

For detection of apoptosis, immune cells were stained with annexin V and 7AAD as per the manufacturer’s instructions (BD Pharmingen). After surface staining for CD11c, cells were resuspended in 1× annexin binding buffer, incubated with annexin V-FITC for 15 min at room temperature in the dark, followed by addition of 7AAD for 10 min, and analyzed as described above.

For detection of CDPs that are restricted to produce classical splenic DCs (cDCs) and plasmacytoid DCs (pDCs), BM cells were gated as follows: Lin<sup>−</sup> (CD19-CD3e-CD4-CD8-NK1.1-B220-TER119-CD11b-CD11c<sup>−</sup>), CD117 (c-Kit)<sup>−</sup>, CD3<sup>−</sup>, CD135 (Flt3<sup>−</sup>), and CD115 (M-CSFR<sup>−</sup>). For detection of pDCs and interferon-producing killer DCs (IKDCs), splenocytes were gated as follows: pDCs (CD11c<sup>−</sup>, CD11b<sup>−</sup>, B<sub>220</sub>−, and Ly6C<sup>low</sup>) and IKDCs (CD11c<sup>−</sup>, CD11b<sup>−</sup>, B<sub>220</sub>−, and Ly6C<sup>high</sup>). For sorting CD11c<sup>high</sup> and CD11c<sup>low</sup> splenic DCs, a single mouse was injected ip once daily for 10 consecutive days with 20 µg human recombinant Flt3 ligand (PeproTech, Inc.) as previously described (Maraskovsky et al., 1997; Pulendran et al., 1997). The Flt3 ligand–treated mouse was harvested on day 11 and the splenocytes were prepared, stained with CD11c<sup>−</sup>, and purified using anti-phycocerythrin beads from Miltenyi Biotec. Cells were sorted for CD11c<sup>high</sup> (> 92% purity) and CD11c<sup>low</sup> DCs (> 86% purity) in sterile sorting buffer using a FACS Aria flow cytometer.
Western blotting. Lysates were prepared from FACS-sorted splenic CD11c<sup>high</sup> and CD11c<sup>low</sup> DCs. Protein concentrations from cell lysates were determined using the Pierce bicinchoninic acid assay from Thermo Scientific (Rockford, IL). Protein lysates (17 µg per lane) were loaded on a 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Invitrogen, Carlsbad, CA) and transferred onto a Polyvinylidene Fluoride membrane (Bio-Rad, Hercules, CA). Anti-AhR antibody (BIOMOL, Plymouth Meeting, PA) was used to specifically detect murine AhR<sup>B</sup> at a dilution of 1:5000 at 4°C overnight. After washes in TBST (Tris-buffered saline with Tween-20), horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA) was used at a dilution of 1:1000 for 1 h at room temperature. The membrane was washed and incubated in enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ). Membranes were visualized and analyzed using a Fujifilm Image Reader (Las 3000, version 2). Similarly, the membrane was also probed for β-actin using anti-β-actin antibody (Abcam, Cambridge, MA) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (SouthernBiotech, Birmingham, AL) as the secondary antibody.

Statistical analysis. Results are presented as the mean ± SEM of six mice unless otherwise indicated. For all experiments, an unpaired t-test was used to compare the mean of the vehicle-treated group with the mean of the TCDD-treated group. Values of p ≤ 0.05 were considered statistically significant.

RESULTS

DC Subpopulations in the Peripheral Immune Tissues

Based on the expression of CD11c, the murine marker for DCs, splenocytes and PBLNs differ in their DC composition. Two distinct subpopulations of DCs, referred to as CD11c<sup>high</sup> and CD11c<sup>low</sup>, exist in the mouse spleen (Fig. 1). In contrast, the peripheral LNs contain a uniform CD11c<sup>+</sup> population with no discernible CD11c<sup>high</sup> and CD11c<sup>low</sup> DCs (Fig. 1). DCs representing an immature phenotype display low levels of accessory molecules such as MHC class II and CD86 and a decreased capacity to activate naïve T cells. In contrast, mature APCs display increased levels of costimulatory molecules and are highly efficient at T-cell stimulation (Sato and Fujita, 2007).

Effects of TCDD on Naive Splenic DC Subpopulations

To assess the potential sensitivity of DC subsets to TCDD, AhR protein levels were evaluated in purified CD11c<sup>high</sup> and
CD11c<sup>low</sup> splenic DCs. As shown in Figure 1B, both splenic DC subpopulations constitutively express comparable levels of AhR. Consistent with previous studies, TCDD exposure caused a decline in the percentage and number of CD11c<sup>high</sup> DCs recovered from the spleen on day 7 (Vorderstrasse and Kerkvliet, 2001; Fig. 2). In the majority of experiments, TCDD had no effect on the percentage or number of splenic CD11c<sup>low</sup> DCs recovered on day 7 (Fig. 2).

**Costimulatory Molecule Expression on Naive Splenic DCs from TCDD-Treated Mice**

Expression of costimulatory molecules such as MHC class II, CD86, CD80, CD40, CD54, and CD11a reflect the activation and/or maturation status of DCs. Therefore, several key activation markers were evaluated on both splenic CD11c populations. TCDD increased the expression of CD86 and CD54 and the frequency of CD11c<sup>high</sup> DCs expressing CD40 (Table 1). The frequency of CD11c<sup>high</sup> DCs expressing MHC class II, CD80, and CD11a was decreased in TCDD-treated mice. Similarly, fewer splenic CD11c<sup>low</sup> cells expressed MHC class II and CD11a in TCDD-treated mice when compared with vehicle-treated controls. Although there was no effect on CD40, the expression of CD86, CD80, and CD54 on CD11c<sup>low</sup> DCs increased following TCDD exposure. All the observed effects of TCDD on the modulation of the markers on both the CD11c<sup>high</sup> and the CD11c<sup>low</sup> DCs were AhR dependent as no TCDD-induced effects were detected on splenic DCs from naive AhR<sup>−/−</sup> mice (Supplementary table 1).

Because distinct DC subsets with unique functions have been recently characterized in the mouse spleen, we examined the effects of TCDD on these DC subsets. CD11c<sup>high</sup> DCs expressing the phenotypic markers CD8α<sup>+</sup>DEC205<sup>+</sup> or CD8α<sup>−</sup>33D1<sup>+</sup> occupy different anatomical positions in the spleen and mediate distinct effector functions (Dudziak et al., 2007). TCDD caused a 40% reduction in the number of splenic CD11c<sup>high</sup>CD8α<sup>−</sup>33D1<sup>+</sup> DCs on day 7 but not on day 3 or 5 (Table 2 and data not shown). This effect was dependent on the expression of the AhR as no loss of CD11c<sup>high</sup>CD8α<sup>−</sup>33D1<sup>+</sup> DCs occurred in AhR knockout mice exposed to TCDD (Table 2). In contrast, the number of CD11c<sup>high</sup>CD8α<sup>+</sup>DEC205<sup>+</sup> DCs was unaffected by TCDD. Consistent with the study by Vorderstrasse and coworkers, the frequency of CD11c<sup>high</sup>CD8α<sup>−</sup>33D1<sup>+</sup> DCs increased by 10% in TCDD-treated mice when compared with the vehicle-treated controls (data not shown).

Additionally, splenic CD11c<sup>low</sup> cells can be segregated phenotypically into two subsets—pDCs, which express CD11c<sup>low</sup>CD11b<sup>+</sup>Ly6C<sup>+</sup>B220<sup>+</sup>, and IKDCs, which express CD11c<sup>low</sup>CD11b<sup>−</sup>Ly6C<sup>−</sup>B220<sup>+</sup>. In our study, neither the percentages nor the numbers of splenic pDCs or IKDCs were affected following TCDD exposure of naive mice (Supplementary fig. 1).

**FIG. 2.** TCDD induces a loss of splenic CD11c<sup>high</sup> but not CD11c<sup>low</sup> DCs. C57Bl/6 mice were treated with vehicle or TCDD (15 μg/kg). Spleens were harvested on day 7, processed, and phenotyped by flow cytometry as described in the “Materials and Methods” section. Histograms represent the percentage of splenic CD11c<sup>high</sup> and CD11c<sup>low</sup> DCs from vehicle- (A) and TCDD-treated (B) mice. Bar graphs represent the total number of splenic CD11c<sup>high</sup> (C) and CD11c<sup>low</sup> (D) DCs from vehicle- and TCDD-treated mice. Data represent mean ± SEM of six mice per treatment group and are representative of four independent experiments. *p ≤ 0.05 for the comparison between vehicle- and TCDD-treated mice.
Effects of TCDD on the Fate of Splenic DCs in Naïve Mice

Because TCDD selectively decreased CD11c<sup>high</sup> DCs in the spleen, we next investigated potential mechanisms responsible for this decline. We hypothesized that TCDD-induced decreases in the splenic CD11c<sup>high</sup> cells may be due to at least three outcomes: (1) increased DC death via apoptosis, (2) decreased DC repopulation from precursors in the BM, or (3) increased DC emigration out of the spleen.

Fas-FasL interactions have been implicated in the TCDD-mediated death of various immune cells including BMDCs (Camacho et al., 2001, 2002; Dearstyne and Kerkvliet, 2002; Rhile et al., 1996; Ruby et al., 2005). Therefore, we examined the potential role of Fas and FasL in the TCDD-induced loss of splenic CD11c<sup>high</sup> DCs. To evaluate this pathway, unimmunized C57Bl/6<sup>+/+</sup> or C57Bl/6<sup>+/−</sup> mice were treated with vehicle or TCDD for 7 days. As shown in Figure 3, TCDD treatment decreased both the percentage and the number of splenic CD11c<sup>high</sup> DCs in both the Fas- and the FasL-deficient mice when compared with the vehicle-treated controls. In a separate experiment, apoptosis was directly evaluated in splenic DCs from wild-type C57Bl/6 mice on day 5 following TCDD exposure using two well-characterized markers of apoptosis, annexin V and 7AAD. TCDD had no effect on the early or late apoptotic staining profiles of splenic CD11c<sup>high</sup> cells when compared with controls (Table 3).

In the steady state, mouse splenic CD11c<sup>high</sup> DCs turn over every 3–5 days and are repopulated by precursor cells that are produced in the BM (Kamath et al., 2000; Merad and Manz, 2009). TCDD affects the fate and function of hematopoietic stem cells in mice but it is not known if downstream progenitor cells in the BM such as committed DC precursors are similarly affected (Singh et al., 2009a). We hypothesized that TCDD reduces the number of CDPs in the BM compartment, ultimately leading to defects in the repopulation of splenic CD11c<sup>+</sup> DCs. To address this hypothesis, we examined Lin<sup>−</sup>CD117<sup>+</sup>CD135<sup>+</sup>CD115<sup>+</sup> CDPs in the BM of mice after treatment with vehicle or TCDD. On day 7 after exposure, no differences existed in the frequency or number of CDPs in TCDD-treated mice when compared with the vehicle-treated controls (Fig. 4). Furthermore, we also evaluated the potential for whole BM cultures from these mice to generate steady-state BMDCs in vitro. Consistent with the lack of adverse effects of TCDD on the CDPs, BM from TCDD-treated mice not only produced similar frequencies of CD11c<sup>+</sup> cells after 10 days in culture but also generated increased numbers of these Flt3 ligand–derived BMDCs (Supplementary table 2).

DCs reside in the periphery and upon encounter with antigen undergo maturation. Maturation of DCs is characterized by phenotypic changes including the induction of the chemokine receptor CCR7 (Forster et al., 1999; Gunn, 2003; Randolph et al., 2005; Sallusto et al., 1998; Sanchez-Sanchez et al., 2006; Scandella et al., 2004). Expression of this receptor allows DCs to home to T-cell-rich areas in draining LNs (Cyster, 1999; Scandella et al., 2002, 2004). We hypothesized that a decrease in splenic CD11c<sup>high</sup> DCs could be due to CCR7-mediated migration. To address this hypothesis, the expression of CCR7 was evaluated on splenic DCs to explore the possibility that increased emigration might underlie the TCDD-induced decline of splenic CD11c<sup>high</sup> cells. CCR7 expression was increased on splenic CD11c<sup>high</sup> DCs in TCDD-treated mice on day 5 (Fig. 5), whereas CCR7 expression on CD11c<sup>low</sup> DCs was unaffected by TCDD (data not shown).

DCs in the Peripheral LNs Are Affected by TCDD Exposure

Because TCDD affects homeostasis of splenic DCs, we next examined if DCs that reside in peripheral LNs are also affected
by TCDD. We evaluated the effects of TCDD exposure on DCs in two combined sets of peripheral LNs, the PBLNs. In contrast to the spleen, the peripheral LNs in mice contain a uniform CD11c<sup>+</sup> DC population with no discernible CD11<sub>chigh</sub> and CD11<sub>clow</sub> DCs (Fig. 1). TCDD did not affect the frequency or the number of CD11c<sup>+</sup> cells in the PBLNs on day 7 (Fig. 6 and Supplementary table 3). As shown in Table 4, TCDD exposure enhanced expression of MHC class II and CD40 but did not affect the expression of CD86 and CD11a or alter the percentage of CD11c<sup>+</sup> DCs that expressed these costimulatory molecules in the peripheral LNs. As with the splenic DCs, modulation of accessory molecules by TCDD was AhR dependent (Supplementary table 4). Interestingly, on days 5 and 7 following treatment, CD11c<sup>+</sup> DCs in the PBLNs of TCDD-treated mice expressed increased levels of CCR7 (Fig. 7 and data not shown).

**DISCUSSION**

DCs play an important role in both the innate and the adaptive arms of the immune system. The effects of TCDD exposure on distinct DC subsets in the spleen and the LNs have not been previously examined. In our experiments, we have evaluated the effects of TCDD on naive splenic DCs, both CD11<sub>chigh</sub> and CD11<sub>clow</sub> cells, in addition to naive DCs in the peripheral LNs. Both splenic DC subpopulations constitutively express AhR and were therefore expected to be sensitive to TCDD.

DCs exist in various differentiation stages referred to as immature, mature, and activated cells, which can be characterized phenotypically by their varying expression of the accessory molecules MHC class II, CD86, and CD40. The seminal publication by Vorderstrasse and Kerkvliet (2001) showed that oral administration of TCDD (15 μg/kg) induced a loss of splenic CD11c<sup>+</sup> DCs in naive mice. Importantly, this study used low-density gradient separation to isolate splenic

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<th>TABLE 3</th>
<th>Exposure to TCDD Does Not Induce Apoptosis&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup>C57Bl/6 mice were treated with TCDD (15 μg/kg) or vehicle for 5 days, and spleens were harvested, processed, and stained for expression of CD11c, annexin V and 7AAD, as described in the "Materials and Methods" section. Mature DCs were identified by gating on the CD11c<sub>chigh</sub> cells from vehicle- and TCDD-treated mice. Early apoptosis (annexin V<sup>+</sup>/7AAD<sup>−</sup>) and late apoptosis (annexin V<sup>+</sup>/7AAD<sup>+</sup>) were evaluated. Data represent the mean ± SEM of four to five mice per treatment.
DCs. This procedure tends to preferentially select for CD11c\textsuperscript{high} DCs from the splenic tissue and can also affect DC activation. Consistent with this published study, we observed a decrease in the frequency and number of CD11c\textsuperscript{+} DCs following TCDD exposure. This decline was concomitant with an AhR-dependent modulation of MHC class II, CD11a, CD80, CD86, and CD40 on the remaining CD11c\textsuperscript{+} DCs in the spleen. Polarization of T-cell differentiation into a T-helper 1 (Th1) or Th2 response can be significantly affected by the nature of the DCs interacting with T cells. A recent study by Dudziak et al. (2007) identified two distinct CD11c\textsuperscript{high} splenic DC subsets in mice based on their expression of select phenotypic markers: CD8\textsuperscript{α}\textsuperscript{DEC205\textsuperscript{+}} DCs and CD8\textsuperscript{α}\textsuperscript{33D1\textsuperscript{+}} DCs. Our results show the selective effects of TCDD in mediating a decline in the splenic CD11c\textsuperscript{high}CD8\textsuperscript{α}\textsuperscript{33D1\textsuperscript{+}} population, which are vital for the activation of CD4\textsuperscript{+} Th1 cells. The selective loss of this splenic DC subset, with the concomitant retention of the Treg-inducing CD8\textsuperscript{α}\textsuperscript{DEC205\textsuperscript{+}} splenic CD11c DCs by TCDD, may predispose naive mice to be more susceptible to blood-borne extracellular pathogens and account for suppression of CD4\textsuperscript{+} T-cell-mediated responses. However, further studies are necessary to specifically evaluate the functional capacity of the splenic DC subsets in response to extracellular blood-borne pathogens in TCDD-treated mice. Several investigators have linked TCDD-induced immunosuppression with the induction of Tregs. AhR activation leads to the generation of adaptive Tregs (Funatake et al., 2005). Additionally, Vogel et al. (2008) have shown an increase in the expression of Foxp3\textsuperscript{+} Tregs in the splenic tissue of TCDD-treated mice. Our present findings provide suggestive evidence that disruption of DC homeostasis following AhR activation may contribute to the generation of Tregs in unimmunized mice.

**FIG. 4.** TCDD does not affect CDP levels in the BM. On day 7 following exposure, BM cells were harvested from vehicle- or TCDD-treated C57Bl/6 mice and CDPs analyzed by flow cytometry as described in the "Materials and Methods" section. Representative histograms show the Lin\textsuperscript{-}CD117\textsuperscript{int}CD135\textsuperscript{+}CD115\textsuperscript{+} CDPs from vehicle- (A) and TCDD-treated (B) mice and the lack of effects of TCDD on the frequencies (C) and numbers (D) of CDPs in the BM. Values shown are the mean \(\pm\) SEM of five to six mice per treatment and are representative of two independent experiments.
In contrast to the CD11c\textsuperscript{high} cells, TCDD did not alter the frequency or the number of splenic CD11c\textsuperscript{low} DCs. However, alteration of activation markers such as MHC class II in CD11c\textsuperscript{low} DCs following exposure to TCDD suggests a modulation in the differentiation status of these cells. CD11c\textsuperscript{low} DCs can be phenotypically and functionally characterized into at least two distinct populations, IKDCs and pDCs. pDCs in mice have been described to possess tolerogenic functions (Rutella et al., 2006). It is therefore possible that the immunosuppressive effects of TCDD on the activation of T cells may be due to changes in the number and frequency of tolerogenic DCs.

Contrary to our expectations, both the frequency and the number of pDCs were unaffected by TCDD. Collectively in the spleen, TCDD-induced decreases in the immunostimulatory, but not the tolerogenic DCs subset on day 7, could contribute to immune suppression.

In sharp contrast to spleen, TCDD did not affect the number or frequency of DCs in the LNs. It did however alter their expression of costimulatory markers such as MHC class II and CD40 in an AhR-dependent manner. This modulation is reflective of alteration of the steady-state differentiation status of DCs in LNs upon TCDD exposure and establishes that TCDD,

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either directly or indirectly, has effects in the peripheral LNs. Modulation of DC differentiation may alter the functional ability of these APCs to interact with T cells and generate successful adaptive immune responses.

Exposure to TCDD has been reported to enhance cellular death in several leukocyte populations. Fas (CD95), a member of the TNF superfamily, is expressed on the surface of DCs as well as other immune cells. Upon binding to FasL (CD95L), a series of downstream signaling cascades leads to the activation of terminal effector caspases and ultimately apoptosis (Adachi et al., 1993; Dearstyne and Kerkvliet, 2002; Lenardo et al., 1999; Nagata and Golstein, 1995; Peter and Krammer, 1998; Scaffidi et al., 1998). Moreover, Fas-FasL interactions have been implicated in the TCDD-induced loss of various immune cells including T cells and DCs (Camacho et al., 2001, 2005; Dearstyne and Kerkvliet, 2002; Kamath et al., 1999; Rhile et al., 1996; Ruby et al., 2005). We used Fas-deficient (lpr/lpr) and FasL-deficient (gld/gld) mice, defective in this extrinsic apoptotic pathway, to evaluate the possible involvement of Fas-FasL interactions in the decline of naive splenic CD11chigh DCs by TCDD. Splenic CD11chigh DCs in both the Fas-deficient and the FasL-deficient mice declined in a manner similar to that observed in the wild-type mice following TCDD exposure, suggesting that Fas-mediated apoptosis is not responsible for the loss of splenic CD11chigh DCs in naive mice. Additionally, using annexin V and 7AAD, no

**TABLE 4**

| Effects of TCDD on the Expression of Accessory Molecules on DCs from Peripheral LNs
<table>
<thead>
<tr>
<th>MFI</th>
<th>CD11c+ DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC class II</td>
<td>Vehicle</td>
</tr>
<tr>
<td>CD86</td>
<td>1803 ± 13</td>
</tr>
<tr>
<td>CD40</td>
<td>814 ± 32</td>
</tr>
<tr>
<td>CD11a</td>
<td>2846 ± 114</td>
</tr>
</tbody>
</table>

*C57Bl/6 mice were treated with TCDD or vehicle for 7 days, and LNs were harvested and processed for flow cytometric analysis. The median fluorescence intensity (MFI) for each activation marker was determined for the CD11c+ DCs. Results are representative of two independent experiments and data represent the mean ± SEM of three mice per treatment.

*p ≤ 0.05 for the comparison between vehicle- and TCDD-treated mice.

**FIG. 7.** Expression of CCR7 is increased on DCs from LNs following TCDD exposure. On day 5 following exposure to vehicle or TCDD, LNs were harvested and analyzed by flow cytometric analysis as previously described. Representative histograms depict the mean fluorescence intensity (MFI) of CCR7 expression on CD11c+ cells in mice treated with (A) vehicle and (B) TCDD. Values represent the mean ± SEM of five mice per treatment group and are representative of two independent experiments. *p ≤ 0.05 for the comparison between vehicle- and TCDD-treated mice.
Evidence of TCDD-induced apoptosis was detected on the CD11c<sup>high</sup> DCs from TCDD-exposed naive mice. Taken together, our results show an overall lack of apoptosis in splenic CD11c<sup>high</sup> DCs following exposure to TCDD.

Homeostasis of splenic DCs in naive mice depends on continuous replacement by hematopoietically derived progenitors from the BM (Merad and Manz, 2009). Repopulation is especially important for CD11c<sup>high</sup> DCs in the murine spleen as they tend to turn over relatively rapidly (every 3–5 days; Kamath et al., 2000). Recently, a study by Liu et al. (2009) showed that DC development progresses via the generation of CDPs in the BM that are committed to producing both cDCs and pDCs. Based on these pieces of information and reports that TCDD can significantly impair the development of lymphoid-derived immune cells via effects on hematopoietic stem cells and/or very immature progenitor cells in the BM (Singh et al., 2009a, b), we assessed the effects of TCDD on CDPs in naive mice. Our results failed to show any effects of TCDD on the frequency or number of CDPs in the BM compartment on day 7 following exposure, a time that corresponded with a significant loss of splenic CD11c<sup>high</sup> DCs. Furthermore, no differences existed in the ability of whole BM cultures from mice treated with vehicle or TCDD to generate lymphoid-derived, steady-state BMDCs <i>in vitro</i>. Taken together, these results suggest that the loss of splenic CD11c<sup>high</sup> DCs that we observed in TCDD-treated mice is not attributable to defects in DC development in the BM. However, it remains to be determined if TCDD alters the ability of CDPs to traffic from the BM to lymphoid tissues such as the spleen, or the potential for CDPs and downstream progenitors to differentiate into splenic CD11c<sup>high</sup> DCs. If either of these defects resulted from exposure to TCDD, then alterations in DC homeostasis might be expected to contribute to some, or all, of the adverse effects that we have observed on splenic CD11c<sup>high</sup> DCs in naive mice.

Alternatively, the TCDD-induced decline in splenic CD11c<sup>high</sup> DCs could be due to induced emigration. CCR7 is a chemokine receptor that plays a prominent role in the migration/trafficking of immune cells including DCs. This receptor specifically binds two ligands, CCL21 and CCL19, which are expressed constitutively by endothelial cells in the lymphatic vessels and high endothelial venules. Immature DCs, which comprise the majority of DCs in an unimmunized animal, also express this receptor and migrate to the LNs (Sanchez-Sanchez et al., 2006). However, in the absence of danger signals, these DCs contribute to peripheral tolerance against self-antigens (Sanchez-Sanchez et al., 2006; Scandella et al., 2004). Premature induction of migration, in the absence
of danger signals, may alter DC homeostasis in the periphery. Specifically for DCs, CCR7 expression increases following activation. Interestingly, CCR7 expression was enhanced on the splenic CD11c<sup>high</sup> DCs following TCDD exposure, suggesting the possibility that these cells were aberrantly being induced to emigrate out of the spleen. Also, CCR7 expression increased on DCs that resided in the peripheral LNs in unimmunized mice treated with TCDD. The possibility exists that TCDD induces the release of proinflammatory cytokines by resident cells in the LNs leading to upregulation of CCL21 on the lymphatic endothelium. However, no alteration in the frequency or the number of LN DCs was observed with TCDD. It is possible that increased CCR7 expression on LN DCs could induce their emigration to the source of CCR7 ligands. The role of TCDD-induced increased CCR7 expression on LN DCs remains to be identified. However, additional experiments are necessary to establish if TCDD-induced migration of DCs is dependent on alterations in the expression of CCR7 or its ligands.

A seemingly plausible model emerges considering the progressive increase in CD11c expression, along with modulation of accessory molecules on DCs as differentiation proceeds (Fig. 8). It is possible that TCDD selectively affects a subset of splenic CD11c<sup>high</sup> DCs increasing the CCR7 expression on these DCs, ultimately leading to their emigration out of the spleen. Because DCs acquire enhanced migratory abilities as they undergo differentiation, aberrant migration of splenic DCs may underlie the ability of TCDD to disrupt DC homeostasis in lymphoid organs. Alteration of DC differentiation and homeostasis by TCDD may, in turn, generate dysfunctional DCs that lead to immune suppression. Alternatively, a possibility also exists that TCDD-induced splenic DC emigrants may possess specialized functions in the periphery or other immune tissues. Indeed, maturation of DCs in the absence of antigenic stimulation can generate tolerogenic DCs (Steinman et al., 2003). Therefore, it is plausible that immunosuppression in mice exposed to TCDD could be attributable, at least in part, by altered interactions between migratory modulated splenic DCs and (regulatory and/or conventional) T cells or other immune cells. To this end, studies in our laboratory have shown that BMDCs (both steady-state and inflammatory) exposed to TCDD display a regulatory phenotype as defined by an increased expression of indoleamine 2,3-dioxygenase (IDO)-1, IDO-2, and transforming growth factor-β3 (Bankoti, Rase, Simones, and Shepherd, manuscript submitted; Simones, Bankoti, and Shepherd, manuscript in preparation). However, it remains to be determined if similar effects occur in splenic CD11c<sup>high</sup> DCs after TCDD exposure in vivo and if specialized functions are induced in these cells.

In conclusion, we have shown that TCDD affects DC homeostasis by selectively decreasing a distinct subset of CD11c<sup>high</sup>CD8α<sup>+</sup>33D1<sup>+</sup> DCs in the splenic tissue of naive mice in an AhR-mediated manner. This loss of mature DCs was not attributed to Fas-mediated apoptosis or defects in splenic DC repopulation from common DC precursors in the BM. Instead, upregulation of CCR7 on the splenic CD11c<sup>high</sup> DCs provides suggestive evidence that migration of these DCs may be playing a role in the TCDD-induced loss of splenic CD11c<sup>high</sup> DCs. In addition, our results show increased activation of DCs in both the spleen and the LNs following exposure of naive mice to TCDD. Collectively, this study shows the presence of a TCDD-sensitive splenic DC subpopulation in naive mice, suggesting that TCDD may induce suppression of T-cell-mediated immunity by disrupting DC homeostasis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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