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SHP-1 is Directly Activated by the Aryl Hydrocarbon Receptor and regulates BCL-6 in the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

Ashwini S. Phadnis-Moghe[a,b], **Jinpeng Li**[a,b], **Robert B. Crawford**[b,c], and **Norbert E. Kaminski**[b,c,*]

[a]Genetics Program, Michigan State University, East Lansing, Michigan, 48824

[b]Institute for Integrative Toxicology, Michigan State University, East Lansing, Michigan, 48824

[c]Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan, 48824

Abstract

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is a strong AHR agonist, causes significant suppression of human B cell activation and differentiation. The current studies describe the identification of Src homology phosphatase 1 (SHP-1) encoded by the gene *PTPN6* as a putative regulator of TCDD-mediated suppression of B cell activation. Shp-1 was initially identified through a genome-wide analysis of AHR binding in mouse B cells in the presence of TCDD. The binding of AHR to the *PTPN6* promoter was further confirmed using electrophoretic mobility shift assays in which, specific binding of AHR was detected at four putative DRE sites within *PTPN6* promoter. Time-course measurements performed in human B cells highlighted a significant increase in SHP-1 mRNA and protein levels in the presence of TCDD. The changes in the protein levels of SHP-1 were also observed in a TCDD concentration-dependent manner. The increase in SHP-1 levels was also seen to occur due to a change in early signaling events in the presence of TCDD. We have shown that BCL-6 regulates B cell activation by repressing activation marker CD80 in the presence of TCDD. TCDD-treatment led to a significant increase in the double positive (SHP-1^{hi} BCL-6^{hi}) population. Interestingly, treatment of naïve human B cells with SHP-1 inhibitor decreased BCL-6 protein levels suggesting possible regulation of BCL-6 by SHP-1 for the first time. Collectively, these results suggest that SHP-1 is regulated by AHR in presence of TCDD and may, in part through BCL-6, regulate TCDD-mediated suppression of human B cell activation.

*Corresponding author: Norbert E. Kaminski, 1129 Farm Lane, 165G, Food Safety and Toxicology, Michigan State University, East Lansing, MI 48824, Telephone: (517) 353 3786, kamins11@msu.edu.

Conflict of interest

The authors declare no conflict of interest.

Keywords

SHP-1; *PTPN6*; aryl hydrocarbon receptor; human B cell activation; TCDD immunotoxicity

Introduction

Mature B cells are constantly instructed by the B cell receptor (BCR) to make critical cell fate decisions (Niiro and Clark, 2002). The BCR has two major roles: first to transmit signals that regulate B cell activation and differentiation and second, to mediate antigen processing and presentation to T helper cells thereby leading to B cell activation. Ligation of the BCR with cognate antigen leads to activation of the proximal kinases LYN and SYK, which then phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) within Igα and Igβ thus activating kinases- SYK, BTK and a host of other cellular signaling pathways. These upstream signaling networks ultimately lead to activation of the MAPK pathway and NFκB, NFAT transcription factors thereby positively regulating B cell activation. Negative feedback regulation controls excessive B cell activation and proliferation. The paired immunoglobulin-like receptors (PIR) and FcγRIIB mediate negative regulation of BCR signaling. Association of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) with Src-homology phosphatase- 1 (SHP-1) and CD22 lead to inhibition of B cell receptor signaling (Nitschke, 2005). In addition to the BCR, the CD40 ligand expressed on the surface of T cells also stimulates B cells in a T-dependent manner along with cytokines driving B cell activation (Bishop and Hostager, 2001). SHP-1 has recently been implicated in setting the threshold for CD40-induced MAPK activation and for also regulating the CD40 signaling feedback system thereby controlling B cell activation (Khan *et al.*, 2014).

SHP-1 encoded by the gene protein tyrosine phosphatase, non-receptor type 6 (*PTPN6*) in humans is an intracellular phosphatase. The protein structure of SHP-1 is characterized by the presence of two Src-homology 2 (SH2) domains at the N-terminus, a central catalytic domain and a C-terminal domain with tyrosine phosphorylation sites responsible for phosphorylation of SHP-1 (Pao *et al.*, 2007a; Lorenz, 2009). The SH2 domains are critical for activation of the phosphatase (Pao *et al.*, 2007b) and serve as docking sites for several molecules such as receptor tyrosine kinases, cytokine receptors, scaffolding adapters (Grb2, SLAM) and ITIMs. SHP-1 is expressed in hematopoietic stem cells and regulates different stages of lymphoid and myeloid lineage development (Paling and Welham, 2005). One of the main functions of SHP-1 involves attenuation of signaling downstream of the BCR by inhibiting kinases such as LYN, SYK, Jak2 and ERK (Jiao *et al.*, 1996; Dustin *et al.*, 1999).

Environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) continually pose a long-standing concern to human health. Among the myriad toxicities elicited by TCDD, immune toxicity, specifically, suppression of B cell activation and the primary humoral immune response represent a highly sensitive endpoint of TCDD exposure (Holsapple *et al.*, 1991). Several studies have contributed towards identification of the molecular targets underlying TCDD-mediated suppression of the primary humoral immune response in rodents with B cell as the direct target of TCDD (Dooley and Holsapple, 1988).

The specific process that TCDD impairs is the ability of B cells to differentiate into IgM-secreting plasma cells (Morris *et al.*, 1993; Sulentic *et al.*, 1998).

Most of the toxic effects of TCDD are manifested upon diffusion into the cytosol and binding to the Aryl Hydrocarbon Receptor (AHR) (Fernandez-Salguero *et al.*, 1995; Vorderstrasse *et al.*, 2001). AHR is a ligand- activated transcription factor belonging to the Per-ARNT-Sim (PAS) family of proteins (Poland *et al.*, 1976). Binding to AHR leads to dissociation of the chaperone proteins- heat shock protein 90 (hsp90), p23, Ah-associated protein 9 (ARA9) from the cytosolic AHR complex and subsequently translocation of the TCDD-AHR complex to the nucleus where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator protein (ARNT) (Hankinson, 1995; Carver *et al.*, 1998; Bell and Poland, 2000; Petrulis and Perdew, 2002). The TCDD-AHR: ARNT complex can then bind to dioxin-response elements in the regulatory regions of dioxin-sensitive genes thereby altering their expression (Hankinson, 2005).

Our recent studies with human primary cells have made it feasible to study consequences of TCDD exposure on human B cells. CD40 ligand and cytokines IL-2, IL-6 and IL-10 were used to activate human B cells. B cell activation, determined by the expression of B cell activation markers CD80, CD86 and CD69 was significantly reduced upon TCDD treatment (Lu *et al.*, 2011). In a previous study, we identified that BCL-6, a key transcriptional repressor of B cell activation and differentiation is dysregulated in the presence of TCDD (Phadnis-Moghe *et al.*, 2015). Another candidate gene, SHP-1, was initially identified as a potential direct target of AHR through a genome-wide assay (ChIP-on-chip) and subsequently in gene expression microarrays performed using mouse B cells treated with TCDD (De Abrew *et al.*, 2011). The increased AHR binding and up regulation of *PTPN6* expression was observed *in vitro* in human primary B cells. Given the role of SHP-1 in B cell activation, this study explores the role of SHP-1 in regulating BCL-6 and in turn, the process of B cell activation in the presence of TCDD.

Materials and Methods

Chemicals and Reagents

99.1% pure TCDD dissolved in dimethyl sulfoxide (DMSO) was purchased from AccuStandard Inc., (New Haven, CT). Tissue culture grade DMSO was purchased from Sigma Aldrich (St. Louis, MO). Sodium Stibogluconate (SSG) (EMD Millipore, Billerica, MA) also known as sodium antimony gluconate is a potent inhibitor of SHP-1 phosphatase (Pathak and Yi, 2001) and was used at a final concentration of 10μg/ml *in vitro*. SSG was dissolved in water at 70°C and was freshly prepared for all experiments involving the inhibitor.

Cell culture

CD40 ligand-expressing L cells are a mouse fibroblast cell line expressing the human CD40 ligand. This cell line was obtained as a generous gift from Dr. David Sherr and was maintained in Dulbeccos modified eagle's medium (Life Technologies, Carlsbad, CA) supplemented with 10% bovine calf serum (Thermo Scientific, Lafayette, CO) with 100U/ml

penicillin and 100μg/ml streptomycin and 50μM of β-mercaptoethanol and HT supplement (Life Technologies, Carlsbad, CA). The expression of CD40 ligand was monitored every 6 months by flow cytometry using APC anti-human CD40L antibody (clone 89–76) (BD Biosciences, San Jose, CA) to confirm high expression of CD40 ligand on the cell surface of CD40L–L cells. CD40 ligand-L cells were cultured for 4 days and irradiated with 35 Gy X-rays using X-Rad 320 (Precision X-Ray, Inc, CT). Human peripheral blood B cells were isolated from human leukocyte packs as described below and were co-cultured with CD40 ligand-L cells in RPMI 1640 supplemented with 10% human AB serum (serum from human type AB donors that lack antibodies against A and B blood type antigens) (Valley Biomedical, VA), penicillin, streptomycin and 50μM of β-mercaptoethanol. Cells were cultured in 5% CO_2 incubator at 37°C.

Human leukocyte packs and isolation of human B cells

Leukocyte packs were obtained from Gulf Coast Regional Laboratories (Houston, TX). Leukocyte packs that tested negative for HIV, HBV, HCV and HTLV were shipped on ice. After receipt, leukocyte packs were diluted with HBSS and overlaid on Ficoll-Paque Plus density gradient (GE Healthcare, Piscataway, NJ) and centrifuged at 1800 rpm for 40 min with low brake. The peripheral blood mononuclear cells were isolated from the buffy coat post-centrifugation, washed, counted and subjected to a magnetic column-based separation that enables isolation of >95% pure naïve ($CD19^+CD27^-$) B cells. This negative selection was conducted using MACS Naïve human B cell isolation kits (Miltenyi Biotech, Auburn CA) following manufacturer's instructions. Purified B cells at a concentration of 1×10^6 cells/ml were then co-cultured with sub-lethally 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 10U/ml, IL-6 at 100U/ml (Roche Applied Sciences, Indianapolis, IN) and IL-10 at 20ng/ml (BioVision Inc., Milpitas, CA) for a period of three or four days depending on the endpoints assayed

Flow cytometry

Antibodies used for flow cytometry are as follows: PE anti-human/mouse BCL-6 (Clone: 603406) (R&D Systems, Minneapolis, MN), PE anti-human BCL-6 (Clone: K112-91) (BD Pharmingen, San Jose, CA), FITC anti-human SHP-1 (Biorbyt, UK) and FITC anti-human SHP-1 (LS Bio, Seattle, WA). For each staining, approximately $0.5 – 1 \times 10^6$ cells were harvested at the indicated time points and viable cells were identified by Fixable Live/Dead Near-IR dye (Life Technologies, Carlsbad, CA) following manufacturer's instructions prior to cell surface or intracellular staining. Surface FcDReceptors 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 re-suspended in FACS buffer (1X Phosphate buffered saline, 1% bovine serum albumin (BSA) (Calbiochem, San Diego, CA) and 0.1% sodium azide (Sigma, St. Louis, MO) pH: 7.6) in the presence of 20% human AB serum and the specific antibodies were added at the company recommended 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 Cytofix cell fixation buffer (BD Biosciences, San Jose, CA) for 10 min, washed and stored at 4°C until they were ready to be analyzed by flow cytometry. For intracellular

protein staining, cells that were previously fixed after surface staining were permeabilized with 1X BD PermWash buffer (BD Biosciences, San Jose, CA) 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 FACS Canto II using FACSDiva software (BD Biosciences, San Jose, CA) and subsequently analyzed using FlowJo (version 8.8.7, Treestar Software Ashland, OR) or Kaluza (version 1.1 or 1.2, Beckman Coulter Inc., Brea, CA). 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 at day 0 (Resting B cells not stimulated with CD40 ligand or IL-2, IL-6 or IL-10) or unstained cells as appropriate.

Quantitative real-time PCR

RNA was isolated from human peripheral blood B cells using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA from each sample was quantified using Nanodrop ND-1000 Scientific spectrophotometer (Thermo Scientific, Wilmington, DE) and 500ng 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 *PTPN6* (Hs00169359_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, CA) 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 as described (Livak and Schmittgen, 2001).

Electrophoretic mobility shift assays and EMSA-Western analysis

a. Nuclear Protein Preparation—Nuclear protein was isolated from HepG2 cells as previously described (Denison *et al.*, 2002) with a few modifications. Briefly, HepG2 cells were treated with vehicle (0.01% DMSO) or TCDD (30 nM in DMSO) for 2h at 37°C Cells were washed twice with 10 mM HEPES (pH 7.5), incubated at 37°C for 15 min, then harvested in MDH buffer [2 mM $MgCl_2$, 1 mM DTT, 2mM HEPES, pH 7.5, with protease inhibitor (Roche, Indianapolis, IN)] and homogenized using a Dounce homogenizer. The homogenates were centrifuged at 1000g for 5 min, washed twice with MDHK buffer [2 mM $MgCl_2$, 1 mM DTT, 2mM HEPES, pH 7.5, and 100 mM KCl, with protease inhibitor (Roche)] and centrifuged. The crude nuclear pellets were resuspended in HEDGK4 buffer [25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 400 mM KCl, 200 μM phenylmethylsulfonyl fluoride, with protease inhibitor (Roche)] (50 μl per plate of confluent cell) and incubated on ice for 30 min for high-salt extraction followed by centrifugation at 14,000 x g, 4°C for 15 min. The supernatants were ultracentrifuged at 99,000 x g, 4°C for 1 h. The protein concentration in supernatants was determined using the BCA assay (Sigma, St Louis, MO).

b. DRE Oligonucleotides—The consensus DRE has been previously described (Sun *et al.*, 2004). The oligonucleotide sequence for consensus DRE is: GGC TTG CGT GCG A. There are four putative DREs in the promoter region of human *PTPN6* gene based on position weight matrix and matrix similarity score computational method (Sun *et al.*, 2004). The sequence for these four DREs are: −1954: ACT CTG TGC GTG CCA CC TC, −1211: ATT ACA GGC GTG AGC CAC, −246: GTT ATT AGC GTG GGC CAG G, −170:TTC GCA TGC GTG AAG TAT T. Complementary pairs of DRE oligomers were synthesized and HPLC purified (Integrated DNA Technologies), followed by annealing and end labeling using T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P] ATP (PerkinElmer).

c. EMSA-Western—Nuclear extracts (10 μg of protein) were incubated with double stranded poly (dI-dC) (0.5 μg) (Sigma) for 30 min at room temperature. The ^{32}P-labeled DRE oligomer (240,000 – 480,000 cpm) or unlabeled DRE oligomer (10 pmol) was added and incubated for another 30 min at room temperature. The final buffer condition in the binding reaction was: 25 mM Hepes (pH7.5), 1mM EDTA, 1mM DTT, 10% glycerol, 100 mM KCl. Protein:DNA complexes were resolved on a 4% non-denaturing PAGE gel in TGE buffer (25 mM Tris, 380 mM glycine, 2 mM EDTA). The radiolabeled portion of the EMSA gel was dried on 3-mm filter paper, and autoradiographed. The non-radiolabeled portion of the EMSA gel was incubated in soaking buffer (375 mM Tris-HCl, pH 7.5, 1% SDS) for 2 h at room temperature, transferred to nitrocellulose blotting membrane (GE Healthcare Life Sciences) using transfer buffer (30 mM Tris, 240 mM glycine, 20% methanol). The protein:DNA complexes on the blot were blocked in TBST buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat milk for 1 h at room temperature. The anti-human AHR purified (Clone: FF3399, eBioscience) primary antibody was then added at 1:1000 dilution and incubated for 2 h at room temperature. The blot was washed using TBST buffer and incubated with the anti-mouse IgG-HRP (Sigma A3673) antibody in TBST buffer with 5% nonfat milk for 1 h.

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 a one-way ANOVA followed by the Bonferroni's post-hoc test or Dunnett's post-hoc test as appropriate. The Fisher's uncorrected least significant difference (LSD) test was used following a significant one-way ANOVA wherever appropriate. Significant outliers were identified using Grubb's outlier test and eliminated from the analyses. For comparison between just the vehicle (VH) and TCDD groups, a Student's two-tailed t-test was employed.

Results

TCDD-mediated AHR binding to the *PTPN6* promoter

In a previous study performed in mouse B cells, *Shp1* was identified as one of 78 genes which showed a significant increase in gene expression at 8 and 12 h in the presence of TCDD and increased AHR binding at sites within the promoter as determined by gene

expression microarrays and ChIP-on-chip (De Abrew *et al.*, 2010). To further confirm the binding of AHR to the putative DRE sites within the *PTPN6* target region, electrophoretic mobility shift assays were performed. Nuclear protein was isolated from HepG2 cells post-treatment with TCDD or VH for 2h. As a positive control, TCDD-inducible binding activity was also measured at the consensus DRE. The nuclear extracts from the TCDD-treated cells showed increased DNA binding activity at the consensus probe (Figure 1A). In the case of the probes spanning the *PTPN6* promoter region, TCDD-inducible DNA binding activity was seen at the probes harboring a DRE at −1954, −1211 and −170 bp upstream of the transcriptional start site (TSS). No TCDD-inducible DNA binding activity was detected at the probe harboring a DRE at −246. These results suggest the presence of TCDD-inducible nuclear complexes at the putative DREs in the presence of TCDD. To confirm the presence of AHR at the putative DREs within the *PTPN6* promoter, EMSA-Western analysis was performed. The EMSA-Western procedure revealed the presence of AHR at the consensus DRE and at all the DREs within the *PTPN6* promoter at locations −1954, −1211, −246 and −170 upstream of the TSS in the presence of TCDD (Figure 1B). Identical migration pattern of the AHR protein detected through the EMSA-western and EMSA analysis further confirms AHR-binding to the DREs within the *PTPN6* promoter.

Increase in *PTPN6* mRNA levels in presence of TCDD

To confirm that *PTPN6* expression is indeed altered by TCDD in human B cells, qRT-PCR was performed. Figure 2A, shows an increase in *PTPN6* mRNA levels upon B cell activation with CD40 ligand and cytokines at day 2 followed by maintenance of the mRNA levels at the later time points. However, TCDD-treatment led to a significant increase in *PTPN6* mRNA levels on days 2 and 3 as compared to the cells treated with VH. Treatment of activated human B cells with an AHR antagonist, failed to induce *PTPN6* mRNA levels to the same extent as cells treated with TCDD. Moreover, treatment of activated B cells with AHR antagonist and TCDD also failed to induce *PTPN6* mRNA levels (Figure 2B).

Time course and concentration response of SHP-1 protein levels in presence of TCDD

In order to assess if changes in *PTPN6* mRNA levels result in changes in the amount of SHP-1 protein, the percentage of SHP-1^{hi} cells were measured by intracellular flow cytometry on days 2, 3 and 4 following B cell activation and TCDD-treatment. The percentage of SHP-1^{hi} cells increased with activation of B cells but TCDD-treatment further elevated the percentage of SHP-1^{hi} cells as observed by the profile of cells in Figure 3 panel A. The increase in SHP-1^{hi} population of cells and the mean fluorescence intensity (MFI) of SHP-1 was significant at day 3 in presence of TCDD as compared to the VH-treated cells (Figure 3 panels B and C). MFI is indicative of the average amount of protein expressed in the selected population of cells. This increase in SHP-1 protein levels parallels the increase in mRNA levels observed at similar time points. The change in SHP-1 MFI relative to the vehicle control cells was also measured in several TCDD-sensitive human donors. Though the magnitude of change and relative time of increase in SHP-1 levels varied across donors (data not shown), the overall trend observed on day 3 was consistent across several human donors (Figure 3 panel D and E, Supplementary Figure 1). Furthermore, TCDD concentration-response changes in SHP-1 protein levels following TCDD-treatment were

examined on day 3. Figure 4 shows a TCDD-mediated concentration-dependent increase in the percentage of SHP-1^{hi} cells in one representative TCDD-sensitive human donor.

Time of TCDD addition identifies a critical window of sensitivity leading to altered SHP-1 protein expression

qRT-PCR and flow cytometric analysis have shown that an increase in SHP-1 expression occurs on days 2 or 3 following TCDD-treatment in activated primary human B cells. In addition to the DRE-mediated regulation of SHP-1, we hypothesized that the increase in SHP-1 levels in the presence of TCDD would also result from an early signaling event triggered by the TCDD-AHR pathway given the involvement of SHP-1 in proximal B cell signaling. To this end, TCDD was added to primary human B cells either 30 min prior to or at the time of B cell activation or at days 1, 2 or 3 post-B cell activation. SHP-1 protein levels were measured on day 4. Figure 5 panel A and B show that the percentage of SHP-1^{hi} cells is increased in presence of TCDD especially when TCDD is added 30 min before activation or at the time of activation and remain elevated post-activation on day 1. Significant effects of TCDD on the mean fluorescence intensity of SHP-1 were also seen when TCDD was added at the time of B cell activation or one day later (Figure 5 panel C).

Increase in BCL-6^{hi} SHP-1^{hi} double positive cells in the presence of TCDD

Network analysis performed on the 78 genes obtained as direct targets of AHR through the ChIP-chip and gene expression microarray studies showed associations with three key transcription factors Bcl-6, Pax-5 and Blimp-1 involved in B cell differentiation. Of particular relevance to this study is the association of SHP-1 (direct AHR target gene) with Bcl-6 involved in B cell activation (De Abrew *et al.*, 2010). Through an earlier study, we have demonstrated the role of BCL-6 in suppression of B cell activation in presence of TCDD (Phadnis-Moghe *et al.*, 2015). Putative associations between BCL-6 and SHP-1 highlight the functional role of SHP-1 in the presence of TCDD as especially as both BCL-6 and SHP-1 are known to inhibit B cell activation. We measured the protein levels of BCL-6 and SHP-1 in the same population of cells using multi parametric flow cytometry. Figure 6 panel A shows the increase in the percentage of BCL-6 SHP-1^{hi} cells in the presence of TCDD on days 3 and 4. This trend of increase in the BCL-6^{+} SHP-1^{+} population is also seen in five TCDD-sensitive human donors measured at Day 3 (Figure 6 panel B).

Putative cross talk between SHP-1 and BCL-6

Given the role of SHP-1 phosphatase in B cells, we hypothesized that SHP-1 could dephosphorylate BCL-6 thereby increasing stability of BCL-6 causing an increase in BCL-6 levels. Moreover, it has been shown that SHP-1 can translocate to the nucleus and retain its phosphatase activity (Yang *et al.*, 2002). To this end, the SHP-1 inhibitor, SSG, was added to human peripheral blood B cells activated with CD40 ligand plus cytokines. As shown in Figure 7 panel A, an increase in BCL-6 levels was observed in naïve (non-treated) human B cells upon treatment with SSG for 2–3 days. In contrast, naïve cells cultured in absence of SSG failed to show a change in BCL-6 levels. The increase in the percentage and MFI of BCL-6 expressing cells is evident through the graphs in Figures 7 panels B and C.

Discussion

The CD40 ligand-dependent activation model has been pivotal in understanding the mechanism underlying suppression of B cell effector function by TCDD in human cells. TCDD-treatment decreased B cell differentiation specifically by suppressing the primary IgM response in human cells (Lu *et al.*, 2009). A recent study further extended the mechanistic investigation of TCDD immune toxic effects and reported that TCDD attenuated expression of surface B cell activation markers CD80, CD86 and CD69 (Lu *et al.*, 2011). Activation of B cells is a critical prequel to the process of differentiation of B cells into antibody secreting plasma cells (Jelinek and Lipsky, 1983; Schmidlin *et al.*, 2009). Hence, the overall aim of this study was to elucidate the mechanism by which AHR agonists perturb B cell activation. *PTPN6* was identified as a potential transcriptional target of AHR in mouse B cells (De Abrew *et al.*, 2010). In addition, SHP-1 negatively regulates BCR signaling by controlling the threshold for B cell activation (Pao *et al.*, 2007b). Hence this paper focuses on confirmation of the changes in SHP-1 expression and its putative role in TCDD-mediated impairment of human B cell activation.

To verify the binding of AHR within the putative DREs in the *PTPN6* promoter, EMSA and EMSA-Western analysis was performed. TCDD-inducible DNA binding at three putative DREs at locations −1954, −1211 and −170 within the *PTPN6* promoter was observed. Although no TCDD inducible DNA binding was seen at the −246 location in the *PTPN6* promoter by EMSA, EMSA-Western analysis confirmed the binding of AHR at that location and to the other DREs at −1954, −1211 and −170. Importantly, the probe designed surrounding the DRE at −246, harbors an NFκB (p65) and RelA binding site that overlaps with the DRE and likely hindered our ability to detect TCDD-inducible activity at that location in spite of the AHR binding observed. NFκB is a known activator of the *PTPN6* promoter. In addition, the presence of AHR at the multiple DRE locations at 2 h post-TCDD addition also suggests concerted regulation of the SHP-1 promoter region by the TCDD:AHR complex. It is currently unknown whether all four DREs require AHR occupancy to mediate the observed changes in *PTPN6* gene expression and this aspect is beyond the realms of the current study. mRNA time course measurements in activated human B cells using qRT-PCR showed that the expression of *PTPN6* was induced by TCDD on days 2 and 3 post-B cell activation. Consistent with the changes in mRNA levels, SHP-1 protein levels were also elevated upon TCDD-treatment at days 3 and 4 in CD40 ligand plus cytokine-activated human B cells isolated from some donors. The increase in the percentage of SHP-1^{hi} cells as well as change in MFI of SHP-1 was seen in several TCDD-sensitive human donors. The changes in protein levels of SHP-1 were also observed in a TCDD concentration-dependent manner. Collectively, these findings confirm the ChIP-on-chip and gene expression microarray studies and suggest that SHP-1 is indeed a direct target of the TCDD:AHR complex.

To further characterize the mechanism by which TCDD increased SHP-1 in human B cells, time of addition studies were conducted in which TCDD was added to cultured human B cells at specific time points in relationship to cell activation. One group of cells was treated with TCDD 30 min prior to B cell activation, the other treatment groups from the same donor were treated with TCDD either 1h, 2h or 4h post-B cell activation using cytokines

plus CD40-ligand L cells to identify the window of sensitivity of human B cells to a TCDD-mediated increase in SHP-1 protein levels on day 4. A significant increase in SHP-1 protein levels as measured by the MFI and percentage of SHP-1^{hi} cells was evident only when TCDD was added 30 min prior to or at the time of B cell activation. Addition of TCDD one day post-activation also resulted in a modest increase in SHP-1^{hi} cells with no effect seen at the later time points. This suggests that the increase in SHP-1 levels is contingent on the time of TCDD addition and provides insight into the temporal relationship wherein disruption of early signaling events by TCDD could potentially influence SHP-1 levels. Results from the time-of-addition study are also consistent with previous observations in which, TCDD-mediated suppression of B cell activation in human cells occurred only when TCDD was added at the time of activation or 24h post- activation of B cells (Lu, H, unpublished observations). Moreover, this observation is concordant with earlier studies performed in mouse cells employing the sheep RBC (sRBC)-elicited IgM response. Maximal suppression of anti-sRBC IgM antibody forming cell response was observed when TCDD was added to splenic cultures either 60 min prior to or at the time of antigen sensitization or day 1 post-antigen sensitization. The effect diminished when TCDD was added at days 2 – 5 post-antigen sensitization (Tucker *et al.*, 1986). In addition, the suppression of the LPS-induced IgM antibody forming cell (AFC) response was produced when TCDD was added before 3h post- B cell activation (Holsapple *et al.*, 1986). Suppression of early kinase phosphorylation by TCDD, especially kinases associated with the CD40 receptor and cytokine signaling pathways may provide an explanation for the window of sensitivity surrounding the effects observed in human cells. It is well known that kinases in B cells respond very rapidly to activation stimuli and transmit the signal downstream via signal amplification to affect B cell effector functions before returning to normal levels (Irish *et al.*, 2006). It is tempting to speculate that the initial signaling pathway induced via ligation of CD40 and binding of cytokines may have reached peak activity within hours of activation and then decreased back to basal levels thereby the critical window of sensitivity having passed thus rendering the B cells refractory to the effects of TCDD at the later time points. Thus, our study proposes two potential mechanisms by which TCDD leads to alteration of SHP-1 levels: (a) by direct binding of AHR to the *PTPN6* promoter as evidenced by EMSA-Western study; and (b) by altering an early signaling event in the B cells, which ultimately modifies SHP-1 expression, as evidence by the TCDD time-of-addition studies.

To identify which genes among the 78 genes identified by the ChIP-on-chip and gene expression microarrays were involved in B cell differentiation, putative signaling networks were constructed to connect the 78 genes to PAX5, BLIMP-1 and BCL-6, the key regulators of B cell differentiation (De Abrew *et al.*, 2010). Through the network analysis, SHP-1 was seen to negatively regulate STAT1, STAT5 and IRF8, which in turn were regulating BCL-6 expression. Hence given the possibility of SHP-1-mediated regulation of BCL-6, SHP-1 and BCL-6 levels were measured concomitantly in human B cells treated with TCDD. An increase in the percentage of SHP-1^{+} BCL-6^{+} cells was observed in presence of TCDD in human B cells from five TCDD-sensitive human donors.

Elevated SHP-1 levels inhibit activation of B cells through the alteration of upstream kinases. TCDD treatment was shown to decrease the percentage of pERK^{+}p38^{+}pAKT^{+} cells

upon immediate early activation of B cells (Lu *et al.*, 2011). TCDD time-of-addition studies also suggested that changes in SHP-1 expression occur if TCDD is present 30 min before or at the time of B cell activation. Hence it is possible that decrease in levels of B cell kinases may be occurring through elevated SHP-1 levels in human B cells. Moreover, SHP-1 has been reported to inhibit Akt, pSTAT3 and pSTAT5 (Han *et al.*, 2006; Mittal *et al.*, 2011). Also Akt is important for cell survival (Andjelic *et al.*, 2000; Calo *et al.*, 2003). Hence the impaired B cell activation phenotype could be a result of impaired activation of B cells implicated as a result of decreased Akt and decreased survival in presence of elevated SHP-1 levels. SHP-1 and BCL-6 multi-parametric flow cytometry further revealed that SHP-1^{hi} cells also showed elevated BCL-6 levels thus suggesting potential cross-talk between SHP-1 and BCL-6 in presence of TCDD. We further explored if BCL-6 levels were altered in presence of SHP-1 inhibitor, sodium stibogluconate (Pathak and Yi, 2001) in naïve B cells and interestingly observed a decrease in BCL-6 as opposed to no change or stable BCL-6 levels in absence of SHP-1 inhibitor. This is suggestive of a role by SHP-1 in dephosphorylating BCL-6 thereby stabilizing BCL-6 levels in the absence of the SHP-1 inhibitor. These studies show for the first time a relationship between SHP-1 and BCL-6 levels in human primary B cells. BCL-6 mediated suppression of B cell activation markers was recently demonstrated (Phadnis-Moghe *et al.*, 2015). Another mechanism by which SHP-1 putatively regulates BCL-6 levels is through amplification of double-negative feedback loop involving B cell signaling and transcription factors BCL-6 and Blimp-1 (Reth and Brummer, 2004). SHP-1 is known to inhibit Lyn, Syk and Btk kinases downstream of BCR, thereby keeping BCR activation at a minimum. This would lead to a decrease in ERK activation ultimately preventing BCL-6 phosphorylation and degradation. BCL-6 would then suppress B cell activation and differentiation through repression of its target genes such as CD80, CD69 and Blimp-1 (Shaffer *et al.*, 2000). We have already shown that suppression of human B cell activation by TCDD involves altered regulation of BCL-6 (Phadnis-Moghe *et al.*, 2015). Figure 8 illustrates the current model of SHP-1 regulation in presence of TCDD in human B cells. SHP-1 is directly activated by AHR in B cells and could play an important regulatory role in mediating the suppression B cell activation. (a) SHP-1 can directly dephosphorylate kinases associated with the B cell receptor or CD40 receptor ;(b) SHP-1 can dephosphorylate signaling intermediates downstream such as ERK, AKT ;(c) SHP-1 can dephosphorylate BCL-6 in the nucleus. All these processes are not mutually exclusively but can act in a coordinated manner to decrease B cell activation. Thus taken together, our studies suggest that BCL-6 and SHP-1 may, in part, contribute to the impaired B cell activation phenotype observed in the presence of TCDD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

AHR	Aryl Hydrocarbon Receptor
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
DRE	dioxin response element
CD40L	CD40 ligand
BCR	B cell receptor
SHP-1	Src homology phosphatase-1
PTPN6	protein tyrosine phosphatase, non-receptor type 6
BCL-6	B cell lymphoma-6

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Highlights

1. SHP-1 encoded by the gene *PTPN6* is directly activated by the AHR
2. AHR binds to dioxin response elements within the SHP-1 promoter in a TCDD-inducible manner
3. TCDD-mediated increase in SHP-1 levels is observed in primary human B cells
4. Higher SHP-1 levels help in maintaining high BCL-6 levels in presence of TCDD
5. In the presence of SHP-1 inhibitor, decreased BCL-6 levels are observed.

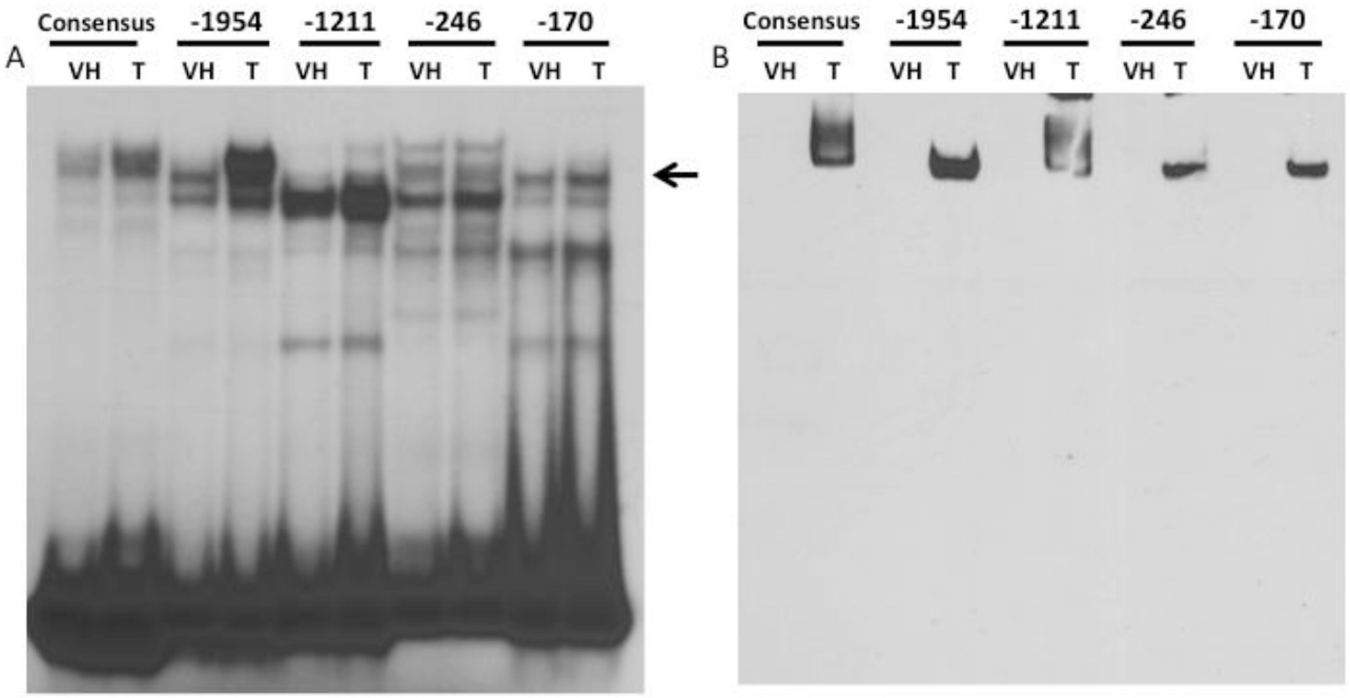

Figure 1. TCDD-induced binding of AHR to putative DRE sites in PTPN6 promoter

Nuclear protein was isolated from HepG2 cells treated with vehicle (VH, 0.01% DMSO) or TCDD (T, 30nM), incubated with ^{32}P -labeled A) or unlabeled B) consensus DRE and four DRE oligomers in *PTPN6* promoter (indicated by the position relative to transcription start site). A) Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, dried and autoradiographed. B) Protein:DNA complexes were resolved on a 4% nondenaturing PAGE gel, transferred to nitrocellulose, and probed with anti-AHR antibody. Arrow indicates specific binding of AHR to DRE oligomers. Results are representative of more than two independent experiments.

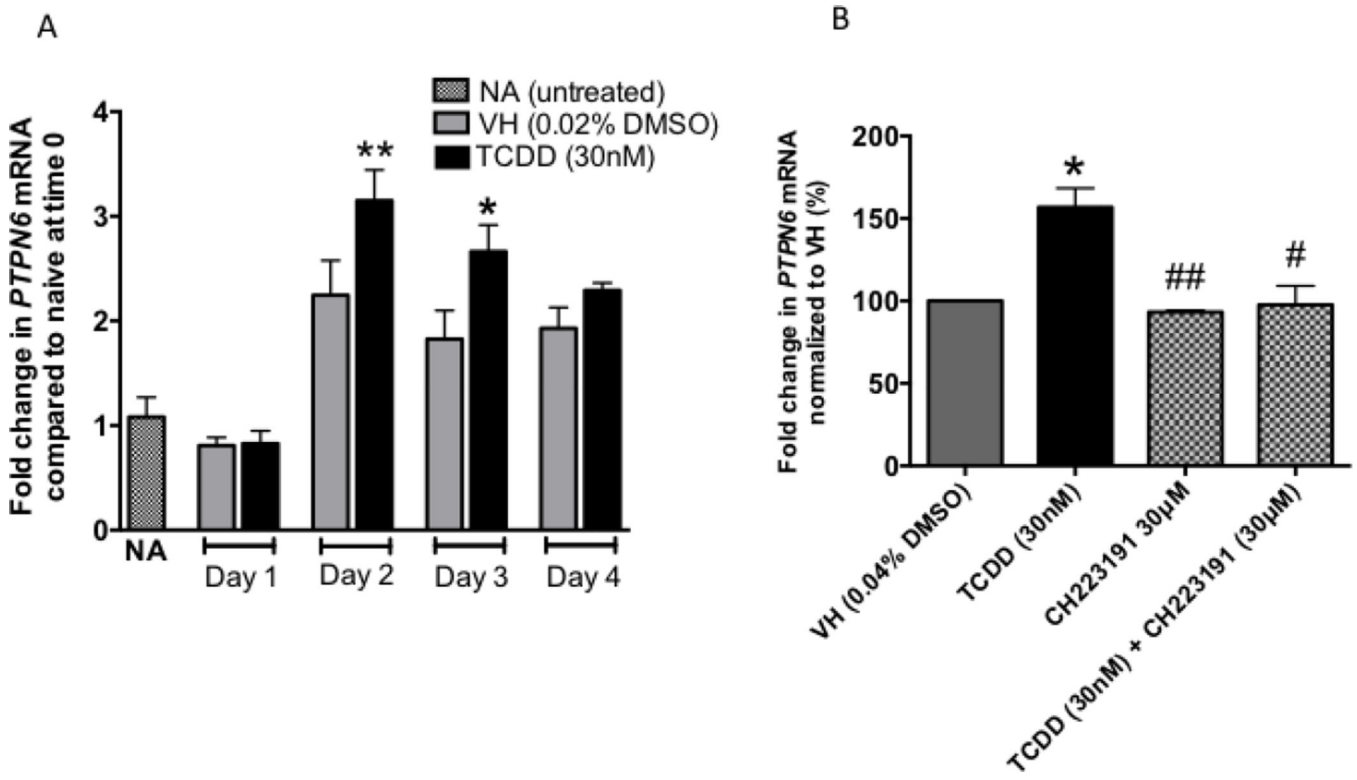

Figure 2. Increase in PTPN6 mRNA levels following TCDD-treatment in human B cells

Human B cells were activated with CD40 ligand plus cytokines and treated with vehicle (VH) 0.02% DMSO or TCDD (30 nM) for four days. *PTPN6* mRNA levels were measured by qRT-PCR at the time points indicated on the graph (A). Naïve cells were activated with CD40 ligand plus cytokines and harvested on day 0. *PTPN6* mRNA levels were measured on day 3, from cells treated with TCDD or AHR antagonist, CH223191. Fold change in *PTPN6* mRNA was normalized to VH control of each donor. Data from two human donors is presented. * $p < 0.05$ comparing the VH to the TCDD-treated group, # $p<0.05$ comparing treatment with CH223191 and TCDD to VH, ## $p<0.01$ comparing CH223191 treatment to VH group.

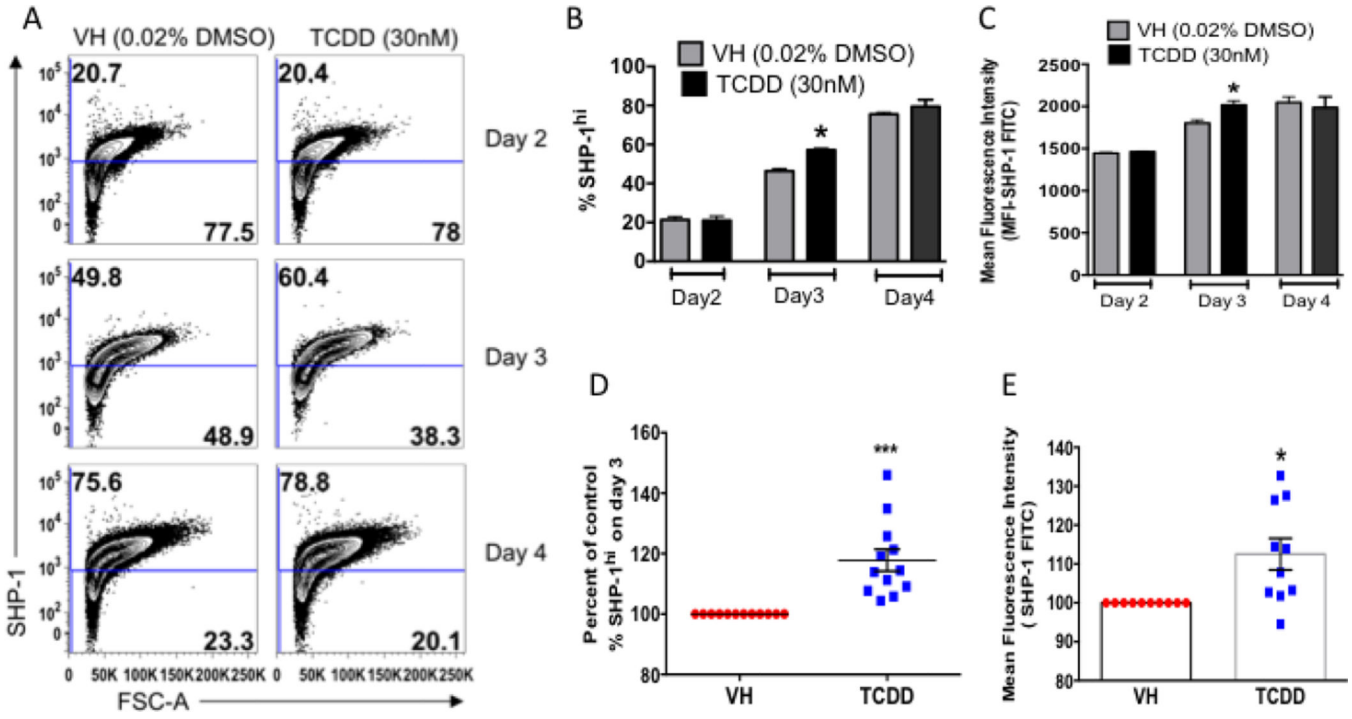

Figure 3. Increase in SHP-1 protein levels in presence of TCDD

A) Human primary B cells were activated with CD40L plus cytokines and treated with TCDD or VH. Cells were harvested at the indicated time points and permeabilized. SHP-1 levels were measured by flow cytometry. The gates mark the SHP-1^{hi} and SHP-1^{lo} populations of cells and the numbers within each gate depict the percentage of SHP-1^{+} cells. Data shown in the figure is representative of fifteen TCDD-sensitive human donors. B) Changes in %SHP-1^{hi} cells at different time points in the same donor as shown in panel A. C). Graph depicts change in SHP-1 MFI in the same donor as shown in panel A. D) %SHP-1^{hi} cells in twelve TCDD-sensitive human donors measured on day 3. E) MFI SHP-1 in ten TCDD-sensitive human donors measured on day 3. Each dot represents one TCDD-sensitive human donor and is normalized to its own vehicle control. Dead cells were excluded from the analysis. * $p < 0.05$ as compared to VH control of each day by one-way ANOVA followed by Fisher's Uncorrected LSD test for panels B and C. * $p < 0.05$, ***$p<0.001$ using Student's unpaired t-test.

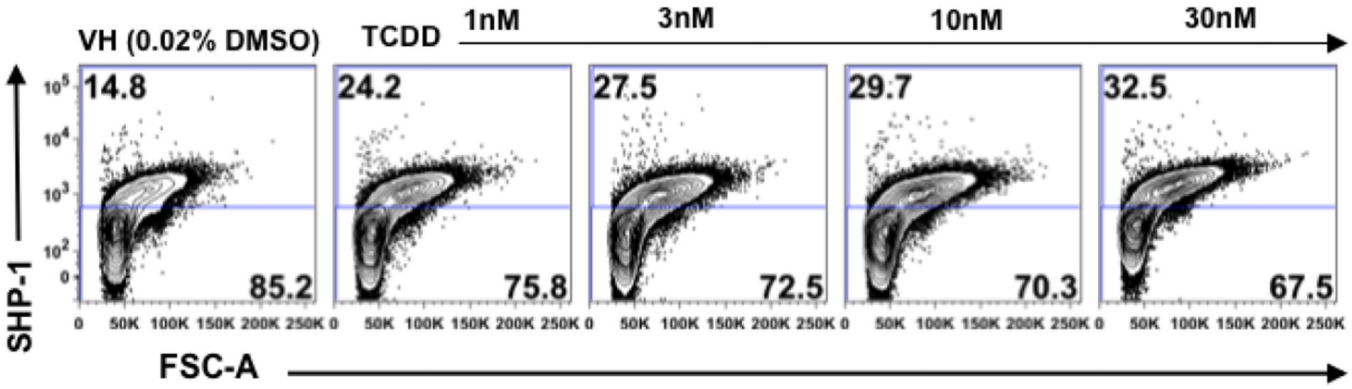

Figure 4. TCDD-concentration dependent increase in SHP-1 protein levels

Primary human B cells were treated with VH or TCDD at the indicated concentrations for a period of three days. Cells were harvested and then permeabilized for staining by flow cytometry. SHP-1 protein levels were measured on day 3. The gates depict the SHP-1^{hi} and SHP-1^{lo} cell populations with the numbers within the gates indicating the percentage of SHP-1 cells. Data shown in the figure is representative of five TCDD- sensitive human donors.

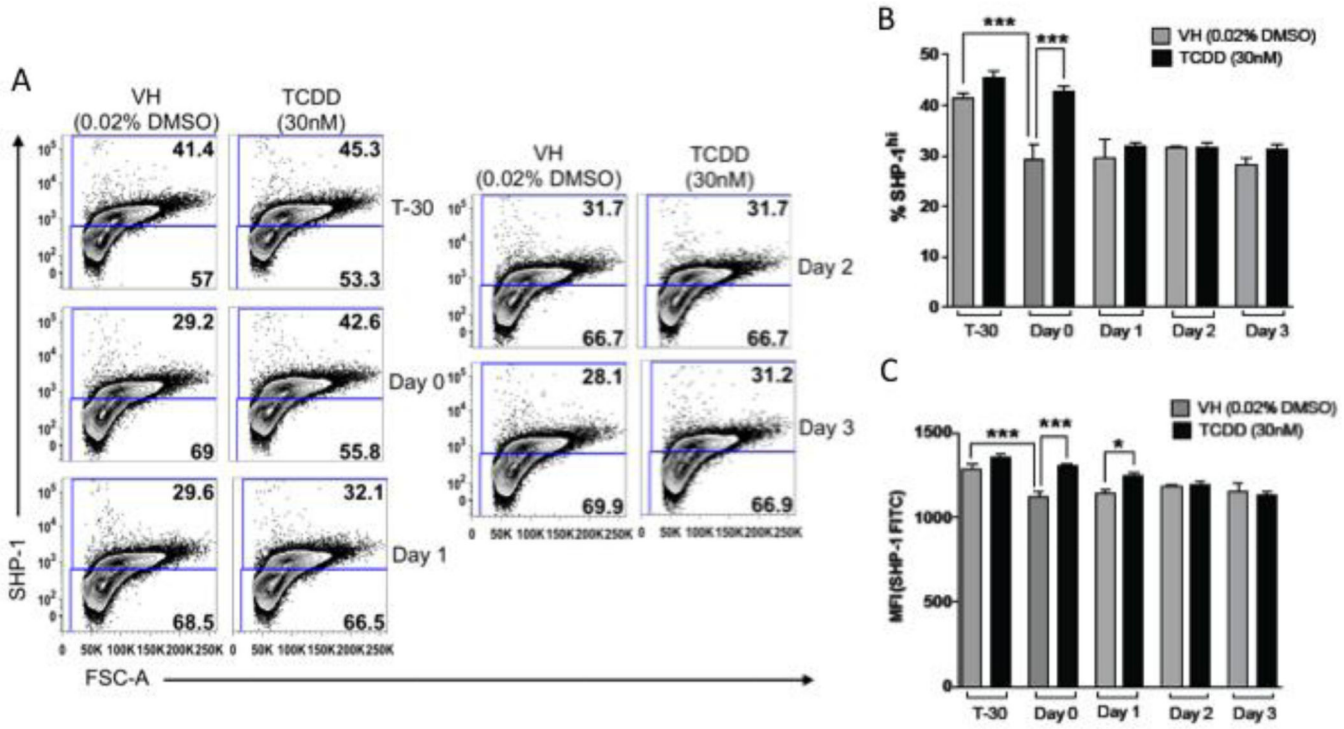

Figure 5. Increase in SHP-1 protein levels is dependent on time of TCDD addition

A) Human primary B cells were activated with CD40 ligand plus cytokines. TCDD was added to the cells either 30 min before activation or at the time of activation or at the indicated time points. Cells were harvested on day 4 and permeabilized in order to measure intracellular SHP-1 protein levels by flow cytometry. Graphs depict the percentage SHP-1^{hi} and SHP-1^{lo} cells as indicated by the gates and the frequency numbers in the corner. B) Percent of SHP-1^{hi} cells in the same TCDD-sensitive human donor as shown in panel A C) MFI of SHP-1 in the same donor as shown in panel A. * $p< 0.05$, ***$p< 0.001$ by one-way ANOVA followed by Fisher's Uncorrected LSD test.

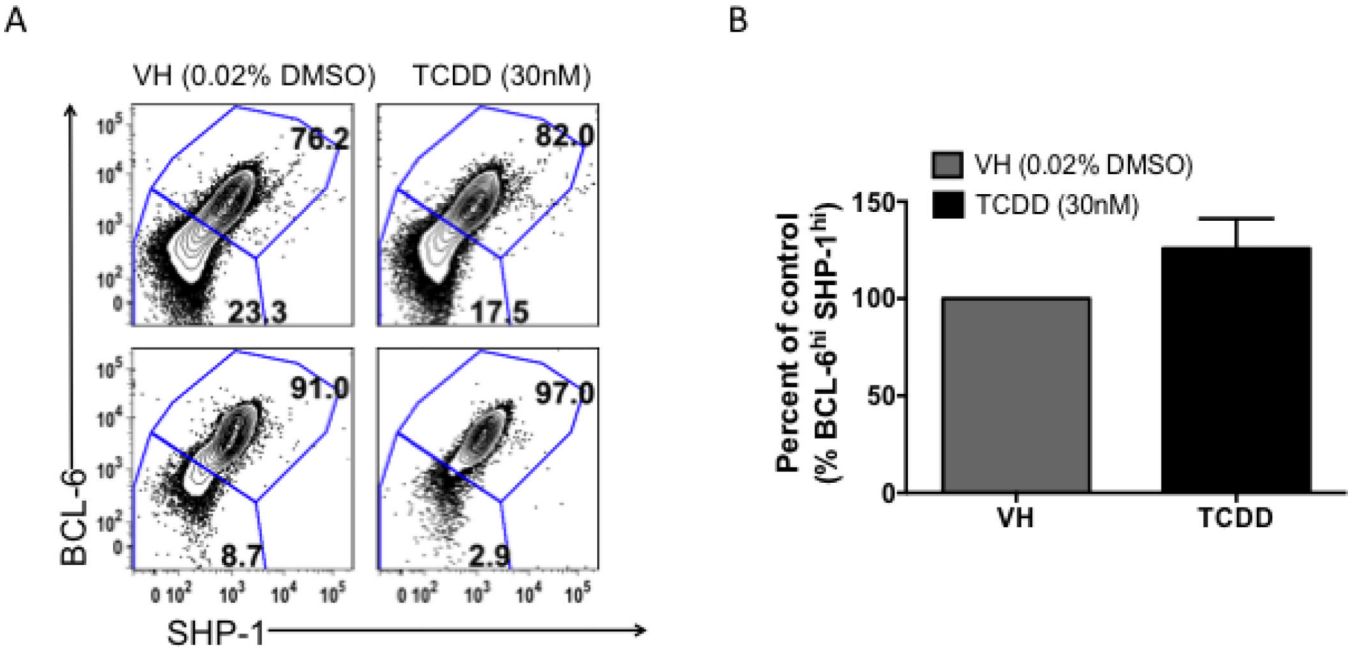

Figure 6. Increase in BCL-6^{hi} SHP-1^{hi} double positive cells in presence of TCDD

A) Human B cells were harvested at Day 3 and Day 4 following activation with CD40 ligand plus cytokines and treatment with TCDD or VH. The gates indicate the BCL-6^{hi} SHP-1^{hi} and BCL-6^{lo} SHP-1^{lo} populations of cells with numbers indicating the percent of double positive cells from one human donor. B) Increase in %BCL-6^{hi} SHP-1^{hi} cells on day 3 shown as percent of VH control. Data pooled from five TCDD-sensitive human donors.

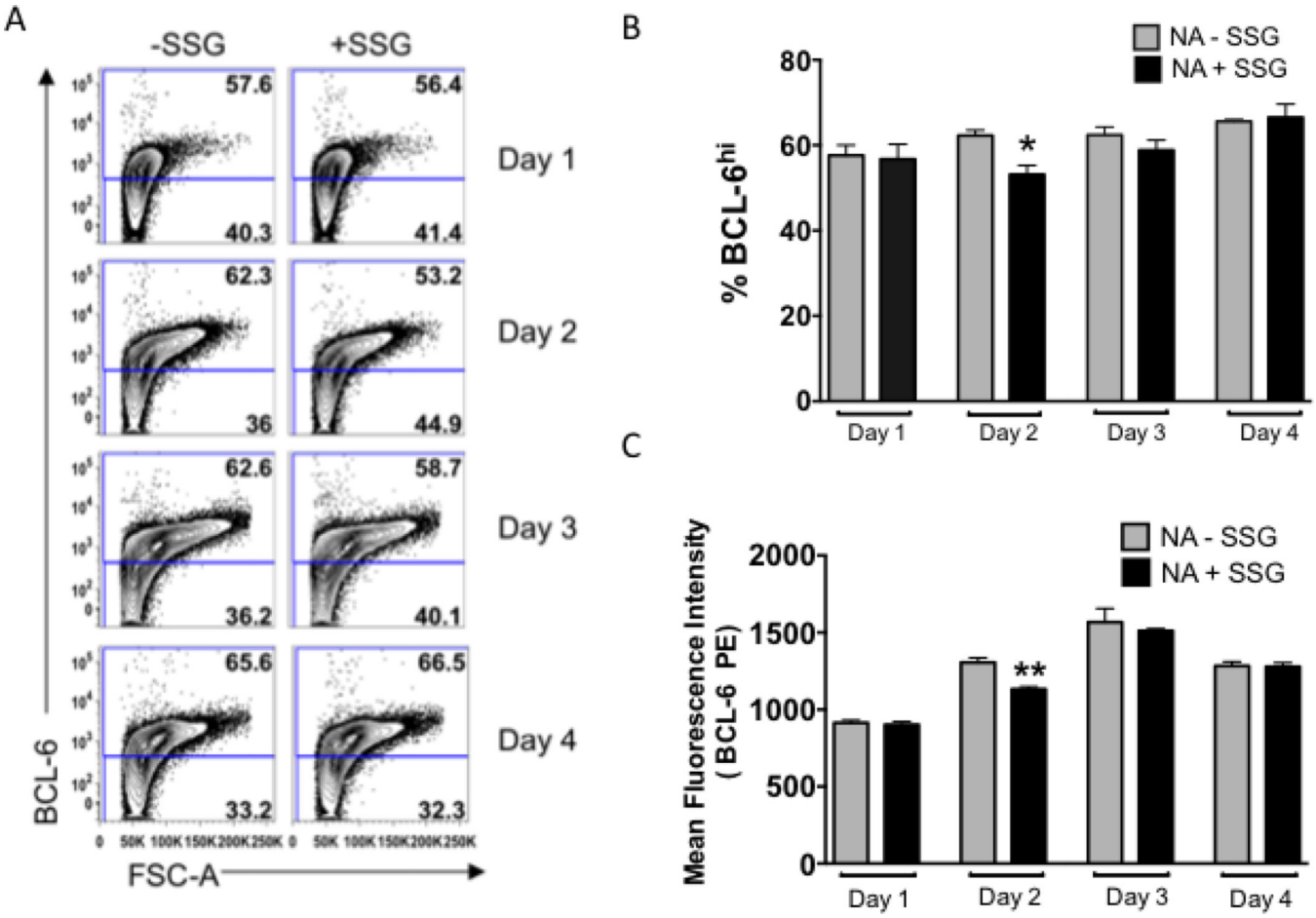

Figure 7. Changes in BCL-6 protein levels in presence of SHP-1 inhibitor

A) Naïve (untreated) human B cells were activated with CD40 ligand plus cytokines and 10μg/ml of SHP-1 inhibitor SSG. Cells were harvested at the indicated time points and stained for BCL-6. Data in the flow cytometry plots is representative of two TCDD-sensitive human donors. B) Changes in percentage of BCL-6^{hi} cells in the same donor as shown in panel A in presence and absence of the SHP-1 inhibitor. C) Changes in MFI of BCL-6 in the same donor as shown in panel A. *$p < 0.05$, **$p < 0.01$ by one-way ANOVA followed by Fisher's Uncorrected LSD test.

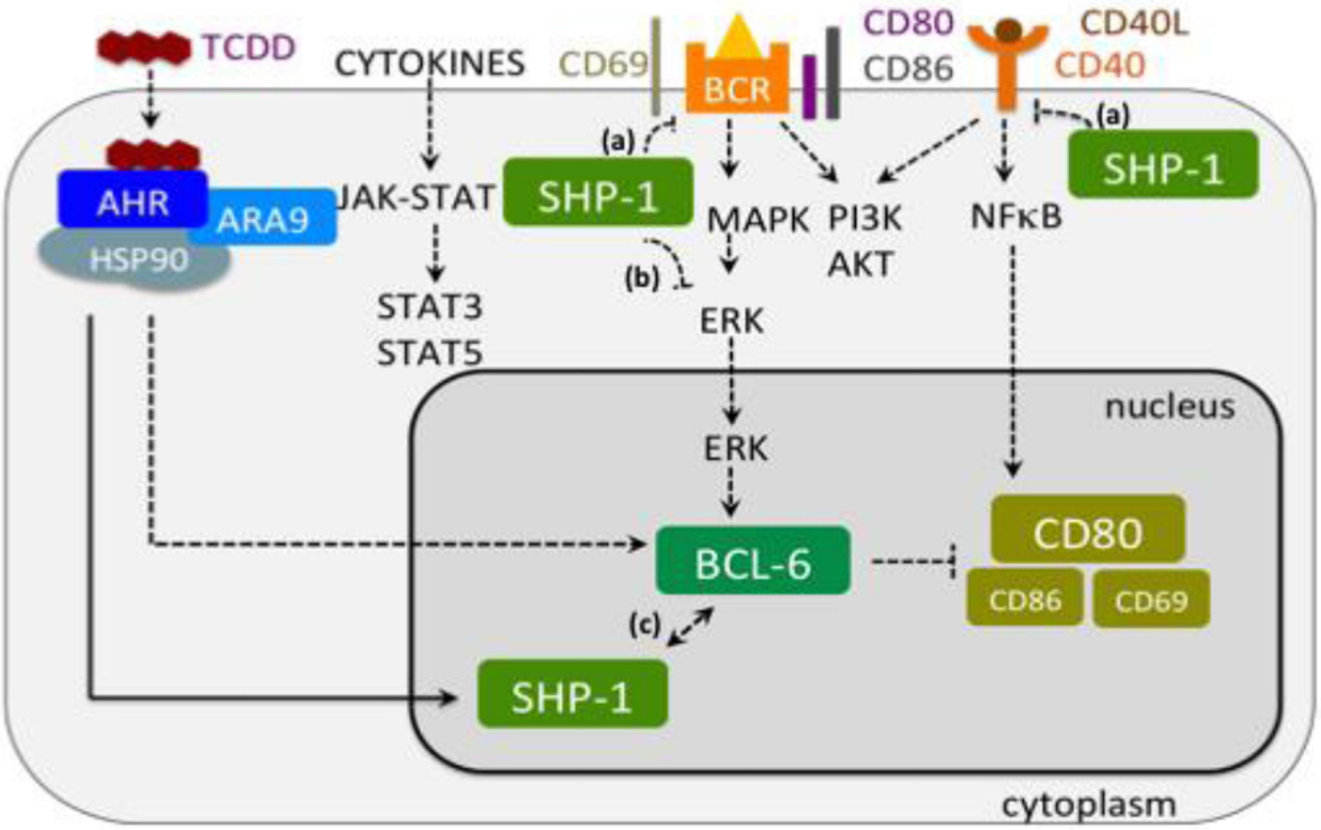

Figure 8. Schematic illustration of the interaction between BCL-6 and SHP-1 in the presence of TCDD

The figure illustrates signaling pathways activation upon ligation of BCR with antigen and upon stimulation of the B cell with CD40 ligand and cytokines. These signaling pathways result in B cell activation exemplified by an increase in CD80, CD86 and CD69 levels. The TCDD:AHR complex results in an increase of SHP-1. SHP-1 potentially regulates BCL-6 and thereby alters B cell activation in the presence of TCDD.