Suppression of Human B Cell Activation by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Involves Altered Regulation of B Cell Lymphoma-6

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ABSTRACT

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) produces marked suppression of the primary humoral immune response in virtually every animal species evaluated thus far. In addition, epidemiological studies performed in areas of dioxin contamination have identified an association between TCDD exposure and an increased incidence of non-Hodgkin's lymphoma (NHL). Recent studies using an *in vitro* CD40 ligand model of human B cell differentiation have shown that TCDD impairs both B cell activation and differentiation. The present study extends these findings by identifying B cell lymphoma-6 [BCL-6] as a putative cellular target for deregulation by TCDD, which may contribute to suppression of B cell function as well as NHL. BCL-6 is a multifunctional transcriptional repressor frequently mutated in NHLs and known to regulate critical events of B cell activation and differentiation. In the presence of TCDD, BCL-6 protein levels were elevated and concurrently the same population of cells with high BCL-6 levels showed decreased CD80 and CD69 expression indicative of impaired cellular activation. The elevated BCL-6 levels resulted in a concomitant increase in BCL-6 DNA binding activity at its cognate binding site within an enhancer region for CD80. Furthermore, a small molecule inhibitor of BCL-6 activity reversed TCDD-mediated suppression of CD80 expression in human B cells. In the presence of a low-affinity ligand of the aryl hydrocarbon receptor (AHR), suppression of B cell activation and altered BCL-6 regulation were not observed. These results provide new mechanistic insights into the role of BCL-6 in the suppression of human B cell activation by TCDD.

Key words: TCDD; immunotoxicity; AHR pathway; impaired B cell activation

Optimal activation of human B cells is critical for humoral immune responses, as poor activation is known to block progression toward B cell differentiation (Jelinek and Lipsky, 1983). Activation of mature B cells is initiated through ligation of either the B cell receptor, the CD40 receptor, or through stimulation of the toll-like receptors and cytokine signaling. Activated B cells then undergo proliferation before ultimately differentiating into antibody secreting cells (Banchereau *et al.*, 1994; Renard *et al.*, 1994). Numerous intracellular signaling pathways are activated upon binding of the CD40 receptor to the CD40 ligand expressed on the surface of activated CD4⁺ T cells which

ultimately result in increased expression of CD80 (B7.1), CD86 (B7.2), and CD69 on the surface of B cells along with an increase in MHCII and ICAM-1 (Bishop and Hostager, 2001). These surface markers are indicative of an activated B cell phenotype and are determinative of the magnitude of B cell activation.

The immune system is a sensitive target organ susceptible to alterations by environmental chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental toxicant and the most potent congener of the group of halogenated aromatic hydrocarbons posing significant immunotoxicity. Suppression of the primary humoral immune

response is a sensitive and well-established consequence of TCDD exposure. TCDD markedly suppresses the T-dependent and T-independent antibody responses as evidenced by a decrease in immunoglobulin M (IgM) secretion; thereby, impairing the process of B cell differentiation (Dooley and Holsapple, 1988). Although several different immune cell types are modulated by TCDD, the B cell is known to be a sensitive and direct target for TCDD-mediated suppression of the primary IgM response (Morris et al., 1993; Wood et al., 1993). Most of the toxic effects produced by TCDD are mediated by the activation of the aryl hydrocarbon receptor (Rowlands and Gustafsson, 1997).

The effects of TCDD on the human immune system are substantiated by data obtained from epidemiological studies performed in areas of dioxin exposure and from early experiments performed using *in vitro* culture of primary lymphocytes (Wood et al., 1993). Decreased plasma antibody levels were observed in Korean veterans from Vietnam War exposed to Agent Orange (Kim et al., 2003) and in people residing in areas contaminated with dioxins in Seveso, Italy (Baccarelli et al., 2002). In addition, many case-control and cohort studies showed a correlation between TCDD exposure and an increased incidence of non-Hodgkin's lymphoma (NHL) (Floret et al., 2007; Kogevinas et al., 1997). It has been recently hypothesized that B cell malignancies could arise from altered AHR expression or activity induced by TCDD (Sherr and Monti, 2013). An examination of the direct effects of TCDD in human peripheral blood B cells activated by CD40 ligand plus cytokines IL-2, IL-6, and IL-10 showed that TCDD suppressed the CD40 ligand-induced primary IgM response in several "TCDD-responsive" human donors whereas some donors were seen to be refractory to TCDD treatment or "TCDD-non-responsive" (Lu et al., 2010). In a follow-up study, Lu et al. (2011) also eluded that suppression of human B cell differentiation by TCDD involved impaired B cell activation; exemplified by suppression of activation markers CD80, CD86, and CD69. Hence, the objective of this study was to investigate the mechanism by which TCDD disrupts B cell activation. Specifically, we hypothesized that TCDD impairs B cell activation by altering the regulation of the transcriptional repressor, B cell lymphoma-6 (BCL-6).

The expression of BCL-6 is tightly regulated in normal B cells but deregulation of BCL-6, mostly through alterations in BCL-6 promoter region (Niu, 2002), is common in lymphomas. A third of NHLs possess a mutation in BCL-6 (Dalla-Favera et al., 1999; Staudt et al., 1999) and a significant proportion of the diffuse large B cell lymphomas (DLBCLs) harbor translocations within the BCL-6 gene (Ye, 2000). BCL-6 blocks B cell differentiation by repressing Blimp-1 whereas Pax-5 maintains BCL-6 expression in mature B cells. Other important target genes of BCL-6 include p53, p21, and p27kip1 that control the cell cycle and CD69 and CD80 that control B cell activation (Shaffer et al., 2000).

Previously, in LPS-activated mouse B cells, TCDD treatment elevated BCL-6 and Bach2 levels, decreased Blimp-1 mRNA and prevented downregulation of Pax-5, thereby suppressing B cell differentiation (reviewed in Sulentic and Kaminski, 2011). Given this existing paradigm and the function of BCL-6 in B cells, we present additional insights into the mechanism by which BCL-6 affects CD80 expression and impairs B cell activation in presence of TCDD.

MATERIALS AND METHODS

Chemicals and reagents. 99.1% pure TCDD in dimethylsulfoxide (DMSO) and 1-chlorodibenzo-*p*-dioxin (monochlorodibenzo-*p*-dioxin [MCDD]) in DMSO were purchased from Accustandard

Inc (New Haven, Connecticut). DMSO was purchased from Sigma-Aldrich (St Louis, Missouri) and was used to dilute the TCDD. The previously characterized BCL-6 small molecule inhibitor 79-6 was purchased from Calbiochem EMD Millipore (Darmstadt, Germany) and used at a final concentration of 100 μ M *in vitro*. DMSO was used as a solvent for the inhibitor 79-6. The specificity and the activity of this inhibitor have been described previously (Cerchiatti et al., 2010).

Cell culture. CD40 ligand-L cells were obtained as a generous gift from Dr David Sherr (Boston University). CD40 ligand-L cells are a mouse fibroblast cell line containing the stably transfected human CD40 ligand. The cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, California) supplemented with 10% bovine calf serum (ThermoScientific, Lafayette, Colorado), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen), 50 μ M of 2-mercaptoethanol, and HT supplement (0.1 mM sodium hypoxanthine and 0.016 mM thymidine) (Invitrogen). CD40 ligand-L cells were thawed at least 4 days prior to use and irradiated with 3500 Gy of x-rays using X-Rad 320 (Precision X-Ray, Inc, North Branford, Connecticut) at least a day prior to coculture with B cells. CD40 ligand-L cells were seeded at a concentration of 1×10^4 cells/ml in 500 μ l of media per well in 48 well tissue culture plates (Invitrogen) for the culture period. The expression of CD40 ligand on the surface of these cells was monitored routinely to select for high CD40 ligand-expressing cells. Human peripheral blood B cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% human AB serum (Valley Biomedical, Virginia), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen), and 50 μ M of 2-mercaptoethanol. In all cases, cells were cultured in 5% CO₂ incubator at 37°C.

Flow cytometry. Antibodies used for flow cytometry were as follows: PE anti-mouse/human BCL-6 (Clone: IG191E/A8), PE/Cy5 anti-human CD80 (Clone: 2D10), PE anti-human CD86 (Clone: IT2.2), PE/Cy7 anti-human CD69 (Clone: FN50) Biolegend (San Diego, California), PE anti-human/mouse BCL-6 (Clone: 603406) R&D Systems (Minneapolis, Minnesota), PE anti-human BCL-6 (Clone: K112-91) BD Pharmingen (San Jose, California). For BCL-6 proliferation assays, Cell Trace Violet Proliferation kit from Invitrogen Molecular Probes (Eugene, Oregon) was used. For every staining, approximately $0.5\text{--}1 \times 10^6$ cells were harvested at the indicated time points and viable cells were identified by Fixable Live/Dead Near-IR dye (Invitrogen) following manufacturer's instructions prior to cell surface or intracellular staining. Surface Fc receptors were blocked using human AB serum before staining for intracellular or extracellular proteins. For surface staining of activation markers CD80, CD86, or CD69, cells were resuspended in FACS buffer ($1 \times$ phosphate-buffered saline, 1% bovine serum albumin [BSA] Calbiochem (San Diego, California) and 0.1% sodium azide Sigma [St Louis, Missouri] pH: 7.6) in the presence of 20% human AB serum and the specific antibodies were added at the company specified concentrations and incubated at 4°C for 15–30 min. Following incubation with antibody, the cells were washed twice with FACS buffer to remove excess antibody and then fixed by incubation in the BD Cytotfix fixation buffer (BD Biosciences, California) for 10 min and stored at 4°C until they were ready to be analyzed by flow cytometry. For intracellular protein antigen staining, cells that were previously fixed after surface staining were permeabilized with $1 \times$ BD PermWash buffer (BD Biosciences) by washing twice and then incubating them for an additional 30 min at 4°C.

Antibodies specific to the intracellular antigens were then added to the cells and allowed to incubate for 30 min at 4°C. No difference was observed in the trends of surface activation markers due to the effect of fixation followed by permeabilization as opposed to just fixation followed by flow cytometry analysis. In all cases, cells were analyzed on BD FACSCanto II using FACS Diva software (BD Biosciences) and subsequently analyzed using FlowJo (version 8.8.7, Treestar Software Ashland, Oregon) or Kaluza (version 1.1, Beckman Coulter Inc, Brea, California). Unless stated, cells were gated on singlets, live (as determined by Live/Dead dye) followed by gating on lymphocyte populations. Gates were drawn on the basis of unstimulated cells (resting B cells not stimulated with CD40 ligand or IL-2, IL-6, or IL-10) or unstained cells as appropriate.

Human leukocyte packs and isolation of human B cells. Buffy coats collected from anonymous platelet donors were obtained from Gulf Coast Regional Laboratories (Houston, Texas). All human leukocyte packs were tested for presence of HIV, HBV, HCV, and HTLV before shipment. No donor used in this study was repeated. For every experiment, blood packs were diluted with HBSS and overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, New Jersey) and centrifuged at 1800 rpm for 40 min with low brake. The peripheral blood mononuclear cells were isolated post-centrifugation, washed, counted, and subjected to a magnetic column-based isolation that enables isolation of untouched (more than 95% pure) CD19⁺CD27⁻ B cells. This negative selection was conducted using MACS Naïve human B cell isolation kits (Miltenyi Biotec, Auburn, California) following manufacturer's instructions. Purified B cells at a concentration of 1×10^6 cells/ml were then cocultured with sublethally irradiated CD40 ligand-L cells (1×10^4 cells/ml) in a 48 well tissue culture plate as mentioned earlier. Cells were cultured in presence of recombinant human cytokines IL-2 at 10 U/ml, IL-6 at 100 U/ml (Roche Applied Sciences, Indianapolis, Indiana), and IL-10 at 20 ng/ml (BioVision Inc, Milpitas, California) for a period of 3 or 4 days depending on the endpoints assayed. Activated B cells were treated with either vehicle (0.05% DMSO) or TCDD (30 nM) or as described in the experiment. Typically, a preliminary assessment of the responsiveness of donors' B cells to TCDD was performed 3 days post-activation and treatment of cells by analyzing the expression of surface activation markers in the presence of vehicle or TCDD. The expression of B cell activation markers typically peaked on day 3 and hence that was the time point chosen. Donors showing a significant suppression of B cell activation markers namely CD80, CD86, and CD69 were further analyzed for the different endpoints. Due to the limited number of cells obtained from one donor, studies employed B cells from a single donor, which were then repeated in multiple donors to confirm the reproducibility of the biological effect.

Quantitative real-time PCR. RNA was isolated from human peripheral blood B cells using the RNeasy Kit (Qiagen, Valencia, California) according to the manufacturer's instructions. Total RNA from each sample was quantified using Nanodrop ND-1000 Scientific spectrophotometer (Thermo Scientific, Wilmington, Delaware) and 500 ng of RNA from each treatment sample was reverse-transcribed using Applied Biosystems High capacity cDNA reverse transcription kit as per the company's protocol. The cDNA was amplified using Applied Biosystems TaqMan Gene Expression Assays inventoried probe binding

to human BCL-6 (Hs00277037_m1). All quantitative real-time PCR reactions were performed on an Applied Biosystems model ABI Prism 7900 Sequence Detection System. 18S ribosomal RNA (Applied Biosystems, Foster City, California) was used as an internal control gene and the fold change in gene expression from the reference was calculated using the $\Delta\Delta C_t$ method.

Electrophoretic mobility shift assays. Human primary B cells were harvested at specific time points and nuclear proteins from TCDD-treated and VH-treated cells were isolated using standardized protocols as previously described (Andrews and Faller, 1991). Five micrograms of nuclear protein as estimated using the BCA protein determination assay (Sigma-Aldrich) was used in the binding reaction containing 0.5 μ g poly dI/dC (Roche Applied Sciences) with a final salt concentration of 180 mM NaCl. Double-stranded DNA probes specific for the enhancer element within the CD80 gene with the BCL-6 binding sequence in bold (5'-AGGGTCATCTTAGAACATGAA-3') were synthesized and end-labeled by γ -³²P-ATP using T4 polynucleotide kinase (New England Biolabs, Ipswich, Massachusetts) and column purified to remove unbound γ -³²P using Illustra Probe Quant columns (GE Healthcare). The nuclear protein and poly dI/dC reaction were incubated on ice for 10 min followed by addition of 45 000 cpm of the BCL-6 radiolabeled probe and incubation for an additional 30 min at room temperature. The protein-DNA complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE buffer (1× = 89 mM Tris, 89 mM borate, and 2 mM EDTA). Following electrophoresis, the gel was dried and exposed overnight for detection. To assess specific DNA binding activity, nuclear extracts were incubated with 100-fold excess of unlabeled probe before addition of the radiolabeled probe. The bands on the autoradiograph were quantified by densitometry using UN-SCAN IT software (Silk Scientific, Orem, Utah). For the supershift analysis, nuclear protein and dI-dC reactions were incubated with BCL-6 antibody (C-19 X) SantaCruz Biotechnology Inc, (SantaCruz, California) for 15 min at room temperature after addition of the radiolabeled probe. IgG from goat serum (Sigma-Aldrich) was used as the control.

Proliferation assay. Isolated human primary B cells were washed and resuspended in 1× HBSS to remove traces of serum and were incubated with 2 μ M Cell Trace Violet Dye (Cell Trace Violet Cell Proliferation Kit, Invitrogen) at 1×10^6 cells/ml for 20 min in dark at 37°C. The labeled cells are then washed twice with complete RPMI and then the cell density is adjusted as desired prior to treatment of cells with TCDD (30 nM) or VH (0.02% DMSO) and activation with CD40 ligand and cytokines IL-2, IL-6, and IL-10. The cells were harvested at day 1, day 2, day 3, and day 4 for intracellular staining.

Statistical analysis. Statistical analysis to obtain mean fluorescence intensity (MFI) of flow cytometry data was performed within the FlowJo software. Data obtained as percentage of gated cells by flow cytometry were log transformed before performing statistical analysis. Unconcatenated samples were used to calculate statistical significance using 1-way analysis of variance (ANOVA) followed by the Bonferroni's post hoc test or Dunnett's post hoc to compare means between treatment groups and respective control groups. Significant outliers were identified using Grubb's outlier test and eliminated. For comparison between just the VH and TCDD groups, Student's 1-tailed t test was employed.

FIG. 1. Increase in BCL-6 protein levels upon TCDD treatment in human primary B cells. A, Human primary B cells were activated with CD40 ligand and cytokines IL-2, IL-6, IL-10, and treated with TCDD (30 nM) or VH (0.05% DMSO). At the time points indicated in the figure, cells were permeabilized and BCL-6 protein levels were measured by flow cytometry. Dead cells were excluded. The numbers in the left and right corners represent percentage of BCL-6^{hi} cells. B, Graph depicts MFI measurements from cells treated with VH and TCDD from the same donor depicted in panel A. ** $p < 0.01$ by 1-way ANOVA followed by Fisher's LSD post hoc test. Data are representative of at least 15 TCDD-responsive human donors.

RESULTS

TCDD Treatment Alters BCL-6 Protein Levels in Primary Human B Cells

Suboptimal activation of B cells leads to a decrease in their viability thereby affecting their proliferation and the primary IgM response (Jelinek and Lipsky, 1983). Previously, it was shown that TCDD impaired human B cell activation as evidenced by a decrease in the expression of B cell activation markers CD80, CD86, and CD69 (Lu et al., 2011). In order to understand the mechanism underlying altered B cell activation by TCDD, the current studies focused on BCL-6 as a putative candidate gene. In naïve resting B cells, the percentage of BCL-6^{hi} cells was low but increased upon activation with CD40 ligand plus cytokines on day 2 and was further increased on day 3 (Fig. 1A). Correspondingly, TCDD treatment produced a 5% increase in the proportion of BCL-6^{hi} cells at day 2 and a 20% increase in the proportion of BCL-6^{hi} cells on day 3. The MFI, which is a measure of the total amount of BCL-6 protein expressed by individual B cells also increased in the presence of TCDD and was significantly higher when compared with the VH-treated control group on day 3 (Fig. 1B). Figure 2 displays the change in BCL-6 MFI relative to the vehicle control as a box and whiskers plot. The levels of BCL-6 were normalized to the vehicle control of each donor with each donor serving as their own internal control. On days 2, 3, and 4, an increase in BCL-6 MFI, relative to the VH control, was observed. The profile of change in BCL-6 levels across the different days in 15 donors can be seen in Supplementary Figure 1. The overall trend shows at least a 10% increase in BCL-6 levels across 15 TCDD-responsive human donors though the magnitude of change in BCL-6 levels and the peak time of BCL-6 induction varied across donors. Due to limitations in the number of B cells that could be obtained from each human donor, all of the time points for each donor could not be assessed. These results show that TCDD treatment induced a higher level of BCL-6 when compared with VH-treated B cells.

The above studies were further extended to examine whether BCL-6 protein levels increase in a concentration-responsive

FIG. 2. Relative change in BCL-6 protein levels in the presence of TCDD. BCL-6 protein levels were measured by flow cytometry in human primary B cells at the indicated time points and the percent change in BCL-6 MFI was plotted by normalizing to the respective VH control for each donor. Human B cells in all donors were activated with CD40 ligand and cytokines and treated with TCDD (30 nM) or VH (0.05% DMSO). The box depicts 95% of the values, the line indicates the median, and the whiskers indicate the lower and the upper 5% of the values.

manner to TCDD treatment. B cells activated in the presence of increasing concentrations of TCDD (1–30 nM) showed an increase in BCL-6 levels, which was only significantly higher at the 30 nM concentration of TCDD (Fig. 3A). A TCDD-mediated concentration-dependent response was not evident owing to differences among individual human donors in background BCL-6 expression. Figure 3B shows changes in BCL-6^{hi} proportion of cells in an individual human donor. The increase in proportion of BCL-6^{hi} cells is seen in a concentration-responsive manner at the higher concentrations of 10 and 30 nM but not at the lower concentrations. mRNA levels of BCL-6 in human primary B cells were also measured; however, no differences were observed between the VH-treated and TCDD-treated groups (Supplementary Fig. 2).

FIG. 3. TCDD-concentration-dependent changes in BCL-6 protein levels in human primary B cells. A, Graph represents percent of BCL-6^{hi} cells at each concentration of TCDD. N=6 for TCDD concentrations 1, 3, 10 nM and N=31 for VH and 30 nM TCDD groups. Dead cells excluded. ** $p < 0.01$ using Kruskal Wallis test followed by Bonferroni's post hoc test. B, B cells were harvested on day 3 after being activated with CD40 ligand and cytokines and treated with the indicated concentrations of TCDD or VH (0.05% DMSO). BCL-6 levels were measured by flow cytometry. Numbers in the top and bottom corners depict the percentage of BCL-6^{hi} and BCL-6^{lo} cells, respectively. Data shown in panel B is representative of 6 TCDD-responsive human donors.

FIG. 4. Decreased expression of CD80 corresponds to higher BCL-6 expression in TCDD-treated human primary B cells. A, BCL-6 and CD80 protein levels were assessed by multiparametric flow cytometry in human primary B cells activated with CD40 ligand plus cytokines and treated with TCDD (30 nM) or VH (0.05% DMSO) at the indicated time points. Data are representative of 5 TCDD-responsive human donors. B, Graph depicts MFI of CD80 in BCL-6^{hi} cells at the time points indicated. C, Graph depicts MFI of CD80 in BCL-6^{lo} cells at the time points indicated. For panels B and C, BCL-6^{hi} and BCL-6^{lo} cells were gated from the quadrant analysis in panel A. Dead cells excluded. D, MFI of CD80 within BCL-6^{hi} cells in 5 TCDD-responsive human donors on day 3. E, MFI of CD80 within BCL-6^{lo} cells in 5 TCDD-responsive human donors on day 3. Data in each panel are normalized to the VH-treated group and depicted as percent of control. Each small triangle represents data from one donor. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ using 1-way ANOVA followed by Fisher's LSD test.

These results are in accordance with previous reports suggesting that alterations in BCL-6 regulation occur at the protein level through changes in recruitment of BCL-6 to promoter of genes that it regulates or through protein modifications such as phosphorylation of BCL-6 (Niu *et al.*, 1998).

Inverse Relationship between Expression of BCL-6 and Activation Markers CD80 and CD69

Owing to the repressive action of BCL-6 on B cell activation, it has been observed in the B cell that the expression of BCL-6 and CD80 is mutually exclusive (Niu *et al.*, 2003). To test whether

BCL-6 and CD80 regulate each other in presence of TCDD, primary B cells from human donors were activated with CD40 ligand plus cytokines and treated with TCDD. BCL-6 and CD80 levels were measured in the same population of cells by multiparametric flow cytometry. As observed earlier, TCDD treatment increased the percentage of BCL-6^{hi} cells on day 3 (Fig. 4A). The cells expressing higher levels of BCL-6 exhibited lower CD80 levels as is evident in the flow cytometry contour images and shown as quadrant plots in Supplementary Figure 3A. Furthermore, Figure 4B shows the MFI of CD80 in BCL-6^{hi} cells (calculated based on gating of BCL-6^{hi} cells) in a representative TCDD-responsive human donor. The expression of activation marker CD80 in the VH group increases upon activation at day 2 and is subsequently higher at day 3 in the BCL-6^{hi} cells. In contrast, cells treated with TCDD showed a slight increase in CD80 levels at day 2 and day 3 but were significantly lower when compared with the VH group at day 3. Conversely, the cells expressing lower BCL-6 levels (calculated based on gating of BCL-6^{lo} cells) (Fig. 4C) showed significantly higher expression of CD80 in presence of TCDD at day 3. A significant decrease in CD80 MFI within the BCL-6^{hi} cells was also observed in 5 TCDD-responsive human donors (Fig. 4D) and trend toward an increase in CD80 MFI was observed within BCL-6^{lo} cells (Fig. 4E). These results indicate that BCL-6 and CD80 exhibit an inverse expression profile suggesting that BCL-6 could potentially play a role in regulating CD80 in presence of TCDD. The activation marker CD69 was seen to be repressed in microarray experiments performed on cell lines deficient in BCL-6 but transfected with BCL-6 overexpressing constructs

(Shaffer *et al.*, 2000). Similar to the observations made in Figure 4, TCDD treatment increased BCL-6 levels with the BCL-6^{hi} cells exhibiting lower CD69 levels (Fig. 5A and Supplementary Fig. 3B). CD69 expression in BCL-6^{hi} cells decreased significantly in presence of TCDD on day 3 (Fig. 5B) and in contrast was higher in BCL-6^{lo} cells (Fig. 5C). The expression of CD69 on the surface of B cells increased as expected over time with activation (Figs. 5A and 5B). CD69 MFI measured in 5 TCDD-responsive human donors was significantly lower in BCL-6^{hi} cells and the same trend was reversed in case of BCL-6^{lo} cells (Figs. 5D and 5E).

Increased TCDD-Inducible DNA Binding Ability of BCL-6 at the CD80 Enhancer Region

To determine whether altered BCL-6 expression regulates CD80 in presence of TCDD, we focused on the CD80 promoter to identify existing BCL-6 binding sites. The expression of human CD80 is regulated by an enhancer element located 3 kb upstream of the transcriptional start site (Zhao *et al.*, 1996). BCL-6 is known to bind within the enhancer region of CD80 at a site slightly different from the consensus-binding site (Niu *et al.*, 2003). Hence, electrophoretic mobility shift assays were performed using a radiolabeled CD80 enhancer probe harboring the BCL-6 binding site. Nuclear proteins were isolated from human peripheral blood B cells treated with TCDD or VH at various time points. Naïve human B cells showed higher binding of BCL-6 on the CD80 enhancer region that was relieved upon activation of B cells on day 1 (Fig. 6, lane 1). An increase in TCDD-induced DNA binding activity was observed on day 2 and day 3 with maximal

FIG. 5. Decreased expression of CD69 corresponds to higher BCL-6 expression in TCDD-treated human primary B cells. A, BCL-6 and CD69 protein levels were assessed by multiparametric flow cytometry in human primary B cells activated with CD40 ligand and cytokines and treated with TCDD at the indicated time points. B, Graph depicts MFI of CD69 in BCL-6^{hi} cells at the time points indicated. C, Graph depicts MFI of CD69 in BCL-6^{lo} cells at the time points indicated. For panels B and C, BCL-6^{hi} and BCL-6^{lo} cells were gated from the quadrant analysis in panel A. Dead cells excluded. Data are representative of 5 TCDD-responsive human donors. D, MFI of CD69 within BCL-6^{hi} cells in 5 TCDD-responsive human donors on day 3. E, MFI of CD69 within BCL-6^{lo} cells in 5 TCDD-responsive human donors on day 3. Data in panel are normalized to the VH-treated group and depicted as percent of control. Each small triangle represents data from one donor. **p* < 0.05, ****p* < 0.001 using 1-way ANOVA followed by Fisher's LSD test.

FIG. 6. TCDD-mediated increase in DNA-binding activity of BCL-6 at the CD80 enhancer region. Electrophoretic mobility shift assay performed on nuclear extracts isolated from human primary B cells treated with TCDD (30 nM) or VH (0.05% DMSO) for the indicated days. Binding reactions were set up with 32 P-labeled CD80 enhancer region probe. For the supershift assay, nuclear protein from day 2 TCDD-treated human B cells was used with the BCL-6 antibody at 0.5 and 1 μ g (lanes 8 and 9, respectively). An isotype control rabbit IgG was used at the same concentration as the specific antibody (lanes 10 and 11). The arrowhead indicates TCDD-inducible BCL-6 binding to the CD80 enhancer region. The numbers to the bottom indicate the intensity of binding relative to Naïve on day 1 as determined by the densitometry scan. EMSA is representative of 5 TCDD-responsive human donors.

binding on day 2 (Fig. 6, lanes 4–7). To assess BCL-6 binding specificity an anti-BCL-6 antibody was employed. Although the anti-BCL-6 antibody did not supershift the DNA-binding complexes in Figure 6, it did produce a decrease in binding intensity. The TCDD-inducible complex was competed upon addition of the BCL-6 antibody but not by a non-specific rabbit IgG (Fig. 6, lanes 8 and 9, 10 and 11). The kinetics of DNA binding activity of BCL-6 demonstrated in the gel-shift assays is consistent with the pattern of decrease in expression of activation marker CD80 in several TCDD-responsive human donors at day 3.

BCL-6 Inhibitor Reverses Suppression of B Cell Activation Markers

The BTB domain mediates most of the repressive effects of BCL-6 and is important in binding to the corepressors recruited by BCL-6 (Ghetu et al., 2008). To further delineate the role of BCL-6 in suppression of B cell activation, a small molecule inhibitor of BCL-6, 79-6, was added to cells *in vitro* in presence of TCDD. This small molecule inhibitor of BCL-6 was designed to specifically bind the BTB domain thereby disrupting the activity of BCL-6 by blocking its interaction with the corepressors (Cerchiotti et al., 2010). To this end, human primary B cells were activated with CD40 ligand plus cytokines IL-2, IL-6, and IL-10 and treated *in vitro* with the inhibitor, 79-6, in the absence or presence of TCDD. As illustrated in Figure 7A, the treatment of cells with the BCL-6 inhibitor (+ BCL-6 INH panel) reversed the suppression by TCDD as the repressive action of BCL-6 on B cell activation markers was blocked. When compared with cells treated with inhibitor, cells treated with the vehicle of inhibitor (+VH INH panel) still showed moderate suppression of activation marker expression. The control cells treated with VH and TCDD alone showed the typical pattern of suppression of activation marker expression as evident from the MFI and the pattern of

the histograms. The effect of BCL-6 inhibitor on multiple TCDD-responsive human donors was also assessed. Reversal of suppressed activation marker expression was marked in case of CD80 and CD69 (Figs. 7B and 7D) and was seen to a lesser extent for activation markers CD86 owing to a vehicle effect (Fig. 7C).

BCL-6 Expression in Highly Proliferating Human B Cells

BCL-6 allows B cells to tolerate rapid proliferation (Shvarts et al., 2002) and high genomic instability by repressing the genes such as ATR, ATM, and p53 in the germinal center (Phan and Dalla-Favera, 2004). We expected that cells expressing high levels of BCL-6 would exhibit greater proliferation owing to the function of BCL-6 in B cells and given its role as an oncogene in lymphomas. Figure 8 shows the proliferation profile of human B cells activated with CD40 ligand and cytokines. As expected, B cells underwent 2–4 rounds of proliferation post-activation. Cells expressing higher levels of BCL-6 comprised the higher proliferating cells as highlighted by the gate in Figure 8 thereby providing a proof-of-principle for the expression of BCL-6 in proliferating B cells. However, the TCDD-treated cells showed a similar proliferation profile with no significant differences in the percentage of the cells proliferating among the BCL-6^{hi} cells at each day when compared with the vehicle control cells (Table 1).

Impaired Down-Regulation of BCL-6 Involves AHR

It is well known that most biological and toxic effects of TCDD are mediated through the AHR (Rowlands and Gustafsson, 1997). The involvement of the AHR in mediating TCDD toxicity has been shown extensively through the use of AHR null experimental models (Vorderstrasse et al., 2001). To determine whether alteration of BCL-6 levels in presence of TCDD involves AHR, we used MCDD a congener of the dibenzodioxins that has minimal binding affinity for the AHR (Shan et al., 2001) and an inability to induce CYP1A1 and ethoxyresorufin-o-deethylase activity. CD40 ligand and cytokine-activated human peripheral blood B cells were treated with VH, TCDD, or MCDD for 3 days. TCDD treatment increased the proportion of BCL-6^{hi} cells compared with the VH control cells at both time points as seen previously (Figs. 9A and 9B). Concurrently, cells treated with MCDD showed a significantly lower proportion of BCL-6^{hi} cells when compared with the VH-treated or TCDD-treated cells (Figs. 9A and 9B). This suggests that the increase in BCL-6 levels is specific to the TCDD-treated group and thereby suggests a role for the AHR pathway in suppression of activation. To further determine whether suppression of activation markers in presence of TCDD involves the AHR pathway, human primary B cells were treated with MCDD or with VH or TCDD for comparison. The magnitude of suppression of activation marker expression by TCDD was the greatest whereas cells treated with MCDD or VH showed no suppression of activation markers CD80, CD86, or CD69 (Fig. 10, panels A–C) for data from 8 donors and Supplementary Figure 4 showing data from 1 individual donor. Due to donor variability in background response and sensitivity to TCDD, we observed that in some donors the suppression of activation markers was in the rank order of TCDD > MCDD > VH.

DISCUSSION

Numerous signaling pathways, feedback loops, and transcriptional circuits regulate immune responses in mammalian systems. Disruption of any of the signals either transcriptional, cell signaling, or both can lead to impaired immune function. Use of environmental contaminants possessing high affinity for AHR, as probes to understand the role of AHR in B cell function has

FIG. 7. BCL-6 inhibitor reverses suppressed activation marker expression in human B cells activated with CD40 ligand and cytokines IL-2, IL-6, and IL-10 in the presence of TCDD. A, B cells were isolated on day 3 post-activation and treatment with TCDD (30 nM) or VH (0.02% DMSO) as shown in Control panel, VH of inhibitor (0.04% DMSO) plus TCDD or VH as shown in +VH INH panel and 100 μ M of BCL-6 inhibitor plus TCDD or VH as shown in +BCL-6 inhibitor panel. Cells were permeabilized and stained for activation markers. Numbers in the left corner depict the MFI for the indicated activation marker. Data shown in the figure are representative of 7 TCDD-responsive human donors. B–D, Graphs depict the MFI of CD80, CD86, and CD69 in the presence and absence of inhibitor along with treatment with TCDD or VH. Data in each graph are normalized to the VH control. Graphs represent percent of control with each dot representing a single donor. Data from 11 donors are shown in panel B whereas data from 12 donors are shown in panels C and D. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, ## $p < 0.01$.

proven effective in determining the importance of AHR from a physiological standpoint. Several recent reports have highlighted the role of AHR in influencing the kinetics, magnitude, and/or direction of immune responses (Stevens *et al.*, 2009). TCDD one of the most toxic environmental contaminants is known to adversely affect primary humoral immune responses by altering the ability of B cells to produce antibody. Using a primary human B cell differentiation model wherein B cells were activated with CD40 ligand in the presence of cytokines IL-2, IL-6, and IL-10, TCDD treatment showed attenuation of the B cell activation phenotype with an overall decrease in the number of active and viable cells that form antibody secreting cells (Lu *et al.*, 2010, 2011). To dissect the molecular mechanism underlying impaired B cell activation by TCDD, we focused on BCL-6 as a putative candidate gene not only due to its role as a key repressor of B cell differentiation but also as a repressor of B cell activation and cell cycle genes.

We have shown that TCDD treatment of activated human B cells induced a sustained increase in BCL-6 protein levels. This TCDD-mediated increase in the percent and in the MFI of BCL-6 was seen in several TCDD-responsive human donors. Although differences were observed in the magnitude and kinetics of BCL-6 levels between human donors, the overall trends were constant irrespective of the donor. Donor-to-donor variability is a significant challenge in using primary human B cells, hence our study employed a larger N for most experiments. This variability among human donors is not unexpected and can be attributed to the inherent differences in genetics, lifestyle, age, race, and numerous other factors which is also characteristic of the human population. The alteration in BCL-6 protein levels was also seen at different concentrations of TCDD with a significant effect at the highest concentration of TCDD. Concentrations higher than 30 nM were not used as typically maximum suppression of the IgM antibody response in human

and rodent species is routinely observed at 30 nM. However, no changes were observed at the mRNA level upon TCDD treatment. This result is not surprising considering the high expression levels of BCL-6 in naïve and activated B cells in our model. Moreover, BCL-6 expression is controlled at the protein level through multiple mechanisms including acetylation of BCL-6, repression through Blimp-1, and STAT5 (Kusam and Dent, 2007). Recently, BCL-6 was found to interact with FBXO11 (Duan et al., 2012), which is an F-box protein regulating degradation of

BCL-6 through the SCF-cullin proteolysis pathway. FBXO11 was also identified in a genome-wide ChIP-on-chip analysis for AHR binding performed in mouse B cells treated with TCDD. However, contrary to our expectations, FBXO11 mRNA levels were elevated upon treatment with TCDD in human B cells (Supplementary Fig. 5) thus suggesting that FBXO11 did not play a role in maintenance of BCL-6 levels. Another mechanism for downregulation of BCL-6 levels involves phosphorylation of BCL-6 by ERK, which triggers BCL-6 degradation through the ubiquitin proteasome pathway (Niu et al., 1998). In a previous study, TCDD treatment significantly attenuated the percentage of p-ERK⁺ p-p38⁺ p-AKT⁺ cells in human B cells 30 min post-activation with recombinant CD40L (Lu et al., 2011). This suggests that a disruption of early B cell signaling by TCDD: AHR pathway may be partially responsible for the maintenance of elevated BCL-6 protein levels in human B cells. Although we have not completely ruled out the possibility that TCDD would lead to a decreased downregulation of BCL-6 by impairing its ubiquitination through the SCF-cullin or ubiquitin pathway, further studies need to be conducted to address this question.

Multiparametric analyses measuring BCL-6 and CD80 or BCL-6 and CD69 levels in the same population of cells suggested an inverse relationship between BCL-6 expression and the amount of CD80 or CD69 expressed by human B cells. This finding is consistent with the observations made in Burkitt's lymphoma cell lines demonstrating mutually exclusive expression patterns of CD80 and BCL-6 (Niu et al., 2003) thus suggesting that BCL-6 may be playing a role in downregulating CD80 expression. CD69 was identified as one of the genes repressed by BCL-6 in BJAB and SUDHL5 cell lines and was also known to possess high affinity BCL-6 binding sites within the promoter region (Shaffer et al., 2000). Furthermore, genetic deletion of BCL-6 in mice caused increased CD69 (Shaffer et al., 2000) and CD80 expression (Niu et al., 2003), therefore supporting our finding that BCL-6^{hi} cells exhibited lower CD80 and CD69 expression in presence of TCDD.

The activation marker CD80 is directly regulated by BCL-6 (Niu et al., 2003). We found that BCL-6 binds to a previously identified consensus BCL-6 binding site within the CD80 enhancer in presence of TCDD *in vitro*. Expression of CD80 in human B cells in the presence of TCDD may also be regulated partly through the action of NFκB and BCL-6 as NFκB sites have been identified upstream of the BCL-6 binding site. TCDD-treatment of human primary B cells leads to a decrease in phospho-p65 and phospho-c-jun levels (Lu Haitian, Crawford Robert, and Kaminski, Norbert, unpublished data). In addition, several studies have established a possible complex interaction between AHR and NFκB signaling pathways, which may contribute to this effect (Vogel and Matsumura, 2009). Hence, it is tempting to speculate

FIG. 8. BCL-6^{hi} cells show increased proliferation. Human primary B cells were stained with Cell Trace Violet Proliferation Dye on day 0 following which cells were treated with TCDD (30 nM) or VH (0.02% DMSO) and activated through CD40 ligand and cytokines. At the indicated time points, cells were permeabilized and stained for intracellular BCL-6 levels. The gate and the numbers in the corner indicate BCL-6^{hi} cells. Dead cells excluded. Data shown are representative of the proliferation of 3 TCDD-responsive human donors.

TABLE 1. Effect of TCDD on CD40 Ligand-Induced Proliferation of BCL-6^{hi} Cells

	Treatments	Division 0	Division 1	Division 2	Division 3	Division 4	Division 5	Division 6	Division 7
Day 2	VH	86.3	3.76	0.48	0.41	0.09	0.02	0	0
	TCDD	84.5	3.01	0.1	0.05	0.02	0.01	0.01	0.01
Day 3	VH	38.9	14.8	20.9	18.1	3.68	0.7	0.05	0.01
	TCDD	29.1	14.4	26.6	23.2	3.66	0.71	0.01	0.01
Day 4	VH	6.26	5.99	10.9	23.9	28.3	16.1	6.62	0.71
	TCDD	5.73	6.44	13	31.6	28.6	10.2	2.68	0.2

Notes: The experimental design was as described in the legend for Figure 8. Human B cells were labeled with Cell Trace Violet Cell Proliferation Dye and treated with VH (0.02% DMSO) or TCDD (30 nM) and cells were harvested at the indicated time points and stained for BCL-6. The table indicates the percentage of proliferating cells gated on the BCL-6^{hi} gate shown in Figure 8. The values in the table are concatenated from 3 experimental replicates per treatment group.

FIG. 9. Impaired BCL-6 regulation in presence of TCDD involves AHR. A, BCL-6 protein levels were measured by flow cytometry on day 3 and day 4 post-activation and treatment with TCDD. The gates and the numbers in the corner depict the percent BCL-6^{hi} and the percent BCL-6^{lo} cells. Data representative of 4 TCDD-responsive human donors. B, Graph shows percent of BCL-6^{hi} cells in VH, TCDD, and MCDD-treated groups on day 3 and day 4 from the same donor as panel A. C, Graph depicts the percent of BCL-6^{hi} cells as a percent of control. Data are normalized to the VH group. Each square represents one donor. #*p* < 0.05, ***p* < 0.01, ****p* < 0.001 by 1-way ANOVA followed by Fisher's LSD test.

that the effect of TCDD on CD80 expression could be due to a combination of the repressive action of BCL-6 and due to decreased NFκB levels in presence of TCDD.

BCL-6 is expressed at high levels in rapidly proliferating cells of the germinal center (Ranuncolo et al., 2007). We too observed that cells expressing higher BCL-6 levels displayed greater proliferation with no effect of TCDD on B cell proliferation similar to reports by others (Lu et al., 2010; Wood et al., 1993). BCL-6 has a broad range of target genes that it suppresses including genes involved in DNA damage and cell cycle. The survival and proliferation of DLBCL or germinal center cells are governed by the lateral groove of BCL-6 BTB domain (Huang et al., 2013). Hence, upon using a small molecule inhibitor targeting the BTB domain reversal of the suppressed expression of BCL-6 target genes CD80 was observed to a greater extent than CD86 or CD69. This suggests that the effect of BCL-6 repression may be strongest on CD80. BCL-6 is just one of the transcription factors controlling the expression of CD86 and CD69 thus suggesting that other transcription factors such as STAT-1 or NFκB in the case of CD86 (Li et al., 2000) may likely play a role in regulating expression of these activation markers. Differences were also observed in the magnitude of response of different donors to the BCL-6 inhibitor. One reason for this could be the differences in basal and activation-induced expression levels of activation markers and BCL-6 in different human donors. In addition, the levels of different corepressors and their ability to be recruited or sequestered from BCL-6 could be different across individuals. Collectively, the data support the conclusion that suppression of activation marker function is, in part, due to elevated expression of BCL-6- and BCL-6-mediated repression.

The AHR is known to contribute at multiple levels to the suppression of immune system function. Activation of human B cells leads to elevated AHR levels (Allan and Sherr, 2005). The increase in BCL-6 levels and the suppression of B cell activation marker expression were dependent on AHR as demonstrated by

using the high- and low-affinity congeners of the dibenzo-*p*-dioxins. Several studies have also suggested an AHR-dependent but a DRE-independent mechanism of action of TCDD in different cell types (Puga et al., 2000). Taken together the results suggest that the TCDD-AHR complex is involved in altering BCL-6 levels, the mechanism of which remains unresolved.

Through this study, we have tried to bring forth a different perspective for the role of AHR in human B cells, in regulating B cell activation and possibly in B cell malignancies owing to the oncogenic potential of BCL-6. Our study provides interesting parallels to the observations made in the epidemiological studies performed in areas of dioxin exposure. This study also presents a framework of investigations into the molecular mechanisms underlying suppression of B cell activation in human cells in the presence of the environmental contaminant, TCDD. A complete understanding of the effects of impaired B cell activation within the context of immune responses will involve a detailed study of the effects of TCDD on B cell-mediated T cell activation, which remain to be investigated.

SUPPLEMENTARY DATA

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

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FIG. 10. Suppression of activation markers by TCDD involves AHR. CD40 ligand and cytokine activated B cells were treated with VH (0.02% DMSO) or TCDD (30 nM) or MCDD (30 nM) and the levels of activation markers CD80, CD86, and CD69, respectively, were measured by flow cytometry. Panels A–C represent MFI of CD80, CD86, and CD69, respectively, depicted as percent of control. Each data point represent one human donor. * $p < 0.05$, ** $p < 0.01$ by 1-way ANOVA followed by Fisher's LSD test.

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