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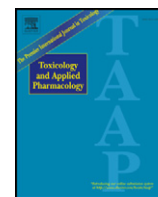

SHP-1 is directly activated by the aryl hydrocarbon receptor and regulates BCL-6 in the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

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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 the presence of TCDD and may, in part through BCL-6, regulate TCDD-mediated suppression of human B cell activation.

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1. 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 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

Abbreviations: 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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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.

2. Materials and methods

2.1. 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 10 μg/ml in vitro. SSG was dissolved in water at 70 °C and was freshly prepared for all experiments involving the inhibitor.

2.2. 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 Dulbecco's modified eagle's medium (Life Technologies, Carlsbad, CA) supplemented with 10% bovine calf serum (Thermo Scientific, Lafayette, CO) with 100 U/ml penicillin and 100 μg/ml streptomycin and 50 μM 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 50 μM of β-mercaptoethanol. Cells were cultured in 5% CO_2 incubator at 37 °C.

2.3. 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 a 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 the 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 the presence of recombinant human cytokines IL-2 at 10 U/ml, IL-6 at 100 U/ml (Roche Applied Sciences, Indianapolis, IN) and IL-10 at 20 ng/ml (BioVision Inc., Milpitas, CA) for a period of three or four days depending on the endpoints assayed.

2.4. 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 the 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× 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 1× 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

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