Quantification of Cytokine Production Following Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) to Determine Potential Role in Suppression of the Immune Response in a Graft versus Host (GvH) Model

By

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent immunosuppressant and a prototypic ligand for the aryl hydrocarbon receptor (AhR). In a graft versus host (GvH) response, treatment with TCDD suppresses a cytotoxic T-lymphocyte (CTL) response by Day 10, concurrent with a T-regulatory (T_{reg}) like phenotype in the donor $CD4^+$ T-cells, observed on Day 2. The phenotype in $CD4^+$ T-cells has been characterized as the following: $CD25^{high}$, $CD62L^{low}$, $CTLA\text{-}4^+$, and $IL\text{-}10^+$. TCDD-mediated suppression may be due to the induction of this T-regulatory cell, which leads to altered cytokine production. In addition to IL-10, IFN-g also plays an important role in T cell differentiation. Previous experiments have measured the effects of TCDD on cytokine production only on day 2 of a GvH response. We were therefore interested in seeing if changes in cytokine production persist through day 3, when the T_{reg} phenotype is still evident. We tested supernatants from prior GvH experiments to determine levels of these cytokines using enzyme-linked immunosorbent assays (ELISA). The results of these assays indicate no significant difference in IL-10 cytokine production between day 2 and day 3. However, on day 3, IFN-g was increased in the supernatants of cells from TCDD-treated animals, indicating that TCDD up-regulated IFN-g production. There are other cytokines also produced by T_{regs}, for example IL-2, IL-27 and TGF-b, which were not quantified and which could be involved in TCDD-mediated immunosuppression seen in a GvH model.

Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent immunosuppressant and a prototypic ligand for the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor (11). The AhR is bound to several co-chaperones within the cell (FIG. 1). When TCDD binds to the AhR, the co-chaperones dissociate, and the TCDD-AhR complex translocates to the nucleus. In the nucleus, the aryl hydrocarbon receptor nuclear translocator (ARNT) is dimerized by the AhR/TCDD structure (11). This combination can lead to altered gene expression and altered cellular function, specifically in T-cells (11).

In a graft-versus-host (GvH) model, Kerkvliet et al. showed that the TCDD-mediated suppression is dependent upon AhR expression in the donor $CD4^+$ T-cell, as well as in the $CD8^+$ T-cell (8). Treatment with TCDD resulted in dose-dependent suppression in a cytotoxic T-lymphocyte (CTL) response (7). Delayed treatment, to day 4 or 5, of TCDD after P815 cells were injected decreased the suppression of the CTL response.

The GvH model involves injecting a host B6D2F1 ($H\text{-}2^{b/d}$) mouse with semi-allogeneic donor cells from a C57Bl/6 ($H\text{-}2^{b/b}$) mouse (FIG. 2). The donor T-cells recognize the MHC disparity and begin the anti-host response (13). As only purified donor T-cells are injected, this model is used to look at T-cells specifically. Once the donor T-cells are activated, they begin to differentiate. There are different types of T-cells that become activated in an immune response. For example, T-helper (Th) cells signal and recruit immune cells like neutrophils, macrophages and other lymphocytes to create an immune response. Th cells activate cells. These $CD4^+$ T cells can become Th1 or Th2 cells (13). Other cells, like those mentioned above, are the T_{reg} and Th17 cells.

Regulatory T-cells (T_{regs}) are T-cell subsets with immunosuppressive functions (13). One T_{reg} phenotype, $CD4^+CD25^+$, is also present in AhR^+ donor T-cells treated with TCDD. Further

characterization of the $CD4^+C25^+$ T_{reg} continued. Treatment with TCDD led to a T-regulatory like phenotype in the $CD4^+$ T-cell, characterized as $CD25^{high}CTLA\text{-}4^+CD62L^{low}$ phenotype, producing IL-10, an immunosuppressive cytokine (10). The cytokine, IL-10, is a key player in immunosuppression (19).

Cytokines, also known as interleukins, are released by immune cells such as dendritic cells and T-cells, and play a role in generating an adaptive immune response, as well as contributing significantly to T-cell differentiation (13). The potent suppression by TCDD may thus be due to the induction of this T_{reg}, which leads to altered cytokine expression. However, this cytokine production is dependent on several factors, including the model being used, as well as the time at which they are measured. Hence, production could vary on different days, dependent upon the resulting adaptive immune response.

More than fifty cytokines are involved in the human immune response mechanism (13). Several of those cytokines are involved in the AhR pathway, including IL-10 and IFN-g (11, 16). IL-10 is an inhibitory factor for cytokine synthesis and associated with T-regs. On day 2 of a GvH, in animals treated with TCDD, $CD4^+$ donor T-cells produced more IL-10 than vehicle (10). IFN-g activates macrophages and is associated with Th1 cell differentiation and function (13). The co-culture of allogeneic T-cells with DCs from mice treated with TCDD increased the production of IFN-g. (16). Therefore, IL-10 and IFN-g were of interest in this study because there are indications that the AhR, following activation by TCDD, may regulate the expression of these cytokines. Additionally, IL-10 was previously only examined on a day 2 GvH. We were therefore interested in seeing if changes in cytokine production persist through day 3.

To examine cytokine production following treatment with TCDD, we first employed a modified mixed lymphocyte model. In a mixed lymphocyte reaction (MLR), semi-allogeneic naïve lymphocytes are co-cultured with two types of cell culture samples. If the two organisms

are non-compatible, the cell culture will proliferate rapidly, and if they are compatible, they will not proliferate. The ones that proliferate react due to foreign major histocompatibility complex (MHC) (13). The MHC is a gene family that plays a significant role in autoimmunity and the immune system. MHCs act as a lookout post for foreign invaders, and alert the immune system. The MHC is divided into two classes: MHC class I and MHC class II. These classes interact with T-cell subsets. MHC class I stimulates $CD8^+$ T-cells, whereas MHC class II stimulates $CD4^+$ T-cells (13). The modified MLR that we used was similar to a GvH in that H-$2^{b/b}$ T-cells from C57Bl/6 mice were cultured with H-$2^{b/d}$ B6D2F1 antigen-presenting cells (APCs). The "b" haplotype from the T-cell recognizes the "d" haplotype from the APC as foreign. Recognizing the d as foreign, the b haplotype sends a signal causing a cascade reaction, including cytokine production (13). The T-cell uses a T-cell receptor to recognize foreign antigen presented by MHC.

We examined IL-10 and IFN-g cytokine production to understand the involvement of these cytokines in TCDD-mediated immunosuppression. We attempted to use a modified MLR to examine the influence of TCDD on IL-10 and IFN-g production in vitro. Additionally, production of IL-10 and IFN-g in day 2 and day 3 GvH supernatants from the Kerkvliet lab was analyzed by ELISA method (FIG. 3).

Materials and Methods

Animals. C57Bl/6J (H-$2^{b/b}$) mice originally purchased from Jackson Laboratory (Sacramento, CA) were bred and maintained in the animal facility at Oregon State University, 97331. B6D2F1 (H-$2^{b/d}$) mice were purchased from Jackson Laboratory. Animals were kept according to the Animal Care and Use Proposal and IACUC guidelines at Oregon State University, 97331. Animals were sacrificed via CO_2 asphyxiation, followed by cervical dislocation.

Reagents

Fetal bovine serum, FBS, was purchased from HyClone (Logan, UT). Gentamicin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), carboxyfluorescein succinimidyl ester, (CFSE), and RPMI 1640 were purchased from Invitrogen (Carlsbad, CA). Ficoll was purchased from GE Healthcare (Waukesha, WI). 35% bovine serum albumin, BSA, was purchased from Amresco, (Solon, Ohio). Spleen Dissociation Medium was purchased from StemCell (Newark, CA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Molecular Sigma Biology (St. Louis, MO).

Splenocyte Preparation. Spleens were aseptically removed. Single cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides into processing media containing 2.5% FBS, 50μg/ml gentamicin, 10mM HEPES, 1X HBSS, and H_2O. Red blood cells were removed by hypotonic water lysis.

Ficoll Gradient. The spleen suspension was carefully layered over 3ml Ficoll gradient. After centrifugation at 3,200 RPM, 4˚C, the upper layer was removed and the lymphocyte layer removed to a clean conical tube. Processing medium equal to three times the lymphocyte layer volume was added and gently mixed via pipette. The mixture was centrifuged again at 3,200 RPM at 4˚C and the supernatant was removed and washed several times.

BSA Gradient. Following red blood cell lysis of the splenocytes preparation, the cell pellet was resuspended in 2.5 ml 35% BSA and overlaid with RPMI. After centrifugation at 9,500g at 4˚C, cells from the interface region were collected and used as the APC fraction.

CD4-APC co-cultures. Using the $CD4^+CD62L^+$ Positive Isolation II kit from Miltenyi, $CD4^+CD62L^+$ T-cells were isolated, and then stained with CFSE from Miltenyi autoMACS (Auburn, CA). APC's were isolated from B6DF1 ($H\text{-}2^{b/d}$) mice using the 35% BSA gradient as described above. T-cells were titrated into APC's at the following T:APC ratios: 10:1, 5:1, 1:1, 1:5, 1:10.

Antibodies. The following primary antibodies were used for flow cytometry analysis: 7-aminoactinomysin D (7-AAD) from Calbiochem (Darmstadt, Germany) and FITC-Mac1 from Caltag (Carlsbad, California). The following antibodies were purchased from BD Biosciences (San Diego, CA): B220-APC-Cy7, GR1-FITC and CD4-PE. In addition, antibodies were also purchased from eBioscience (San Diego, CA): CD8-APC, CD11c-APC, Ter119-FITC, and CD49b-FITC.

Flow Cytometry. After 3 days in culture, cells were transferred to a 96-well plate with wells assigned for samples and compensation controls. Cells were washed with PAB (PBS+, 1% BSA, and 0.1% sodium azide) and resuspended in Rat IgG to block Fc receptors. Surface staining was performed on ice. Viability was measured with 7-AAD. Data analysis and software compensation were performed using WinList from Verity Software House (Topsham, ME).

GvH preparation

Donor spleens were processed as previously described. T-cells ($CD4^+$, $CD8^+$) were obtained with the use of a PanT kit (Miltenyi Biotech). Sex-matched F1 host mice were injected via tail vein with $2x10^7$ B6 donor T-cells. TCDD and vehicle treatments were administrated via gavage to the

mice. After 48 h or 72 h host spleens were harvested for cell culture.

Collection of GvH Supernatants

Following a day 2 or day 3 GvH, splenocytes ($1x10^7$) from host mice (B6D2F1) were cultured overnight in 0.5ml of RPMI 1640 media, supplemented with 10% FBS, 10mM HEPES, 50µg/ml gentamicin, and 50µM 2-Me stock solution. Cells were spun down and supernatant collected and then stored at -80˚C.

Cytokine assays

The concentration of IL-10 and IFN-g in culture supernatants was measured using cytokine-specific enzyme-linked immunosorbant assays (ELISAs). Mouse IFN gamma ELISA Ready-SET-Go!® and Mouse IL-10 ELISA Ready-SET-Go!® were purchased from eBioscience (San Diego, CA) and assays were performed following the manufacture's recommended protocol. Supernatants for day 3 IFN-g were diluted twice, thus values were multiplied by 2.

The basic methodology of the ELISA consisted of four steps. First, the sample bound to the support and any antigen present bound to the capture antibody. Second, a primary antibody was added, which bound to the antigen. After this step several washes were carried out. Third, a secondary antibody-enzyme conjugate was added and this conjugate bound to the detection antibody. Again, several washes were repeated. Fourth, a substrate solution was added and converted to a detectable form to be quantified.

The Ready-Set-GO ELISA protocol that was conducted for the purpose of cytokine analysis was done over a three-day period. On day one, a 96-well plate was coated with capture antibody in Coating Buffer, which was part of the reagent set. The plate was sealed and incubated overnight at 4˚C. On the second day, wells were aspirated and washed with Wash

Buffer (1000mL PBS, 0.05% Tween) five times. Wells were then blocked with Assay Diluent and incubated at room temperature for an hour. Wells were aspirated and washed five times again. Standards were diluted in Assay Diluent by a two-fold dilution and supernatant samples were added to the indicated wells. A plate design was made to organize sample distribution. The plate is resealed and incubated at 4˚C overnight. On day three, wells were aspirated and washed five times. A detection antibody, which was diluted in Assay Diluent give dilution factor, was added and the plate was sealed and left at room temperature for 1 hour. Wells are then aspirated and washed five times. The detection enzyme, Avidin-HRP, was added. Avdin-HRP was diluted in Assay Diluent. Wells are aspirated and washed seven times. Tetramethylbenzidine (TMB), the substrate solution, is added to each well and the plate is left to incubate at room temperature for 15 minutes. Then, stop solution is added to each well. The plate is read at 450nm in a spectrometer.

Statistical analysis. Results presented are the mean ± SE of 3-5 mice per group. To demonstrate statistical significance, the Student's t-test, with $p < 0.05$ considered to be statistically significant, was used.

Results

Isolation of APC's for use in a modified MLR

In an MLR, $CD4^+$ T-cells are activated by semi-allogeneic APC's and predominantly by $CD11c^+$ dendritic cells. CD11c is a lineage marker for dendritic cells, which are considered professional APCs. $CD4^+$ T-cells were purified above 90%, but the isolation of APC's could not be accomplished by using the same principle of magnetic separation. In order to enrich the APC

fraction, both a Ficoll and a 35% BSA gradient were tested. To determine the percent of APC's (dendritic cells and B-cells) and contaminating cell subsets (T-lymphocytes, red blood cells and granulocytes), flow cytometry was used. Fluorescent conjugated antibodies specific to lineage markers of the different cell subsets were used for flow cytometric analysis (Table 1). When APCs were enriched using a Ficoll gradient, there was a high percent of contaminating red blood cells (30%), while less than 5% of cells expressed $CD11c^+$ (FIG. 4A). Expression of $GR1^+$, $CD4^+$ and $CD8^+$ cells was below 5%.Though T lymphocytes were removed, the Ficoll gradient was inefficient at removing $B220^+$ cells, which were enriched to 60%. Thus, we concluded that Ficoll was not appropriate for preparing APCs.

A 35% BSA gradient has previously been used to enrich APCs, such as B-cells, macrophages, and dendritic cells (16). In the method preparation of DCs by Voderstrasse et al. splenic cells were spun over a BSA gradient and the resulting APC fraction was used in an MLR. In the BSA gradient, expression for $CD11c^+$ cells increased to 28%, while enrichment of $B220^+$ cells was at 58%, similar to the enrichment seen with the Ficoll gradient. However, the Ter119 population decreased to only 9%, $GR1^+$, $CD4^+$ and $CD8^+$ cells were expressed at 15%, 27% and 15% (FIG. 4B). Though the percentage of contaminating red blood cells decreased, the 35% BSA gradient was unsuccessful in efficiently removing T-lymphocytes and granulocytes. A confounding factor is the potential of co-expression of lineage markers. For example, GR1+ and B220 can co-express. However, these measurements were analyzed individually, and therefore did not account for co-expression. This explains why the individual percentages can add up to more than 100%.

Given that the 35% BSA gradient appeared to selectively enhance the $CD11c^+$ population, this method was used for the T:APC co-cultures.

Semi-allogeneic APC's do not support T-cell proliferation

To mimic cytokine production during a semi-allogenic GvH, a modified MLR was employed. Host APCs (H-$2^{b/d}$) were cultured with naïve semi-allogeneic $CD4^+$ T-cells (H-$2^{b/b}$). The co-cultures were set up in various ratios (10:1, 5:1,1:1, 1:5, and 1:10), and after 3 days of culture were stained to measure culture proliferation (CFSE) and activation (CD25) of CD4+ T-cells. Figure 5A shows excellent activation and proliferation of semi-allogeneic $CD4^+$ T-cells, activated with enriched $CD11c^+$ host cells, demonstrated by clear CFSE dilution and increased CD25 expression. However, in the modified MLR assay, none of the ratios tested resulted in activation or proliferation of $CD4^+$ T-cells (FIG. 5B). CFSE had not diluted, indicating a complete lack of cell division. Additionally, low expression of CD25 suggested the T-cells were not activated. These results suggest that the T:APC *in vitro* assay does not result in T-cell proliferation, and is not a viable method to examine cytokine production as $CD4^+$ T-cells must be activated to be sensitive to TCDD effects (4, 14). Thus, TCDD-mediated effects on cytokine production cannot be examined by this in vitro approach.

IL-10 is produced during a GvH

Because we were unable to generate activated T cells in the MLR in vitro, we decided to examine cytokines in supernatants collected from prior GVH experiments in the Kerkvliet laboratory. Following adoptive transfer, host mice (H-$2^{b/d}$) were treated with either TCDD or vehicle (VEH). Host mice were sacrificed on either a day 2 or day 3 GvH and the spleens were removed. Splenocytes were cultured overnight in culture media and the supernatants were collected for cytokine analysis. IL-10 protein levels on day 2 and day 3 of the GvH were quantified by ELISA to determine the presence of this cytokine.

The day 2 GvH supernatants had similar IL-10 protein levels in VEH compared to TCDD

(FIG. 6A). Day 3 GvH supernatants had no significant difference in IL-10 production between VEH and TCDD (FIG. 6B). Therefore, treatment with TCDD in a day 2 and day 3 GvH did not appear to increase IL-10 production.

IFN-g is produced in a GvH

The assay was carried out to measure IFN-g production on day 2 and day 3 of a GvH using an ELISA. The same method that generated IL-10 samples was used for these samples. On day 2, IFN-g protein levels were equivalent in TCDD and VEH-treated mice (FIG. 7A). Quantification of IFN-g from a day 3 GvH found a significant increase in TCDD over VEH ($p=0.011$), indicating that TCDD up-regulated IFN-g production on day 3 of a GvH (FIG. 7B).

Discussion

Cytokines influence T-cell differentiation. For example, IFN-g drives Th1 cells, IL-4 drives Th2 cells, IL-6 and TGFb1 drive Th17, and TGFb1 drives T_{reg} (11). All these cells have different functions. The T_{reg} is a moderating cell that focuses on the suppression of the immune system (13). Thus, knowing that TCDD-activated AhR alters cytokine production, AhR may influence $CD4^+$ differentiation via altered cytokine production (7). Cytokines can be quantified from supernatants that have been produced during a GvH. However, it is costly to perform an *in vivo* experiment if supernatants are the objective of the collection. If an *in vitro* MLR assay could be developed, it would be both cost and time effective.

We attempted to develop a semi-allogeneic MLR to examine cytokine production *in vitro*, that would mimic a semi-allogeneic GvH. Host APCs were cultured with naïve donor CD4+ T-cells. The co-cultures were set up in various ratios and after 3 days of culture were stained to measure culture activation (CD25) and proliferation (CFSE) of $CD4^+$T-cells. After

completion of the culture period all the cultures failed to activate and proliferate $CD4^+$ T-cells. Previous studies have indicated that host B cells have little effect on the induction of an acute graft-vs-host disease (GVHD). B-cells alone cannot stimulate proliferation (3). In our experiment, approximately 50-60% of our APC fraction was $B220^+$, indicating a high percentage of B-cells. It is the host DCs that influence and mediate GVHD (3). MHC class II-positive B cells were insufficient to activate $CD4^+$ T-cells, while host DCs activate donor $CD4^+$ T-cells in MHC II-deficient mice (3). In addition, host DCs stimulate donor $CD8^+$ T-cells to induce GVHD (3). Though there were host DCs in the MLR assay, there needs to be fewer B-cells, as these may prevent the T-cells from "discovering" the DCs. If this is the case, another fraction could be investigated; a T:DC co-culture could be set up.

When the mixed MLR did not function and therefore cytokines could not be collected, prior GvH supernatant samples were collected from the Kerkvliet lab. The day 2 GvH supernatants had similar IL-10 levels in both TCDD and VEH treatments. Likewise, the day 3 GvH supernatants showed with no significant difference between TCDD and VEH IL-10 production. Nor was there a difference between day 2 and day 3 GvH samples, indicating that IL-10 between day 2 and day 3 was produced at a constant rate. Previous studies have indicated that on day 2 of a GvH response, the phenotype of the donor $CD4^+$ T-cell was characterized by increased secretion of IL-10 in TCDD-treated animals (10). This discrepancy may have been due to technical error. Additionally, Apetoh et al, found that AhR stimulates secretion of IL-10 during the differentiation of type 1 regulatory T-cells (Tr1), indicating that IL-10 production is regulated following activation by TCDD. However, this was not observed in our study (2). A critical difference that could have affected the results is distinction in protocols to culture T-cells. In Apetoh et al, Tr1 cells were induced by TGF-b and IL-27 (2). Also, these were *in vitro* cultures. Our T-cell cultures were not induced by an endogenous cytokines and were *in vivo*

cultures.

In contrast to the IL-10, there were increased IFN-g protein levels in TCDD-treated mice when analyzed via ELISA. IFN-g production on day 2 GvH, showed similar IFN-g protein levels in TCDD and VEH-treated mice. In day 3 GvH, there was a significant increase in TCDD over VEH in IFN-g production ($p=0.011$) indicating that TCDD up-regulated IFN-g production. In a prior study, IFN-g production also increased in the presence of co-cultured T-cells and semi-allogeneic DCs in TCDD-treated mice from day 2 to day 3 (16). In this study, the presence of IFN-g was greater in TCDD-treated mice than VEH-treated mice on both days (16).

In conclusion, the *in vitro* mixed MLR could not produce cytokines because it was unable to proliferate and activate $CD4^+$ T-cells. When prior supernatant GvH samples from the Kerkvliet lab were used, IL-10 production showed no difference between between day 2 and day 3 production of IL-10 nor VEH and TCDD-treated mice. The secretion of IFN-g significantly increased on day 3 GvH in TCDD-treated mice compared to VEH.

If able to continue experimentation on GvH supernatants, it would be of interest to look at IL-2. In previous study by Kerkvliet et al. it was mentioned that excess IL-2 inhibited IFN-g production in a GvH model (7). Thus, we may want to look at day 2 and day 3 GvH production of IL-2. In addition to looking at other cytokines, it would be pertinent to look into other detection techniques for cytokines besides ELISA, such as the intracellular cytokine staining method. Cytokine production is not always fixed on one cell type (6). The method described by Jung et al. quantifies IL-2 and IFN-g via flow cytometry and eliminates the process for cell sorting in order to determine cytokine-producing cells in a mixed population. There are other experiments that could be conducted to analyze cytokine production, such as the radioimmunoassay. It has already been established that T_{regs} are an important part of regulation of the immune response by suppression.

To conclude, the immunosuppressive function of TCDD bound to AhR continues to be investigated. There are biochemical and molecular markers that are still not understood. A component of understanding this mechanism can be inferred by looking cytokine production as a result of T-cells exposed to TCDD.

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Protein	Cell subset
B220	Primarily expressed on B-cells
CD4	Expressed by T-helper cells
CD8	Primarily expressed on cytotoxic T-cells
CD11c	The lineage marker for dendritic cells
GR1	Lineage marker expressed by granulocytes
Ter119	The lineage marker for erythrocytes

TABLE 1. The lineage markers for cell subsets used in both the Ficoll and 35% BSA gradients.

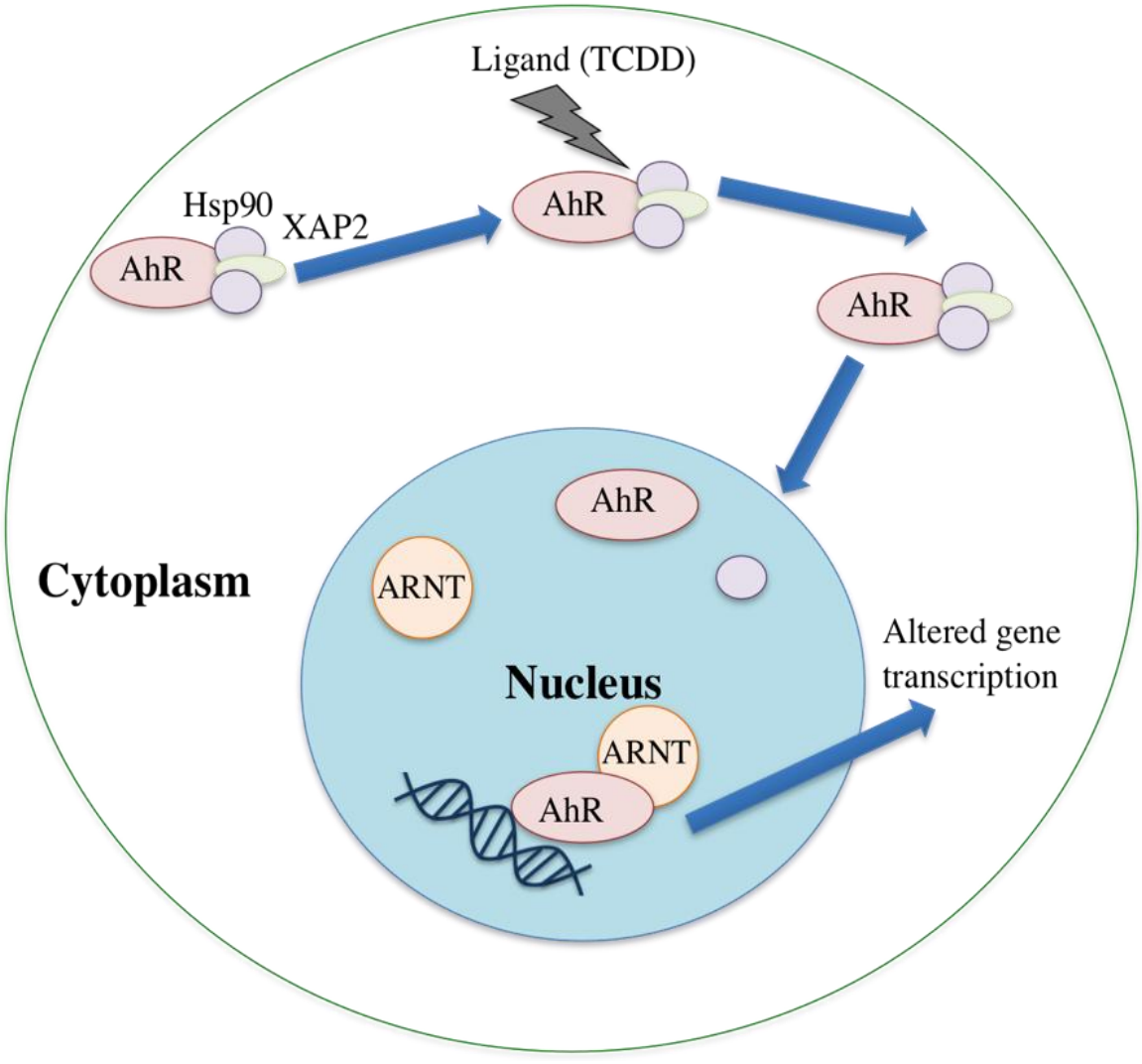

FIGURE 1. The interaction of AhR with co-chaperons, TCDD and ARNT, as it translocates to nucleus from the cytoplasm.

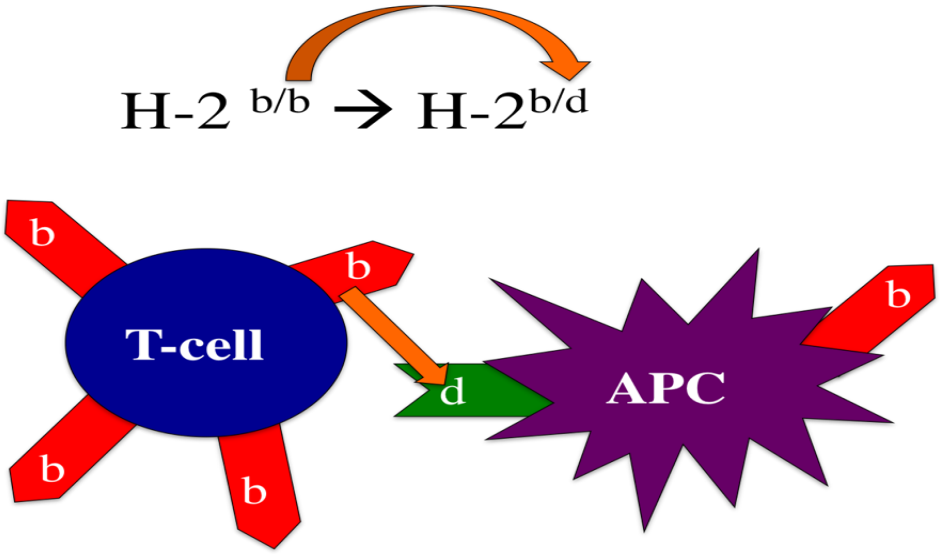

FIGURE 2. A depiction of a GvH model; the T-cells from a host B6D2F1 (H-$2^{b/d}$) mouse are injected into C57Bl/6 (H-$2^{b/b}$) mouse. This causes host T-cells to recognize the donor APCs as foreign and begin a CTL response.

A. **A.**	**B.** **D.**
C. **C.**	**D.** **B.**

FIGURE 3. Process of the enzyme-linked immunosorbent assay (ELISA) technique to detect protein levels in a sample. A. The sample binds to the support and any antigen present will bind to the capture antibody. FIGURE 3B. A primary antibody is added, which binds to the antigen. Several washes are carried out. FIGURE 3C. A secondary antibody-enzyme conjugate is added and this conjugate binds to the detection antibody. Several washes are repeated. FIGURE 3D. The tetramethylbenzidine (TMB) substrate solution is added and converted to a detectable form (5).

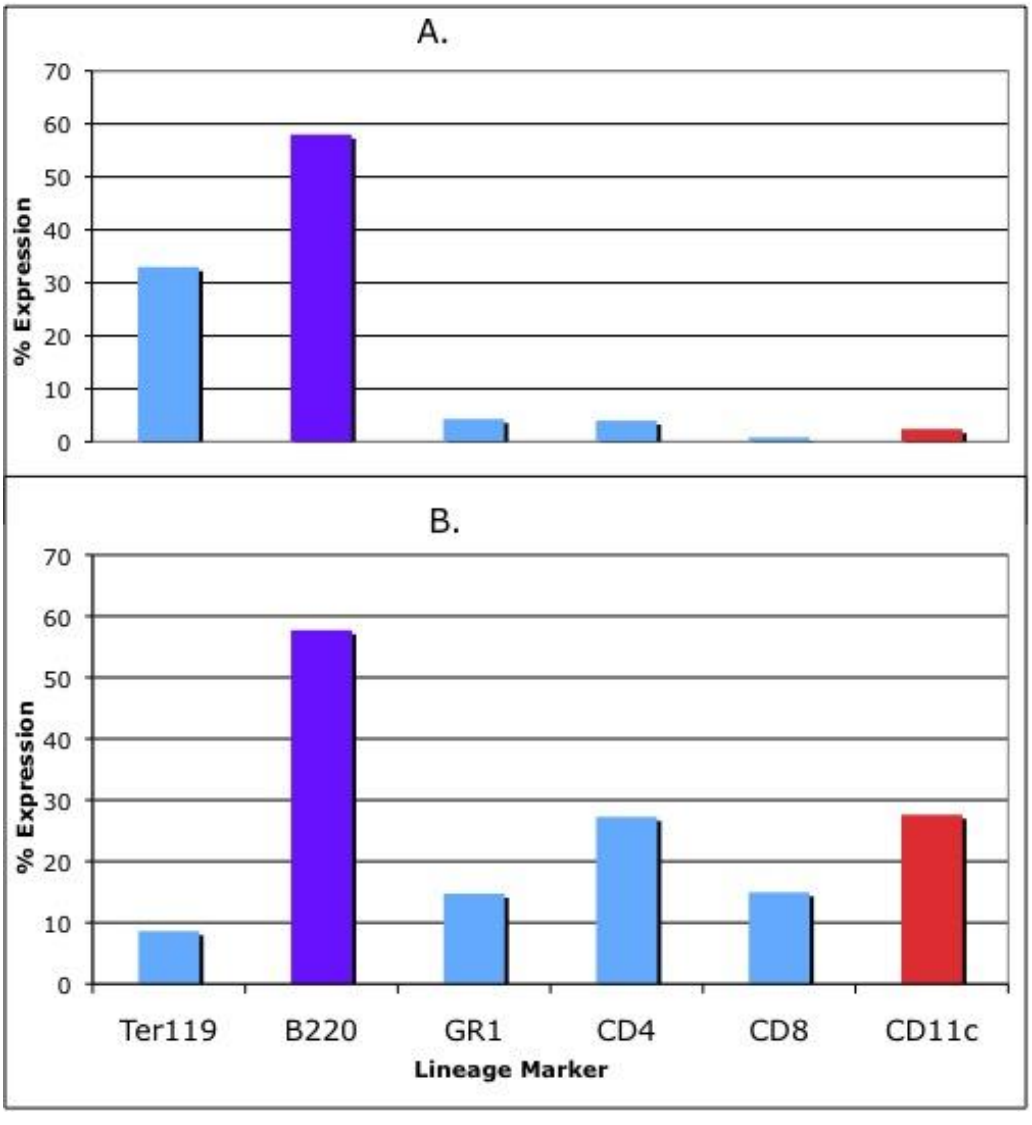

FIGURE 4. The percent expression of lineage markers in two different gradients. *A*, Ficoll gradient. *B*, 35% BSA gradient.

5:1

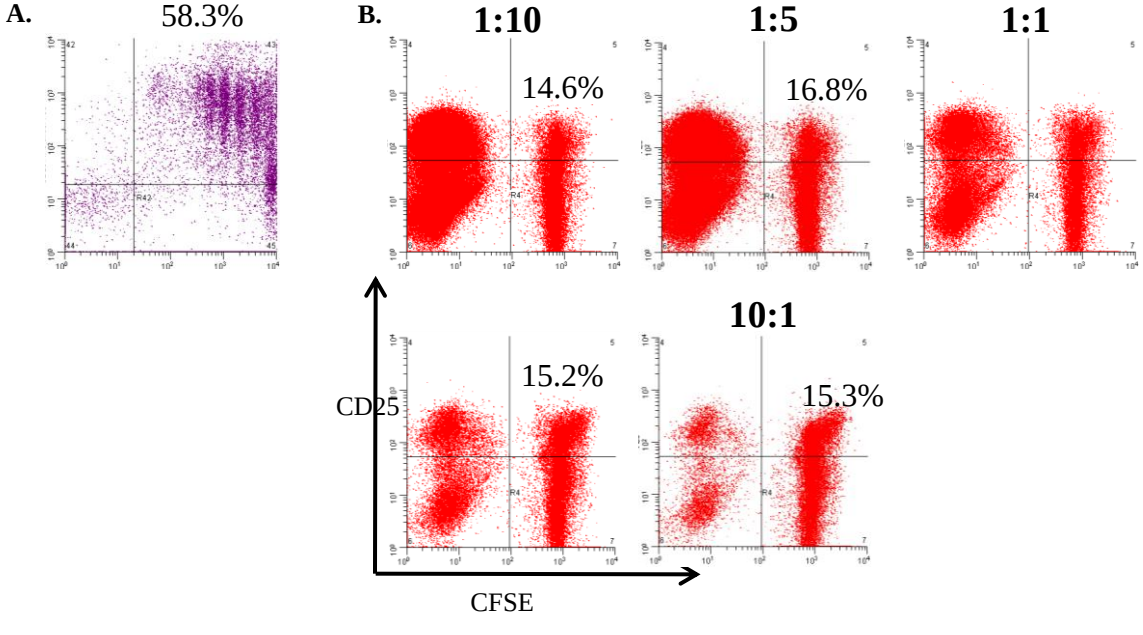

FIGURE 5. Depiction of activation and proliferation of $CD4^+$ T-cells measured on the flow cytometer. *A,* Activated and proliferating $CD4^+$ T-cells can be seen by increase CD25 expression and dilution of CFSE. $CD4^+$ cells were activated by $CD11c^+$ host cells. *B,* Ratios of different CD4:APC co-cultures were evaluated for activation and proliferation of $CD4^+$ T-cells.

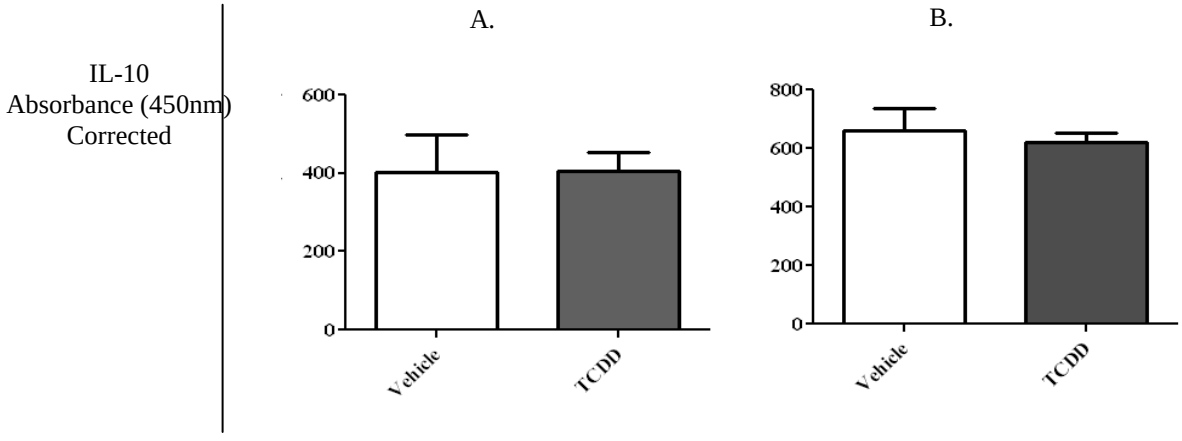

FIGURE 6. IL-10 cytokine production was measured by ELISA. *A*, Production of IL-10 in both VEH and TCDD were measured on supernatants from a day 2 GvH. An ELISA assay determined cytokine production. *B*, Supernatants from VEH and TCDD were quantified on a day 3 GvH for IL-10.

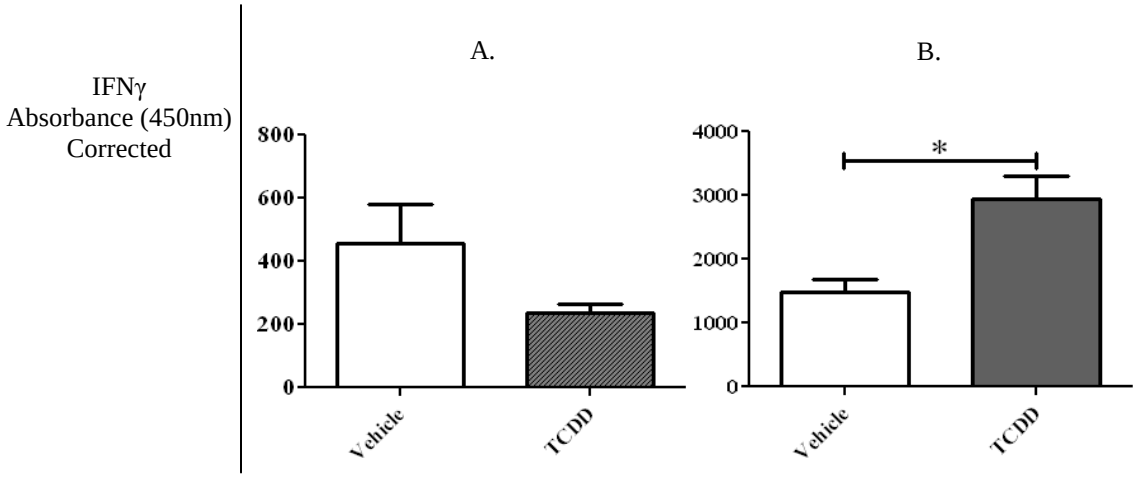

FIGURE 7. Analysis of IFN-g via ELISA. *A*, Production of IFN-g protein levels in both VEH and TCDD were measured on supernatants from a day 2 GvH. *B*, Supernatants from VEH and TCDD were quantified on a day 3 GvH for IFN-g. *Difference from VEH to TCDD-treated mice ($p=0.011$).

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