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Suppression of Acute Graft-Versus-Host Response by TCDD Is Independent of the CTLA-4-IFN-γ-IDO pathway

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Activation of the aryl hydrocarbon receptor (AhR) by its prototypic ligand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), induces potent suppression of an acute graft-versus-host (GVH) response and prevents GVH disease (GVHD). Suppression is associated with development of a regulatory population of donor CD4$^+$ CD25$^+$T-cells that express high levels of cytotoxic T-lymphocyte antigen 4 (CTLA-4). However, a direct link between these AhR-induced Tregs (AhR-Tregs) and suppression of GVHD remains to be shown. CTLA-4 is a negative regulator of T-cell responses and is associated with the induction of tolerogenic dendritic cells (DCs) that produce indoleamine 2,3-dioxygenase (IDO). We hypothesized that AhR-Tregs mediate suppression via their enhanced expression of CTLA-4, which, in turn, induces IFN-γ and IDO in host DCs. Subsequent depletion of tryptophan by IDO leads to termination of the donor T-cell response prior to development of effector CTL. Here, we show that despite increased expression of *Ifng*, *Irf3*, *Irf7*, *Ido1*, and *Ido2* in the lymph nodes of TCDD-treated host mice, inhibition of IDO enzyme activity by 1-methyl-tryptophan was unable to relieve TCDD-mediated suppression of the GVH response. Furthermore, treatment with an anti-CTLA-4 antibody that blocks CTLA-4 signaling was also unable to alleviate TCDD-mediated suppression. Alternatively, we investigated the possibility that donor-derived AhR-Tregs produce IFN-γ to suppress effector CTL development. However, suppression of GVHD by TCDD was not affected by the use of *Ifng*-deficient donor cells. Together, these results indicate that neither overexpression of CTLA-4 nor production of IFN-γ by AhR-Tregs plays a major role in the manifestation of their immunosuppressive function *in vivo*.

Key Words: **TCDD; IDO; CTLA-4; GVHD; T-regulatory cell.**

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that is known to mediate potent immunosuppression upon activation by the prototypic ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Marshall and Kerkvliet, 2010). The ability of TCDD to suppress the development of several autoimmune diseases in association with an increase in the frequency of Foxp3$^+$ Tregs has sparked an interest in the AhR as a therapeutic target (Benson and Shepherd, 2011; Kerkvliet *et al.*, 2009; Quintana *et al.*, 2008; Zhang *et al.*, 2010). Direct activation of AhR in CD4$^+$ T cells also induces Foxp3-negative Tregs (AhR-Tregs). These AhR-Tregs were first described in an acute parent-into-F1 graft-versus-host (GVH) model and were characterized by their high levels of CD25 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) and potent *in vitro* suppressive activity (Funatake *et al.*, 2005). Additional studies have shown that AhR-Tregs express higher levels of several genes that have been associated with Treg function such as IL-10, granzyme B, and CD39 (Marshall *et al.*, 2008). These AhR-Tregs are postulated to mediate suppression of the allo-CTL response and protection from GVH disease (GVHD) in TCDD-treated animals. However, the mechanisms of suppression used by AhR-Tregs remain poorly characterized.

CTLA-4 is an inhibitory receptor that shares 30% homology with CD28, a costimulatory receptor on T cells. Reaching maximal expression 48 h after T-cell activation (Walunas *et al.*, 1994), CTLA-4 competes with CD28 for the shared ligands CD80 and CD86, expressed on dendritic cells (DCs). As CTLA-4 has a higher affinity for these ligands relative to CD28, CTLA-4 may prevent CD28 signaling (Alegre *et al.*, 2001). Therefore, although engagement of CD28 promotes T-cell proliferation and differentiation, engagement of CTLA-4 attenuates T-cell responses. Animals deficient in CTLA-4 succumb to a severe T-dependent lymphoproliferative disease, resulting in death at 3–4 weeks of age (Alegre *et al.*, 2001) underscoring the necessity for CTLA-4 in T-cell regulation.

In addition to T-cell regulation, CTLA-4 can also influence DC function by interaction with CD80/86. This interaction can induce a tolerogenic phenotype through the induction of IFN-γ and indoleamine 2,3-dioxygenase (IDO) (Munn *et al.*, 2004). IDO catalyzes the rate-limiting step of tryptophan degradation to kynurenine, resulting in depleted tryptophan levels and suppression of T-cell responses (Mellor and Munn, 2004). Interestingly, TCDD has been shown to alter IDO expression and function. For example, increased expression of the genes *Ido1* and *Ido2* has been observed in several DC subsets cultured

with TCDD *in vitro*, including DCs derived from the human U937 monocytic cell line, murine bone marrow–derived DCs, and plasmacytoid DCs (pDCs) (Bankoti *et al.*, 2010; Benson and Shepherd, 2011; Mezrich *et al.*, 2010; Vogel *et al.*, 2008). This process appeared to be dependent on AhR, as use of BMDC from AhR$^{-/-}$ mice, or use of an AhR antagonist prevented the TCDD-dependent increase in IDO expression (Bankoti *et al.*, 2010; Vogel *et al.*, 2008). IDO enzyme activity was also increased by TCDD in U937-derived DCs (Vogel *et al.*, 2008). In other studies, IDO was implicated in the induction of Foxp3^{+} Tregs by kynurenine, another known AhR ligand (Mezrich *et al.*, 2010; Nguyen *et al.*, 2010). In one study, AhR in the DCs was required for Treg induction (Nguyen *et al.*, 2010), whereas in the other, AhR expression in the CD4^{+} T cell was necessary (Mezrich *et al.*, 2010).

The studies reported here have addressed the hypothesis that the TCDD-mediated increase in CTLA-4 expression on donor CD4^{+} T cells induces IFN-γ and ultimately IDO in host DCs, thereby suppressing the allospecific CTL response (see Fig. 1). To address this hypothesis, we examined the effect of TCDD on the CTL response while independently blocking CTLA-4 and inhibiting IDO enzyme activity.

MATERIALS AND METHODS

Animals. B6 (H-2$^{b/b}$), B6.129s7-*Ifng*^{tm1Ts}/J (*Ifng*$^{-/-}$, H-2$^{b/b}$), and B6D2F1 (H-2$^{b/d}$) mice were purchased from the Jackson Laboratory. B6.PL-Thy1a/CyJ (Thy1.1^{+}, H-2$^{b/b}$) mice (originally purchased from the Jackson Laboratory) were maintained as a breeding colony on-site. Animals were housed at the specific pathogen-free Laboratory Animals Resource Center. All experimental procedures and treatments were approved by the Institutional Animal Care and Use Committee at Oregon State University.

TCDD preparation. TCDD (Cambridge Isotope Laboratories; 99% purity) was dissolved in anisole (J.T. Baker) and further diluted in peanut oil. Host B6D2F1 mice were given 15 µg/kg of TCDD or vehicle control following adoptive transfer of donor cells on day 0.

Preparation of donor cells. Spleens and lymph nodes from donor C57Bl/6 (H-2$^{b/b}$) mice were aseptically removed and processed into single-cell suspensions by pressing the organs through a 70-µm nylon mesh cell strainer (BD Falcon). Red blood cells were removed via hypotonic water lysis. For experiments involving injection of whole splenocytes, CD4^{+} and CD8^{+} T-cell purity was determined by flow cytometry. Splenocyte suspensions were resuspended in injection buffer (Hank's balanced salt solution [HBSS], 1.5mM HEPES, and 50 µg/ml gentamicin). A maximum of 4×10^{7} donor T cells were injected by tail vein injection into B6D2F1 (H-2$^{b/d}$) hosts.

Where noted, CD4^{+} and CD8^{+} T cells were isolated from donor splenocytes using a negative selection PanT kit with autoMACS magnetic isolation (Miltenyi autoMACS). Purity was routinely above 90%. In some experiments, the donor T cells were labeled with 2µM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes) prior to adoptive transfer.

Anti-CTLA-4 treatment. LEAF-purified anti-mouse CD152 (CTLA-4) (9H10) and LEAF-purified Syrian hamster immunoglobulin G (IgG) isotype control (SHG-1) were obtained from BioLegend. Following adoptive transfer, host mice received 100 µg of either anti-CTLA-4 antibody or isotype control via ip injection. Injections were repeated on days 1 and 2 after adoptive transfer. A similar treatment regimen resulted in increased proliferation of antigen-specific T cells following immunization with A20HA tumor cells (Sotomayor *et al.*, 1999).

1-Methyl-tryptophan treatment. 1-Methyl-tryptophan (1-MT) was prepared as previously described (Saxena *et al.*, 2007). Briefly, 1-MT was dissolved in 0.05N NaOH to a concentration of 20mM. The solution was adjusted to pH 7 with concentrated HCl. To achieve a final concentration of 2 mg/ml, the 1-MT

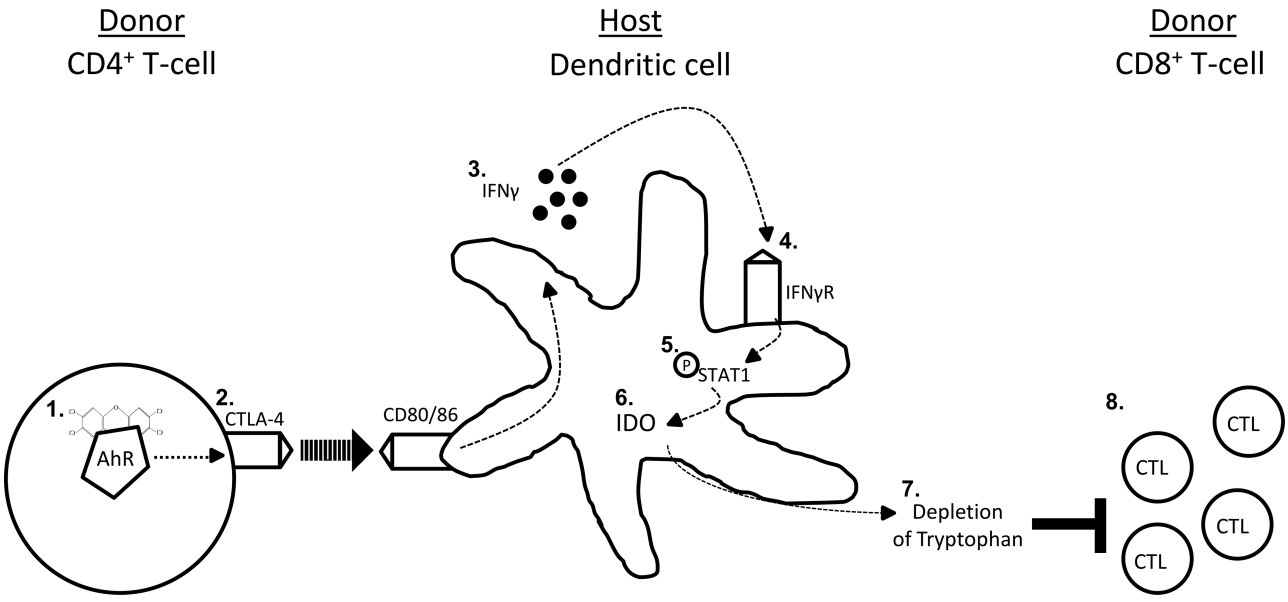

FIG. 1. Depiction of TCDD-induced CTLA-4 on AhR-Treg activating the tolerogenic IDO pathway and suppressing the acute GVH response. AhR is activated by TCDD in donor CD4^{+} T cells (1). CTLA-4 is upregulated in an AhR-dependent manner in donor CD4^{+} T cells and ligates CD80/86 on host DCs (2). The activated DCs produce IFN-γ (3). IFN-γ feeds back in an autocrine manner through the IFN-γ receptor (IFN-γR) (4). IFN-γ signaling through the IFN-γR phosphorylates STAT1 (5). Phosphorylated STAT1 directly induces IDO (6), which catalyzes the rate-limiting step of L-tryptophan metabolism to L-kynurenine, depleting local tryptophan levels (7). Lack of tryptophan suppresses the development of effector CTL (8).

solution was diluted in autoclaved, distilled water, sweetened with one packet aspartame per 2 l of water. Beginning on day 3, all animals received sweetened water *ad libitum*. 1-MT supplemented water was administered in light-protected water bottles and given *ad libitum* to mice a day prior to adoptive transfer. Control animals received sweetened water. Water bottles were checked daily to ensure mice were consuming approximately 4 ml (8 mg) of 1-MT daily.

Quantification of IFN-γ production. Splenocytes were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 μg/ml gentamicin, 10mM HEPES, and 50μM β-mercaptoethanol. The cultures were incubated overnight (~18 h) at 37°C and 5% CO_2 and supernatants collected for ELISA analysis. IFN-γ was detected using the IFN-γ Ready-Set-Go ELISA kit from eBioscience.

Flow cytometry. Host splenocytes were processed as described for donor cell preparation. Following red blood cell lysis, splenocytes were aliquoted into 96-well V-bottom plates (Corning) and washed twice with PAB (PBS, 1% bovine serum albumin, and 0.1% sodium azide). Samples were resuspended in rat IgG (Jackson ImmunoResearch) and incubated on ice prior to surface staining. For detection of donor cells, cells were stained with monoclonal antibodies (mAbs) to Thy1.1 (OX-7 or HIS51; BD PharMingen or eBioscience) or H-2D^d (KH95; BioLegend) in conjunction with either CD4 (GK1.5) or CD8 (53-6.7) (BD PharMingen). mAbs to CD25 (PC61.5), CD19 (eBio1D3), and CTLA-4 (UC10-4B9) were purchased from eBioscience. For detection of CTLA-4, samples were resuspended in fixable viability dye efluor 780 (eBioscience) for 20 min following surface staining for CD4, CD8, Thy1.1, H-2D^d, CD25, and CD19. Intracellular staining was performed following fixation and permeabilization protocols from BD Biosciences.

Fluorescence-minus-one samples, in which all antibodies except the one of interest are included, were used as staining controls. A minimum of 10,000 donor CD4$^+$ events or 1×10^6 total cells were collected per sample on a Beckman Coulter FC-500 flow cytometer. Data were compensated and analyzed using WinList (Verity Software, Version 6.0).

RNA extraction and qPCR. RNA was isolated from pooled axial, brachial, and cervical lymph nodes of host mice using the RNeasy Mini Kit #74104 (Qiagen), with the on-column DNase digestion repeated twice to ensure removal of genomic DNA. RNA integrity was assessed by using a Bioanalyzer 2100 (Agilent). Reverse transcription was performed using the Superscript III first-strand synthesis supermix (Invitrogen), following the manufacturer's instructions. For all qPCR reactions, SYBR Green/Rox qPCR Master Mix (SA Bioscience) was mixed with 10 ng cDNA per reaction, plus the appropriate primers. An ABI PRISM 7500 Real-Time PCR system (Applied Biosystems) was used for all qPCR reactions. Primers for *Ido1* (NM_008324.1), *Ido2* (NM_145949.2), and *Ifng* (NM_008337.1) were obtained from SA Biosciences and used according to the manufacturer's instructions. All other primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank). Forward and reverse primers were purchased from Invitrogen and validated. The PrimerBank ID codes were as follows: *E2-2*: 1001888a1; *Irf3*: 8393627a1; *Irf7*: 8567364a1; *Lag3*: 6678654a1; *Siglech*: 30520121a1; *Tlr7*: 18875360a1; *Tlr9*: 13626030a1. Data were analyzed using 7500 Software (v2.0.1) (Applied Biosystems).

Quantification of IDO enzyme activity. Splenocytes (1×10^7) were cultured in 0.5 ml 1× HBSS without phenol red (Sigma), containing 100μM L-tryptophan (Alfa Aesar, CAS #73-22-3), and incubated at 37°C and 5% CO_2 as previously described (Hwu *et al.*, 2000). After 4 h, the supernatant was removed and stored at −80°C for analysis of L-kynurenine. A standard curve of L-kynurenine (Sigma Aldrich, CAS #2922-83-0) (0.1nM–10μM) was prepared and run in tandem with the experimental samples. An Agilent SB-C18 RRHD 1.8 μm (2.1 mm × 150 mm) column was used for UPLC analysis on a Shimdazu Nexera Liquid Chromatograph, using the following eluents: H_2O + 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient elution was 5% B for 2 min, followed by 90% B for 4 min, and 5% B for 2 min for a total duration of 8 min. The eluents were directed to an ABSciex Triple TOF 5600 for MS analysis of L-kynurenine.

Statistical analysis. All results are presented as the mean ± SEM of biological replicates (n = 3–5). For comparisons between two treatment groups, Students t-tests were used, with statistical significance as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Where indicated, the Mixed procedure with the Satterthwaite option was performed in SAS (version 9.3).

RESULTS

Activation of AhR by TCDD Increases Expression of CTLA-4, IFN-γ, and IDO

Prior studies have shown that activation of AhR by TCDD during an acute GVH response induces a Treg phenotype (CD25$^+$CTLA-4$^+$) in alloresponding donor CD4$^+$ T cells (Funatake *et al.*, 2005). Based on additional studies that have linked TCDD with increased expression of IDO under various conditions, we hypothesized that increased expression of CTLA-4 on these AhR-Tregs drives IDO production in the DCs via induction of IFN-γ. Secretion of excessive IDO then leads to depletion of tryptophan and suppression of the differentiation of effector CD8$^+$ CTL (Fig. 1). To test this hypothesis, we first determined if the increased expression of CTLA-4, known to occur on day 2 of the GVH response, was also observed on day 3. At the same time, we evaluated the expression levels of genes for *Ifng*, *Ido1*, and *Ido2*. *Ido2* is a paralog of *Ido1* (Ball *et al.*, 2007) that has previously been shown to be upregulated by TCDD (Vogel *et al.*, 2008). Because IDO production has been associated with pDCs, we also analyzed the expression of other genes associated with these cells, including *E2-2*, *Tlr7*, *Siglech*, *Tlr9*, *Lag3*, *Irf3*, and *Irf7* to determine if additional functions of the pDCs may be affected by TCDD *in vivo* (Matta *et al.*, 2010).

The gating strategy for identifying donor CD4$^+$ T cells in host spleen by flow cytometry is shown in Figure 2A. The percentage and number of donor CD4$^+$CD25$^+$CTLA-4$^+$ cells were significantly increased in TCDD-treated animals relative to vehicle on days 2 and 3 of the GVH response (Figs. 2B and 2C). The expression of CD25 was also significantly increased on a per cell basis (MFI) on day 2; however, this difference was lost on day 3 as the expression level of CD25 declined in both treatment groups (Fig. 2D). In contrast, the MFI of CTLA-4 was significantly increased by TCDD on both days (Fig. 2C).

Expression levels of *Ifng*, *Irf3*, *Irf7*, and *Ido2* were significantly upregulated in the lymph nodes of TCDD-treated host mice on day 2 (Table 1). On day 3, both *Ido1* and *Ido2* expression levels were increased over 10-fold in TCDD-treated mice, along with increased expression of *Ifng* and *Irf7*. IFN-γ protein levels were also significantly increased by TCDD as measured in supernatants of host splenocytes on day 3 (Fig. 3). On the other hand, the level of secreted L-kynurenine in day 3 supernatants was not significantly different between vehicle- and TCDD-treated cultures, suggesting that the overall enzymatic activity of IDO was not affected by TCDD.

Role of IDO in TCDD-Mediated Suppression

Although no difference in IDO activity was observed in spleen cell culture supernatants from vehicle- and TCDD-treated mice,

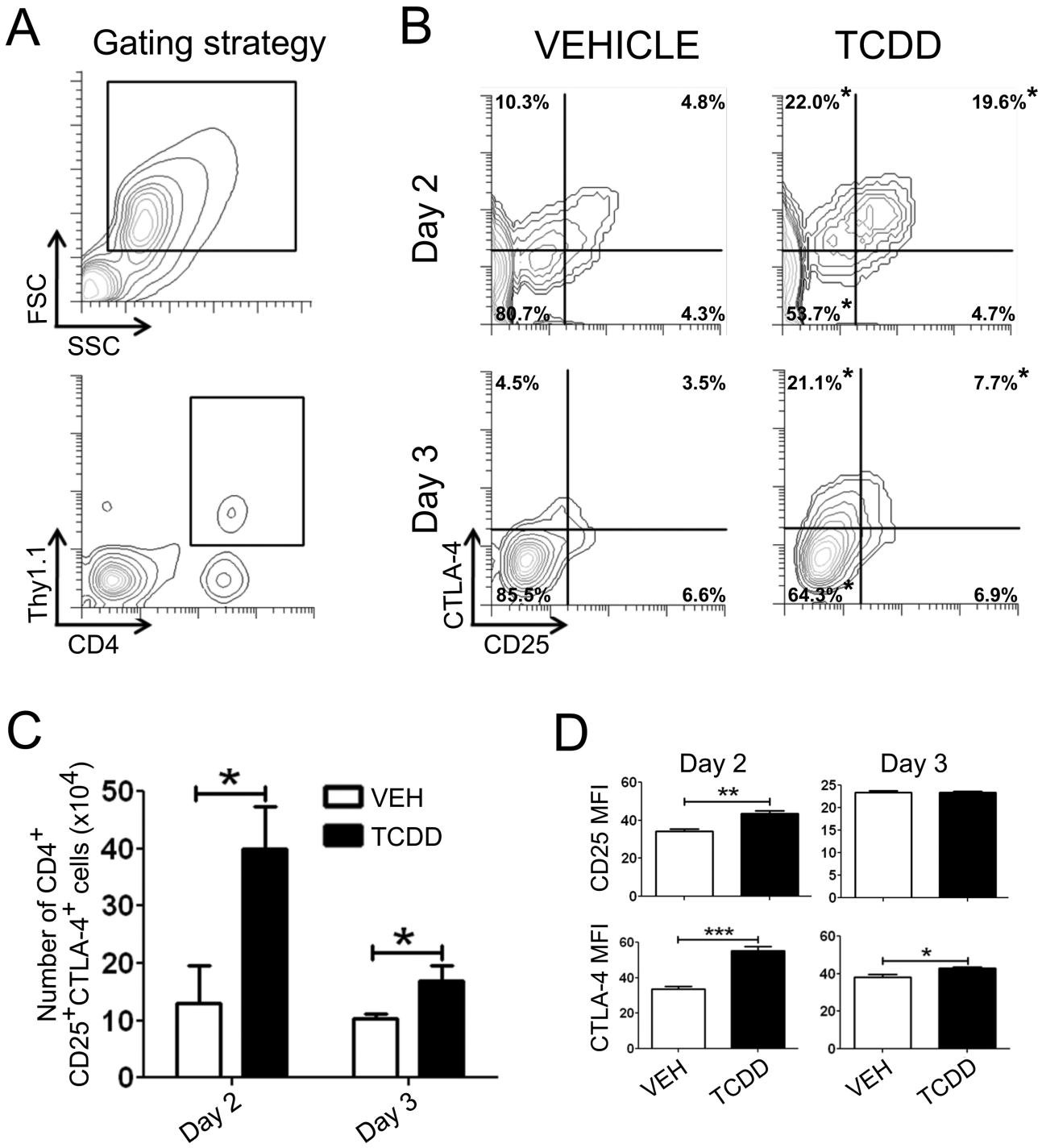

FIG. 2. Influence of TCDD on CD25 and CTLA-4 expression in donor CD4⁺ T cells on day 2 or 3 of the acute GVH response. GVHD was initiated by adoptive transfer of purified donor CD4⁺ and CD8⁺ T cells. (A) Using the forward scatter (FSC) versus side scatter (SSC) profile, lymphocytes were identified. Donor CD4⁺ T cells were identified by CFSE dilution (day 2) or the congenic marker Thy1.1 (day 3). (B) Flow cytometric analysis of CD25 and CTLA-4 expression on donor CD4⁺ T cells in vehicle- or TCDD-treated animals 2 or 3 days after adoptive transfer. (C) Total number of donor CD4⁺ T cells expressing CD25 and CTLA-4. (D) Median fluorescence intensity (MFI) of CD25 and CTLA-4 in donor CD4⁺ T cells. Statistical differences, calculated by Student's *t*-test, are denoted by asterisks. Error bars represent the mean ± SEM, $n = 4–5$ biological replicates.

the bulk measurement may not reveal differences in IDO activity at the interface of DC-T cell interaction. To directly evaluate the functional role of IDO in TCDD-mediated suppression of the GVH response, host mice were given 1-MT, a pharmacological inhibitor of IDO's enzymatic activity, in the drinking water beginning 2 days before injection of donor cells and

continuing for 15 days thereafter (Fig. 4A). Water consumption per cage was monitored daily to estimate intake of 1-MT. The average dose of 1-MT in vehicle-treated mice was 277 mg/kg/day compared with 324 mg/kg/day in TCDD-treated mice, both within the range of 1-MT doses shown to be effective at suppressing T-cell responses (Kwidzinski *et al.*, 2005; Sakurai *et al.*, 2002; Uyttenhove *et al.*, 2003).

The effect of 1-MT on the GVH response was monitored by body weight loss and donor $CD8^+$ T-cell engraftment in the spleen of host mice 15 days after adoptive transfer. Donor $CD8^+$ T cells were identified by the absence of $H\text{-}2D^d$ expression, and CTL activity was estimated based on the percentage of $CD19^+$ host B cells in the spleen. As summarized in Figure 4, vehicle-treated mice lost a significant amount of body weight beginning on day 9 of the GVH response, and treatment with 1-MT did not influence this loss. In agreement with previous studies (Kerkvliet *et al.*, 2002), TCDD treatment prevented the loss of body weight associated with the GVH response, and this was also unaffected by 1-MT. Donor $CD8^+$ T-cell engraftment was reduced in TCDD-treated mice and the increased percentage of host B cells in the spleen of TCDD-treated mice validated suppression of the allo-CTL response. Treatment with 1-MT did not affect donor cell engraftment or CTL activity in either vehicle- or TCDD-treated mice. The absence of effects of 1-MT on these endpoints suggests that increased IDO activity is not a primary mechanism for suppression of the GVH response by TCDD. However, this conclusion must be tempered by the lack of an effect of 1-MT on the GVH response in vehicle-treated mice as well.

TABLE 1
Influence of TCDD on Expression of Genes Associated With the CTLA-4-IFN-γ-IDO Signaling Pathway

	Fold change (TCDD/VEH)	
Gene	Day 2	Day 3
Cyp1a1	208*	300*
Ido1	1.6	12.6*
Ido2	3.0*	10.7*
Ifng	3.0†	1.9
Irf3	1.9*	0.8
Irf7	2.6†	2.7*

Note. Expression of *Cyp1a1* was queried as an indicator of AhR activation. Genes associated with pDCs were also evaluated, including *E2-2*, *Siglech*, *Lag3*, *Irf3*, *Irf7*, *Tlr7*, and *Tlr9*. RNA was isolated from the cervical, brachial, and axial lymph nodes from host mice on days 2 or 3 of the GVH response and used to query gene expression. Fold change ($2^{-\Delta\Delta Ct}$) was calculated relative to vehicle (VEH). n = 4–5 biological replicates. Student's t-test was used to calculate significance.
†$p = 0.1$, *$p < 0.05$.

Functional Requirement for CTLA-4 in TCDD-Mediated Immunosuppression

Although the lack of effect of 1-MT on TCDD-induced suppression did not support the CTLA-4-IFN-γ-IDO hypothesis, CTLA-4 can also mediate suppression of T-cell responses by pathways independent of IDO. To determine if increased CTLA-4 expression was directly involved in the suppression of the GVH response by TCDD, we tested whether blockade of CTLA-4 could prevent TCDD-induced suppression of GVHD. Host mice were treated with anti-CTLA-4 antibody on days 0, 1, and 2 relative to donor T-cell transfer (Fig. 5A), and GVHD pathology was assessed on day 11 by analyzing donor $CD8^+$ T-cell engraftment and host $CD19^+$ B-cell depletion. Relative to vehicle-treated animals, treatment with TCDD suppressed donor T-cell engraftment and preserved the host B-cell population as expected. Anti-CTLA-4 treatment had no effect on GVHD pathology in either vehicle- or TCDD-treated animals (Figs. 5B and 5C). These results suggest that enhanced expression of CTLA-4 on donor $CD4^+$ T cells following treatment with TCDD does not play a functional role in the suppression of GVHD by TCDD.

Effect of Donor-Derived IFN-γ in TCDD-Mediated Immunosuppression

Because neither IDO nor CTLA-4 appeared to be required for TCDD-mediated immunosuppression, a potential independent

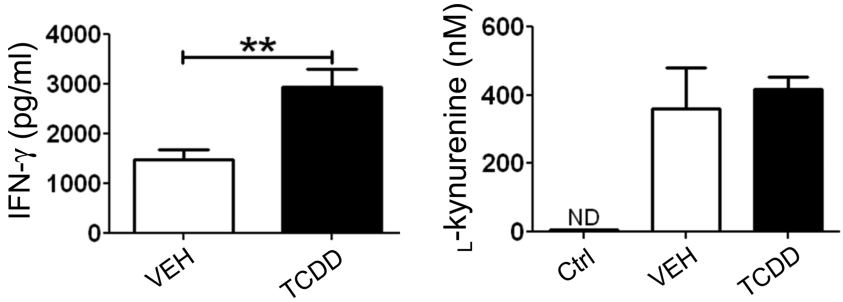

FIG. 3. Effect of TCDD on IFN-γ production and IDO enzyme activity. On day 3 of the GVH response, host splenocytes were isolated and cultured overnight for analysis of IFN-γ levels in the supernatant using an IFN-γ Ready-Set-Go ELISA (eBioscience). Host splenocytes were also prepared for quantification of IDO enzyme activity on day 3. Splenocytes were resuspended in 1× HBSS without phenol red and cultured with excess tryptophan (100μM) for 4 h. The concentration of L-kynurenine was quantified using liquid chromatography-mass spectrometry as a measure of IDO enzyme function. CTRL = background level of L-kynurenine in 1× HBSS alone. Error bars represent the mean ± SEM, n = 4–5 biological replicates. Student's t-test was used to determine significant differences between treatments. **$p < 0.01$. ND = not detected.

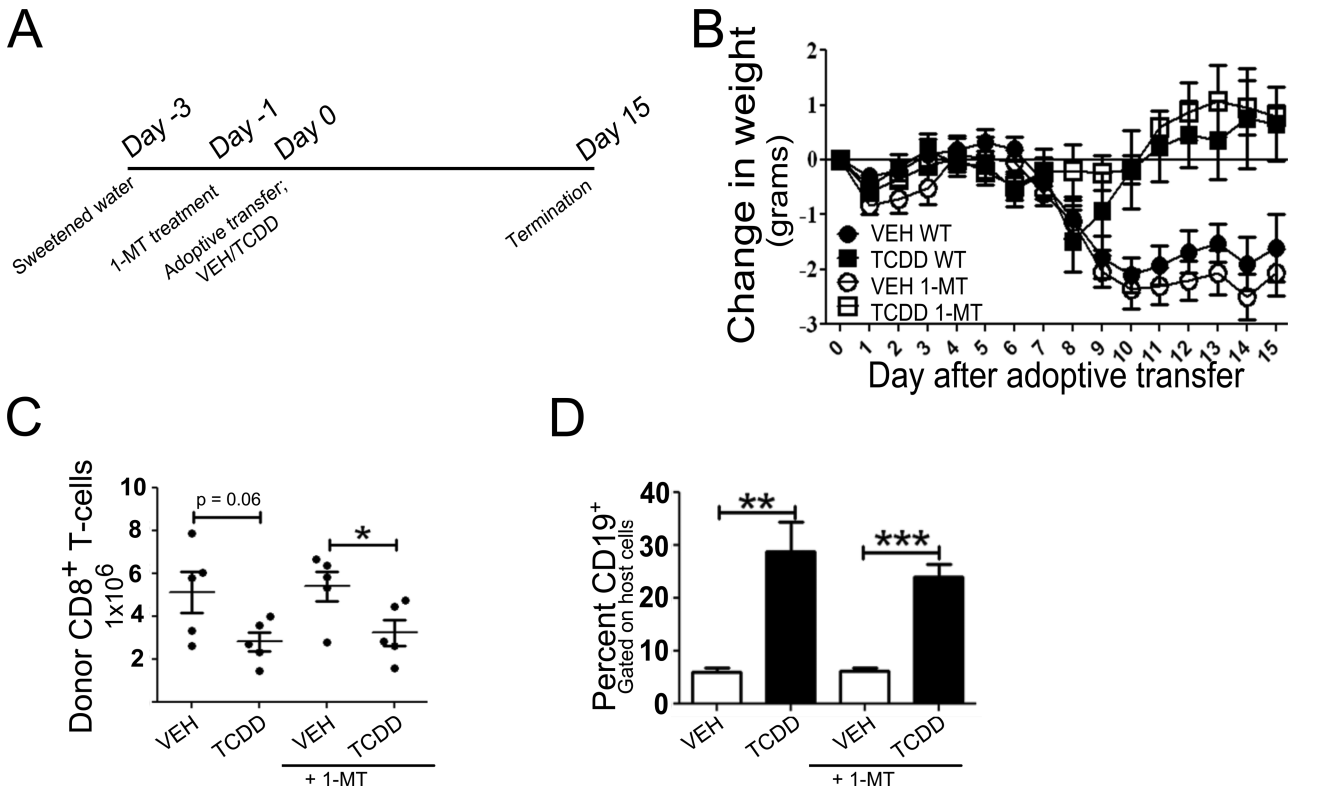

FIG. 4. Effect of inhibition of IDO enzyme activity on suppression of the GVH response by TCDD. (A) Schematic of experimental design. Three days prior to adoptive transfer of donor splenocytes, mice were given autoclaved, distilled water sweetened with aspartame. On day 1, treatment with 1-MT, a pharmacological inhibitor of IDO, was begun. (B) Change in host body weight following adoptive transfer. (C) Using flow cytometry, the percent of H-2D^{d} negative donor cells was determined and used to calculate the total number of donor cells from the total number of splenoctyes. The percent of H-2D^{dneg}CD8^{+} T cells was multiplied by the number of donor cells to calculate the total number of engrafted donor CD8^{+} T cells in the spleen of host animals. (D) CTL function was assessed as a measure of host Bcell depletion. Host B cells were identified by coexpression of H-2D^{d} and CD19. Error bars represent the mean ± SEM, $n = 5$ biological replicates. Treatment-related differences were determined using Student's t-test. *$p < 0.05$, **<0.01, ***$p < 0.001$.

role for IFN-γ was investigated. Based on the fact that nTregs from IFN-γ-deficient animals have attenuated suppressive function (Koenecke *et al.*, 2012; Markees *et al.*, 1998; Sawitzki *et al.*, 2005; Wei *et al.*, 2010), it is possible that AhR-Tregs also utilize IFN-γ to mediate suppression of the CTL response. If true, lack of IFN-γ in the donor cells should result in evidence of GVHD in TCDD-treated mice. To directly evaluate the role of donor-derived IFN-γ, host mice were injected with donor splenocytes obtained from C57Bl/6 (WT) or IFN-γ-deficient (*Ifng*$^{-/-}$) mice, which were then treated with vehicle or TCDD.

Vehicle-treated host mice that received *Ifng*$^{-/-}$ donor cells exhibited early onset and more severe body weight loss (Fig. 6A) consistent with previous results (Ellison *et al.*, 1998). Mice that received *Ifng*$^{-/-}$ donor cells also had significantly fewer splenocytes than mice with WT donor cells (Fig. 6B) yet a much higher proportion of B cells ($p < 0.001$) (Fig. 6C). These results are consistent with a prior study showing a selective loss of Fas-mediated killing of host B cells that is promoted by IFN-γ (Puliaev *et al.*, 2004). We also found that donor CD4^{+} and CD8^{+} T-cell engraftment was significantly reduced when the donor cells lacked IFN-γ (Figs. 6E and 6F). This was not due to a difference in the number of T cells injected in the initial donor cell inoculum (Fig. 6G).

Treatment of host mice with TCDD prevented body weight loss (Fig. 6A), decrease in splenocyte numbers (Fig. 6B), and donor T-cell engraftment (Figs. 6E and 6F) associated with GVHD, independent of the *Ifng* status of the donor cells. These results indicate that AhR-Tregs do not use IFN-γ for suppression of the GVH response.

DISCUSSION

Activation of AhR by TCDD during an acute GVH response induces Tregs with a suppressive capacity greater than that of natural Treg (Marshall *et al.*, 2008). These AhR-Tregs do not express Foxp3, but express high levels of other Treg-associated markers such as CD25 and CTLA-4 (Funatake *et al.*, 2005), suggesting that AhR functions as an autonomous ligand-dependent transcription factor for induction of Tregs. However, the mechanism by which AhR-Tregs mediate suppression of

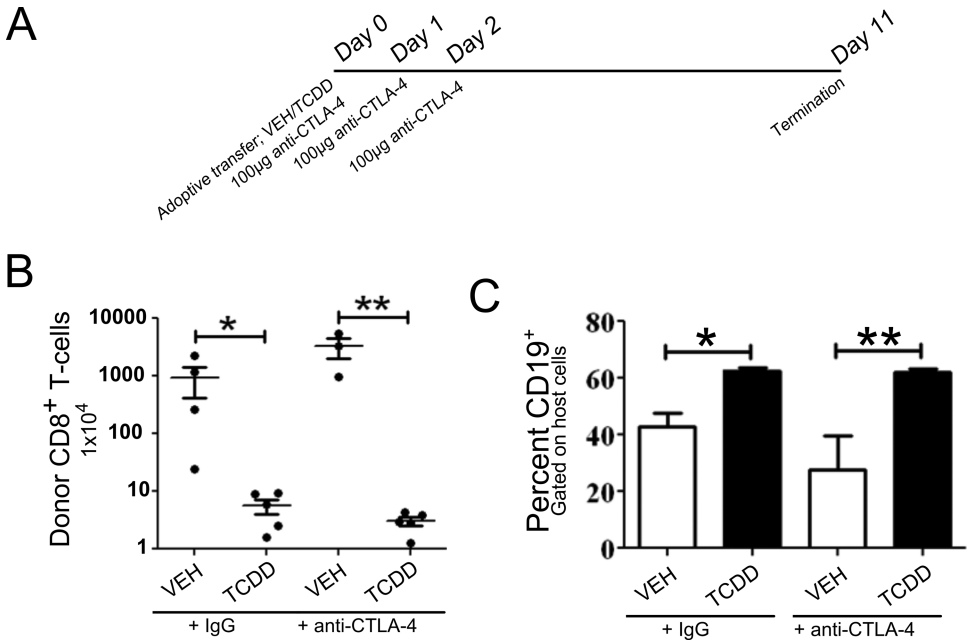

FIG. 5. Effect of blocking CTLA-4 during donor CD4⁺ T-cell differentiation on suppression of GVHD by TCDD. (A) Schematic of experimental design. One hundred micrograms of anti-CTLA-4 or control IgG was administered on days 0, 1, and 2 following adoptive transfer of purified donor T cells and treatment with either vehicle or TCDD. (B) Using flow cytometry, the percent of Thy1.1⁺ donor cells was determined and used to calculate the total number of donor cells from the total number of splenocytes. The percent of Thy1.1⁺CD8⁺ T cells was multiplied by the number of donor cells to calculate the total number of engrafted donor CD8⁺ T cells in the spleen of host animals. (C) CTL function was assessed as a function of host B-cell depletion. Host B cells were tracked by expression of CD19. Error bars represent the mean ± SEM, $n = 3–5$ biological replicates. For statistical analysis, the mixed procedure with the Satterthwaite option was used. $*p < 0.05$ and $**p < 0.01$.

immune function remains unclear. The studies presented here were designed to test the hypothesis that the enhanced expression of CTLA-4 induced by TCDD on donor CD4⁺ T cells activates the IDO pathway, resulting in suppression of the CTL response (see Fig. 1). However, although treatment with TCDD significantly upregulated expression of *Ifng*, *Ido1*, and *Ido2* in the lymph nodes and increased production of IFN-γ in spleen, blockade of CTLA-4 or IDO enzyme activity did not alleviate TCDD's suppressive effects. Furthermore, the use of IFN-γ-deficient donor cells did not influence suppression suggesting that AhR-Tregs do not rely on IFN-γ for their immunosuppressive activity.

We initially tested the role of IDO by blocking its catalytic activity with the pharmacological inhibitor 1-MT. However, treatment with 1-MT had no effect on the day-15 GVH response in either control or TCDD-treated animals. The lack of effect of 1-MT in control animals was initially surprising because IDO-deficient animals were reported to show increased GVHD-associated mortality (Jasperson *et al.*, 2008). However, the enhanced mortality in IDO-deficient mice was not due to excessive T-cell activation but rather to lack of upregulation of IDO in gut epithelial cells that reduced inflammatory injury in the colon. Taken together with our results, IDO and its associated depletion of tryptophan do not appear to play a prominent role in the regulation of T-cell responses in GVHD.

The inability of 1-MT to alter suppression of the GVH response in TCDD-treated animals indicates that the enzymatic activity of IDO is not involved in the suppressive mechanism of AhR-Tregs. This was surprising given the consistent upregulation of IDO by TCDD in this and other studies (Bankoti *et al.*, 2010; Vogel *et al.*, 2008). However, IDO has recently been shown to have signaling properties that are distinct from its catalytic function and are unaffected by 1-MT (Pallotta *et al.*, 2011). In this study, TGF-β-conditioned pDCs were shown to use phosphorylated IDO as a signal transducer that activated p52-RelB to promote TGF-β production, which in turn promoted the induction of Foxp3⁺ Tregs. This IDO signaling pathway could be involved in the generation of Foxp3⁺ Tregs by TCDD-activated AhR that has been reported in several different autoimmune disease models (Benson and Shepherd, 2011; Kerkvliet *et al.*, 2009; Quintana *et al.*, 2008). Interestingly, we have recently observed that treatment with TCDD induces Foxp3 expression in both donor and host CD4⁺ T cells on day 15 of the GVH response (Diana Rohlman, Castle Funatake, Sumit Punj and Nancy I. Kerkvliet, in preparation). However, when the induction of Foxp3⁺ expression was blocked, it did not affect suppression of the GVH response by TCDD. Thus, although IDO may play a role as a signaling molecule to upregulate Foxp3 expression, these Foxp3⁺ T cells are not involved in TCDD-mediated suppression of acute GVH responses.

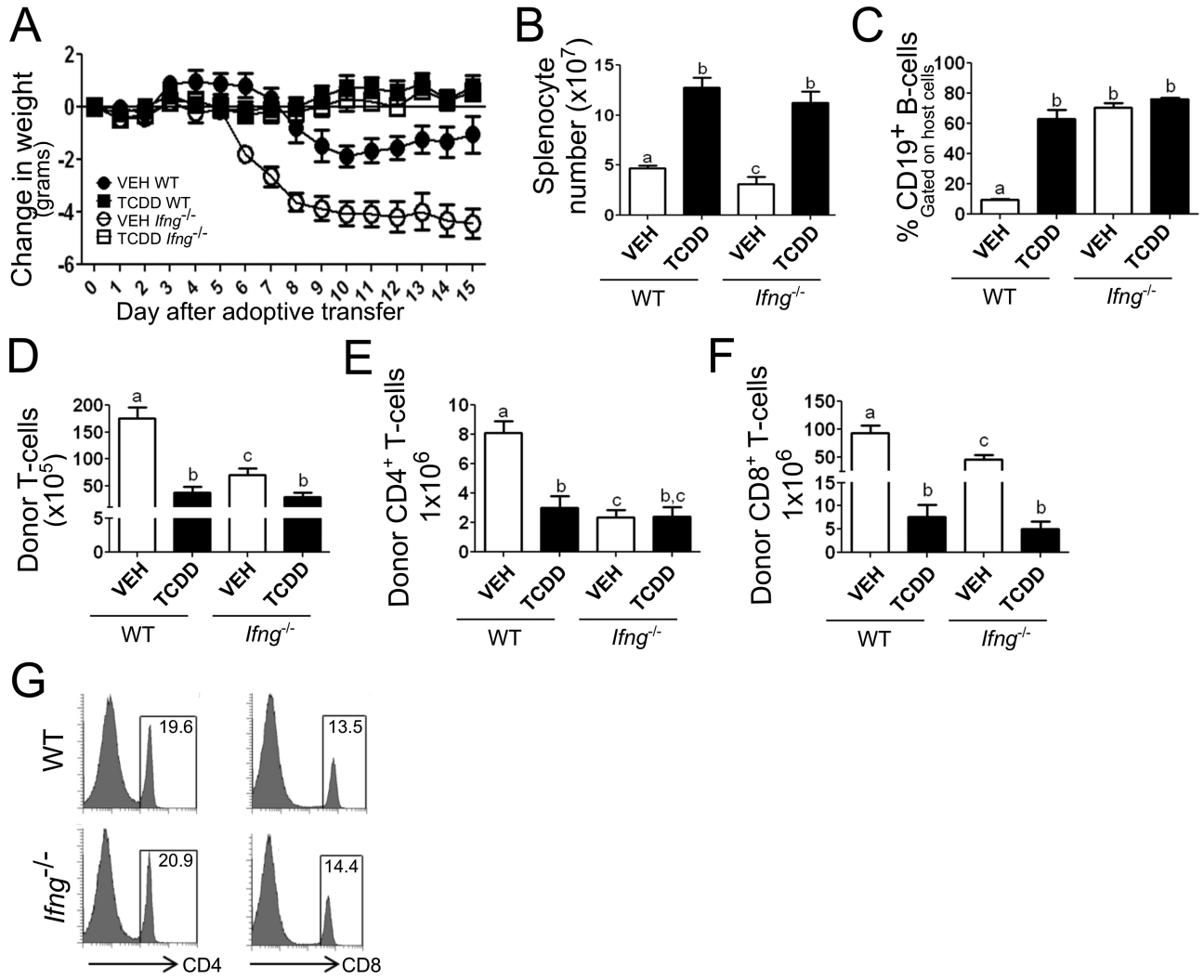

FIG. 6. Absence of IFN-γ in donor cells does not affect suppression of the CTL response by TCDD. (A) Change in host body weight following adoptive transfer. (B and C) Total splenocyte numbers on day 15. (C) Percent of $CD19^+$ B cells remaining in the host ($H\text{-}2D^{d+}$) fraction. (D) Total number of donor ($H\text{-}2D^{d\text{-}neg}$) T cells present in the spleen of host animals. Number of donor (E) $CD4^+$ or (F) $CD8^+$ T cells in the spleen of host animals. (G) Percent of $CD4^+$ and $CD8^+$ T cells in the donor inocula prior to adaptive transfer of splenocytes on day 0. Significant differences between treatment groups were calculated using Student's *t*-test ($p < 0.05$) and are denoted by different alphabetical characters. Error bars represent the mean ± SEM, $n = 5$ biological replicates.

Because IDO has been associated primarily with a subset of tolerogenic pDCs, we looked at expression of several genes associated with pDC phenotype and function in the lymph nodes of TCDD-treated host mice. Of the genes examined, expression of *Irf3* and *Irf7* were significantly increased by TCDD. *Irf3* and *Irf7* code for transcription factors involved in regulation of expression of type I IFNs (IFN-α/β) (Honda *et al.*, 2005). TCDD was previously shown to increase *Irf3* expression in cultured U937 cells, and the mechanism was shown to be AhR and RelB dependent (Vogel *et al.*, 2007). Increased production of type I IFNs by pDCs is a downstream consequence of IDO signaling (Pallotta *et al.*, 2011), suggesting that increased expression of *Irf3* and *Ido* may be linked in TCDD-treated pDCs. Although type I IFNs have long been known to play critical roles in virus defense, they have recently been shown to promote the development of $Foxp3^+$ T cells and enhance their suppressive function (Gonzãlez-Navajas *et al.*, 2012). The involvement of this pathway in AhR-mediated immunoregulation remains to be shown.

Apart from altered interactions with host DCs, upregulation of CTLA-4 on T cells could play a direct role in suppressing effector T-cell activation and/or proliferation by outcompeting CD28 signaling. However, when host mice were treated with an antibody that neutralizes the function of both the full-length and soluble isoforms of CTLA-4 (Ward *et al.*, 2013), there was no effect on the suppression of the GVH response by TCDD. There was also no effect of antibody treatment on the GVH response in vehicle-treated mice. A similar treatment regimen in a murine tumor model resulted in increased expansion of antigen-specific T cells in response to HA-expressing tumor cells but did not prevent tolerance to tumor antigen (Sotomayor

et al., 1999). In addition, although the same anti-CTLA-4 antibody has been shown to exacerbate symptoms of disease in colitis, diabetes, and experimental autoimmune encephalomyelitis (Luhder *et al.*, 1998; Perrin *et al.*, 1996; Watanabe *et al.*, 2008), these studies used repeated dosing throughout the course of disease development. Because we specifically targeted CTLA-4 blockade to the first 3 days of the GVH response, during the window of susceptibility to TCDD and coincident with AhR-Treg differentiation, any effects of CTLA-4 blockade on the CTL effector phase of the immune response would have been spared. In fact, the half-life of the anti-CTLA-4 mAb (9H10) that we used has been shown to be 3–4 days (Allison, personal communication). This explanation is consistent with previous studies showing that antibody blockade of CTLA-4 in regulatory T cells alone was insufficient to enhance antitumor responses, whereas blockade of CTLA-4 in effector cells increased antitumor activity (Peggs *et al.*, 2009). Thus, the inability of CTLA-4 blockade to affect TCDD-mediated suppression of GVHD indicates that increased expression of CTLA-4 on AhR-Treg is not involved in their suppressive function.

The apparent lack of involvement of CTLA-4 and IDO in TCDD-mediated suppression of GVHD led us to consider alternate pathways that would address the increase in IFN-γ seen in TCDD-treated mice (Fig. 3). Based on recent studies that demonstrated an autocrine role for IFN-γ in the suppressive mechanism of nTreg in Th1-mediated diseases (Beeston *et al.*, 2010; Koenecke *et al.*, 2012), we hypothesized that AhR-Tregs produce IFN-γ to mediate suppression of GVHD. *Ifng*$^{-/-}$ donor cells were used to initiate GVHD and the effects on the CTL response in the presence or absence of TCDD were examined. In vehicle-treated mice, GVHD was exacerbated by the lack of IFN-γ in the donor cells, in agreement with prior reports (Ellison *et al.*, 1998; Koenecke *et al.*, 2012; Yang *et al.*, 1998). Splenocyte numbers were decreased, but percent of B cells was not diminished. This phenomenon has been attributed to the loss of IFN-γ-induced Fas expression on host B cells, preventing their selective targeting for FasL-mediated killing by CTL (Puliaev *et al.*, 2004). Non-Fas-mediated pathways of cytotoxicity (i.e., perforin) were not affected by the lack of IFN-γ (Puliaev *et al.*, 2004). However, our data indicate that donor T-cell engraftment is altered by the lack of IFN-γ, because significantly fewer CD4$^+$ and CD8$^+$ donor T cells were present in the spleen of mice receiving *Ifng*$^{-/-}$ donor cells. This is surprising, given their exacerbated symptoms of GVHD. The exacerbation of GVHD in mice that received *Ifng*$^{-/-}$ donor cells has been attributed to loss of suppressive activity of nTreg (Koenecke *et al.*, 2012). Unlike the nTreg, AhR-Tregs apparently do not use IFN-γ as a suppressive mediator, because TCDD-treated animals did not lose weight and showed minimal evidence of a CTL response.

In summary, we found no evidence to support the hypothesis that AhR-Tregs induced by TCDD during a GVH response rely on the tolerogenic IDO pathway, CTLA-4 *per se*, or IFN-γ for suppression of the GVH response. This is in contrast to previous studies implicating IDO in Treg induction by several AhR ligands including TCDD (Mezrich *et al.*, 2010; Nguyen *et al.*, 2010; Vogel *et al.*, 2008). However, these studies have focused on the expression of Foxp3 as a marker of Treg induction, which may arise from direct AhR-dependent induction of IDO in pDCs to drive Foxp3$^+$ Treg expansion. The present study also confirms *Irf3* and identifies *Irf7* as potential AhR targets for induction of Foxp3$^+$ Tregs. However, these Foxp3$^+$ Tregs induced by TCDD are distinct from the Foxp3^{neg} Tregs that emerge early in the GVH response and are dependent on AhR expression in the CD4$^+$ T cell itself (Funatake *et al.*, 2005). These AhR-Tregs share characteristics with Tr1-like Tregs including increased IL-10 and granzyme B expression (Gandhi *et al.*, 2010; Marshall *et al.*, 2008). The mechanisms of suppression used by AhR-Tregs *in vivo* remain to be defined.

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