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CYP1A1 in polycyclic aromatic hydrocarbon-induced B lymphocyte growth suppression ☆

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Abstract

The AhR is a ligand-activated transcription factor that mediates immunosuppression by environmental PAH. Previous studies demonstrated that activation of mature human B cells up-regulates AhR expression, suggesting that human B cells are direct PAH targets. To test this hypothesis and to determine the metabolic requirements for PAH toxicity in a human model, the effects of a prototypic PAH, B[*a*]P, on B cell growth were evaluated. B[*a*]P and its proximal (B[*a*]P-7,8-dihydrodiol) and terminal (B[*a*]P-7,8-dihydrodiol-9,10-epoxide) metabolites inhibited growth in a dose-dependent manner. A poorly metabolized AhR ligand had no effect, suggesting that biotransformation is required for growth inhibition. Inhibition of the CYP1A1 monooxygenase completely blocked growth inhibition induced by B[*a*]P or B[*a*]P-7,8-dihydrodiol, but not by B[*a*]P-dihydrodiol-9,10-epoxide, indicating that CYP1A1-dependent metabolism of B[*a*]P into the terminal B[*a*]P-7,8-dihydrodiol-9,10-epoxide metabolite is required for growth inhibition. These studies show for the first time the metabolic requirements for PAH-mediated suppression of human B cell growth.

Keywords: Human B cells; Transcription factors; Cell proliferation

The aryl hydrocarbon receptor (AhR), a member of the Per-ARNT-Sim family of bHLH transcription factors, is best known for its role in the regulation of cytochrome P450 (CYP) enzymes, e.g., CYP1A1 and CYP1B1. These enzymes are responsible for the oxidation of a variety of environmental AhR ligands and, in some cases, the production of biologically active metabolites. The most potent and most thoroughly studied AhR ligands are environmental pollutants such as polycyclic aromatic hydrocarbons (PAH) and halogenated aromatic hydrocarbons (HAH). Many of these chemicals are immunosuppressive and carcinogenic, likely through mechanisms involving AhR activation and the ensuing transcription of AhR-regulated genes. Among the AhR-regulated genes implicated in malignant transformation are those encoding the CYP1 monooxygenases [1,2].

With regard to AhR-mediated immunosuppression, the developing immune system is particularly sensitive to AhR ligands. For example, administration of prototypic AhR ligands to animals profoundly reduces bone marrow cellularity [3] and induces thymic atrophy [4–7], apparently through the induction of apoptosis [8] and the suppression of precursor T cell generation [9]. Similarly, PAH treatment of bone marrow cultures results in pre- and pro/pre-B cell apoptosis, although this activity is mediated indirectly through AhR^+ bone marrow stromal cells [3,10–15]. Of relevance to the present study, biotransformation of the parent PAH into constituent metabolites is required to

☆ *Abbreviations:* AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; HAH, halogenated aromatic hydrocarbon; B[*a*]P, benzo[*a*]pyrene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 1-PP, 1-(1-propynyl)pyrene; 4,5-diol, B[*a*]P-*trans*-4,5-dihydrodiol; 7,8-diol, B[*a*]P-*trans*-7,8-dihydrodiol; BPDE, B[*a*]P-7,8-dihydrodiol-9,10-epoxide.

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induce a stromal cell-derived death signal which is delivered to the bone marrow B cells [13].

Mature T and B cells also are adversely affected by PAH. Animals treated with a prototypic PAH display decreased cytotoxic T cell responses, alloantigen-specific mixed-lymphocyte reactions, mitogen-dependent T cell proliferative responses, and cytokine production [16–18]. Similarly, PAH decrease splenic B lymphocyte numbers, blunt antibody production, and reduce the number of antigen-specific responder B cells [19–23]. These outcomes likely explain the decreased resistance of PAH-exposed animals to infectious agents and transplantable tumors [20,24]. It has been suggested in some [25–27], but not all [28], rodent studies that PAH metabolism plays an important role in inhibiting B cell-dependent responses. However, macrophages, presumably required for antigen presentation to B cells, and not B cells themselves, appeared to be the immediate targets of the immunosuppressive PAH metabolites in those early studies. Indeed, the level of PAH metabolites produced by B cells from naïve mice was only slightly above detectable levels. Extension of these kinds of studies to human model systems is critical given the potential for AhR ligands to significantly suppress human immune responses [29,30] and the uncertainty of extrapolating results from animal models to human systems, particularly in view of the well-documented differences in murine and human AhR inducibility [31,32]. While at least one study demonstrated that transformed human B lymphoma cells undergo apoptosis on exposure to relatively high doses of B[*a*]P or its metabolites [33], no published studies have evaluated the effects of PAH or their metabolites directly on purified primary populations of human B lymphocytes that have been activated in a fashion which accurately mimics adaptive immune responses. Similarly, none have demonstrated a critical role for AhR-regulated CYP1A1 in mediating changes in human B cell function following PAH exposure.

We directly determined the potential impact of exogenous PAH on activated B lymphocytes, and evaluated the possible contributions of AhR-dependent CYP1A1 and CYP1A1-generated metabolites. To model physiological B cell activation, we exploited a method for expanding non-transformed human B cell populations in vitro. B cell activation by $CD40L^+$ T helper cells during adaptive immune responses is modeled in this system by culturing primary B cells with polyvalent CD40 ligand (CD40L) and IL-4 [34]. Importantly, activation of B cells through the CD40 receptor profoundly increases AhR expression [34], suggesting that it is the responding antigen-specific B cells that are the most sensitive to environmental AhR ligands. This possibility was tested by evaluating the expansion of activated B cell populations in the presence of B[*a*]P. Furthermore, we tested the possibility that metabolites, generated by CYP1A1, are an important effector of B[*a*]P toxicity by evaluating the ability of proximal and terminal B[*a*]P metabolites to inhibit B cell growth in the presence and absence of a CYP1A1 inhibitor. The results demonstrate that primary human B cells behave differently than transformed lymphoma cells following PAH exposure and provide important insights into the biochemical mechanisms through which PAH compromise the effectiveness of human B cells which have up-regulated AhR expression during their response to antigenic stimulation.

Materials and methods

Chemicals and reagents. Benzo[*a*]pyrene (B[*a*]P) (Sigma, St. Louis, MO) and metabolites of B[*a*]P, including B[*a*]P-*trans*-4,5-dihydrodiol, B[*a*]P-*trans*-7,8-dihydrodiol, and B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE), obtained from NCI Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO), were dissolved in dimethylsulfoxide (DMSO; Sigma). CYP1A1 inhibitor, 1-(1-propynyl)pyrene (1-PP) (kindly provided by Dr. Cornelis Elferink, University of Texas Medical Branch, Galveston, TX), also was prepared in DMSO. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (Ultra Scientific, Inc., North Kingstown, RI) was dissolved in DMSO and working dilutions were made in acetone. When using solutions made up in DMSO, cells were dosed from a 1000× stock so that the end-concentration was never more than 0.1%.

Cell culture and conditions. CD40L-transfected L cells (American Type Tissue Culture Collection, VA) were maintained at 37 °C in 10% CO_2 in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 5 μg/ml Plasmocin (Invivogen, San Diego, CA), and 1× HT. All culture reagents were obtained from Cellgro (Mediatech, Herndon, VA) unless otherwise indicated.

Peripheral blood mononuclear cells (PBMCs) were prepared from individual donor blood (New York Biologics, Inc., New Jersey, NY) by centrifugation through Ficoll (Amersham Biosciences, Uppsala, Sweden). To remove excess platelets, PBMCs were washed three times in Hanks buffered salt solution (HBSS) and centrifuged at 1000 rpm for 15 min. PBMCs were depleted of T cells by sheep red blood cell (ICN Biomedicals, Aurora, OH) rosetting for 10 min at 37 °C followed by one hour on ice in RPMI. Following a second centrifugation through Ficoll, cells were stained with FITC-labeled CD20-specific antibody and purified by fluorescence activated cell sorting (MoFlo, Dako Cytomation). Approximately 10^7 B cells (>99% $CD20^+$) were recovered per donor.

Approximately 10^7 Ficoll-enriched PBMCs or 4×10^6 sorted ($CD20^+$) B cells were cultured on confluent monolayers of irradiated (3000 Rads from a ^{137}Cesium gamma cell irradiator; Gammacell 40, Canada) CD40L-transfected L cells in 6-well plates in Iscove's media (Gibco/Invitrogen), supplemented with 5% heat-inactivated, human AB serum (ICN), 50 μg/ml human transferrin (Gibco), 0.5% human serum albumin (Aventis Behring, Kanakakee, IL), 5 μg/ml human insulin (Sigma), and 25 μg/ml Plasmocin, 50 ng/ml human rIL-4 (Research Diagnostics, Inc., Flanders, NJ). For experiments in which activated B cells were generated from PBMCs, cells were cultured in the presence of 0.55 μM cyclosporin A (Sigma) to deplete T cells. Expanding B cell populations were transferred to fresh, irradiated CD40L-transfected L cell monolayers every 3–4 days. Unless otherwise indicated, all experiments were performed using activated B cells that had been cultured between one and two weeks on CD40L-transfected L cells with rIL-4. Approximately 10^7 PBMCs proliferated to yield several hundred million activated B cells in that time period (>97% $CD19^+$). Cell viability was determined by trypan blue and propidium iodide exclusion by light microscopy and flow cytometry, respectively.

CFSE staining. Sorted naïve B cells ($4–5 \times 10^6$) were washed in PBS containing 0.1% BSA, resuspended in 1 ml PBS/0.1% BSA with 2.5 μM CFSE (Molecular Probes, Eugene, OR), and incubated for 15 min at 37 °C. Labeled cells were then washed in PBS/0.1% BSA, resuspended in medium, and cultured on monolayers of CD40L-transfected L cells for 3 days. Activated B cells ($0.5–1 \times 10^6$) were transferred to 24-well plates with fresh CD40L cells and cultured for an additional 3 days in the presence of

vehicle (DMSO) or 10^{-6} M B[*a*]P. Cells were harvested, washed and the profile of cell divisions was determined by measuring CFSE fluorescence by flow cytometry (BD Biosciences). Data were analyzed using ModFitLT analysis software (BD Biosciences).

Ethoxyresorufin–o-deethylase activity assay. CD40L-activated B cells (10^6) were cultured in 48-well plates pre-coated with anti-CD40 antibody (Ancell, Bayport, MN). After 24 h, cells were treated with DMSO (0.1% v/v), TCDD (10^{-9} M), B[*a*]P (10^{-6} M), and/or 1-PP (10^{-5} M) for an additional 24 h. Harvested cells were washed once with PBS, and then buffer (50 μl of 25 mM Hepes, pH 7.5, 1.5 mM EDTA, and 10% (v/v) glycerol) was added to cell pellets. Cells were lysed by freeze-thawing at −80 °C and cell lysates were transferred into 96-well plates. 7-Ethoxyresorufin (20 μM in 25 μl/well) in 100 mM sodium phosphate, pH 7.8, was added and the plate was warmed to 37 °C. The reaction was initiated by the addition of 25 μl/well of 4 mM NADPH in phosphate buffer. Fluorescence was read at 2 min intervals for 20 min at 37 °C in a Cytofluor 4000 (Millipore, Inc., Bedford, MA) plate reader using a 530-nm excitation wavelength and a 620-nm emission wavelength. Results were compared to a standard curve of resorufin in sodium phosphate buffer. All chemicals were purchased from Sigma Chemical, Inc. The protein concentration for each sample was determined using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA) and used to normalize ethoxyresorufin–*o*-deethylase (EROD) activity to obtain (pmol/min/mg).

Relative quantitative real-time PCR assay. Total RNA was prepared from washed B cell pellets using RNA STAT-60 (Tel-Test Inc. Friendswood, TX). Total RNA (2–5 μg) was reverse-transcribed using Superscript II (Invitrogen). Real-time PCR was performed using the Applied Biosystems SYBR Green PCR Master Mix (Foster City, CA) and analyzed on an ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster, CA). To avoid amplification of any contaminating genomic DNA, one of the primers in each pair was placed at the junction between two exons. The primer sequences used were as follows *CYP1A1* (5′-ACAAAGACACAACGCCCCTT-3′/5′-CACCATCCCCCACAGCAC-3′) and *β-actin* (5′-TCATGAAGTGTGACGTGGACATC-3′/5′-CAGGAGGAGCAATGATCTTGATCT-3′). The cDNA was diluted 1/2 to 1/5 (corresponding to approximately 0.2 μg/μl initial RNA) in nuclease-free water and combined with master mix and primers. Each sample analysis was performed in triplicate. At the end of the amplification run (initial denaturing step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min) the products were heat dissociated to confirm that only a single PCR product was generated and detected by SYBR Green Dye. The threshold PCR cycle number (*Ct*), corresponding to the point of amplification at which the exponential growth of the PCR product starts to be detected, was used to obtain quantitative values. The relative mRNA levels in each sample were normalized to their corresponding β-actin content. The fold change, relative to untreated samples, was determined with the formula: $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$.

Proliferation assays. Following one week of culture on CD40L-expressing cells, CD40L-activated B cells were plated at a density of 10^5 cells/well onto a fresh monolayer of irradiated CD40L-expressing cells (10^4 cells/well) and treated for 1 h with either DMSO or 10^{-5} M 1-PP. Cells were then treated with either vehicle or the indicated concentrations of B[*a*]P, TCDD or B[*a*]P metabolites for an additional 20 h. [^{3}H]thymidine (1 μCi/well) was added and plates were incubated for an additional 18 h. Cells were harvested onto filter strips using a cell harvester (Brandel, Gaithersbug, MD) and radionucleotide incorporation was measured using a liquid scintillation counter (Wallac, Turku, Finland). For each donor, B cell treatments were performed in triplicate. The means of the triplicate radioactivity counts per minute (cpm) from each donor were used to obtain an average for each indicated data point.

Statistical analyses. The Student's paired *t* test and one-factor ANOVA were used to analyze the data using Statview (SAS Institute, Cary, NC). For ANOVA, the Dunnett's (to compare with vehicle-treated groups) or the Tukey/Kramer (to compare all possible combinations of groups) multiple comparisons test was used to determine significant differences.

Results

Effect of AhR engagement with exogenous ligands on the growth of AhR^{high}, activated human B cells

In vitro stimulation of human peripheral B cells with CD40L and IL-4, modeling B cell activation during adaptive T cell-dependent immune responses, induces greater than a 5-fold increase in AhR expression compared to resting cells [34]. Consequently, it was postulated that these activated B cells would be sensitive to an environmental AhR ligand such as B[*a*]P. To test this hypothesis, B cells grown for one week on a monolayer of CD40L-transfected L cells in the presence of rIL-4 were treated with vehicle or 10^{-5}–10^{-7} M B[*a*]P. [^{3}H]thymidine was added after 21 h and cells were cultured for another 18 h, after which [^{3}H]thymidine incorporation was determined. Proliferation of CD40L-activated B cells was significantly reduced at B[*a*]P doses as low as 10^{-7} M (Fig. 1A). This dose is 100-fold lower than that required to induce apoptosis in a transformed B cell lymphoma [33]. Furthermore, the decrease in [^{3}H]thymidine incorporation was not the result of apoptotic cell death as the percentage of cells in the sub G_0/G_1 peak following permeabilization and propidium iodide staining (<10%) did not increase following treatment with 10^{-5}–10^{-6} M B[*a*]P for the duration of the proliferation assay (data not shown). Similar data were obtained with another prototypic PAH, 7,12-dimethylbenz[*a*]anthracene (DMBA) (data not shown). These results indicate that decreased cell division, rather than increased apoptosis, accounts for the suppressed [^{3}H]thymidine incorporation.

To determine how B[*a*]P impacts the number of cell divisions over time, purified naïve B cells were labeled with CFSE, activated on CD40L-cells for three days, and then re-cultured on fresh CD40L-cells in the presence of either vehicle or 10^{-6} M B[*a*]P for an additional three days. The cell division profiles of vehicle- or B[*a*]P-treated cells were determined six days later. Up to six generations were typically observed, with the majority of cells present in generations 2–4 (Fig. 1B). Consistent with the studies described above with the [^{3}H]thymidine incorporation assay (Fig. 1A), a significantly smaller fraction of B cells reached the later generations when cultures were treated with B[*a*]P (Figs. 1B and C). From these studies it is concluded that B[*a*]P slows cell division.

Many PAH, including B[*a*]P, are metabolized into reactive metabolites that can adduct onto DNA and proteins thereby altering cell function in general [35] and suppressing immune responses in particular [16,18,25]. PAH metabolism is mediated by AhR-regulated cytochrome P450 enzymes. In order to determine if AhR activation alone is sufficient for human B cell growth inhibition, cells were treated with TCDD, a high affinity AhR agonist which is resistant to CYP1A1- or CYP1B1-mediated metabolism. CD40L-activated B cells were treated with vehicle or 10^{-8}–10^{-12} M TCDD and proliferation assays were

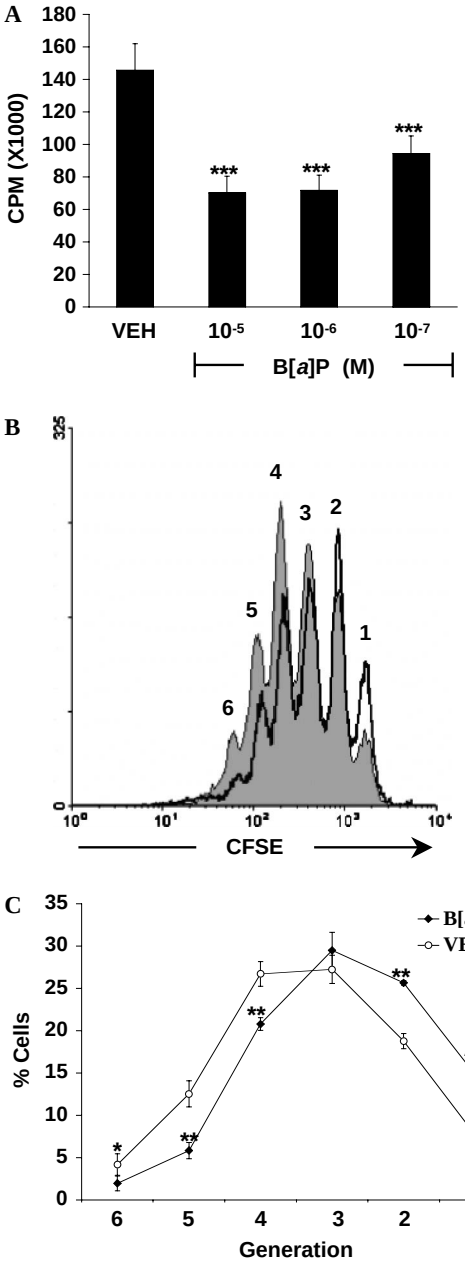

Fig. 1. B[*a*]P suppresses division of CD40L-activated B cells. (A) Activated B cells were treated for 21 h with vehicle (VEH) or 10^{-5}–10^{-7} M B[*a*]P in triplicate wells. [^{3}H]thymidine was added and cells were cultured for another 18 h, harvested, and analyzed for [^{3}H]thymidine incorporation. Data are presented as means ± SE from at least eight donors. A triple asterisk (***) indicates a significant decrease in [^{3}H]thymidine incorporation as compared with vehicle controls ($p < 0.001$). (B) Purified naïve CFSE-labeled B cells were cultured on CD40L-transfected cells for 3 days and transferred to fresh CD40L cell cultures with rIL-4. Cells then were treated with vehicle (VEH) or 10^{-6} M B[*a*]P, cultured for an additional 3 days, and analyzed for CFSE fluorescence by flow cytometry. A representative cell division histogram is presented in which vehicle-treated cells are shown in gray (solid fill) and B[*a*]P-treated cells in bold overlay (no fill). Cell generations are labeled 1–6. The first generation is defined by the level of fluorescence observed in freshly stained cells (not shown). (C) The percentage of B cells in each division was determined using ModFitLT software. Results are presented as means ± SE from five donors. Asterisk(s) indicate a significant difference as compared with vehicle-treated cells ($^{**}p < 0.01$/$^{*}p < 0.5$; paired *t* test).

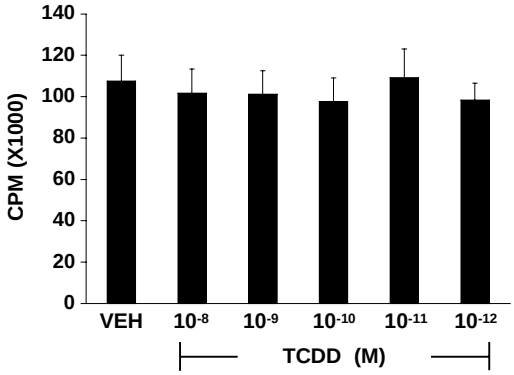

Fig. 2. TCDD does not affect proliferation of CD40L-activated B cells. CD40L-activated B cells were treated in triplicate wells with vehicle (VEH) or 10^{-9}–10^{-12} M TCDD and proliferation assays were performed as described in Fig. 1. The means of the triplicate counts from each donor were then averaged for each indicated data point. Data are presented as means ± SE from at least six donors.

performed. Unlike B[*a*]P, TCDD had little or no effect on the proliferation of CD40L-activated cells (Fig. 2), despite its ability to induce CYP1A1 mRNA and enzyme activity (see below). These results indicate that, while AhR activation and CYP1A1 induction may be important, they are not sufficient for environmental chemical-induced suppression of B cell growth.

Inhibition of CYP1A1 with 1-(1-propynyl)pyrene protects B cells from B[a]P-induced growth suppression

As noted above, the differential sensitivity of activated human B cells to B[*a*]P and TCDD may reflect differences in B[*a*]P and TCDD metabolism. This hypothesis seemed highly plausible since CD40L activation of human B cells induces modest levels of CYP1A1 activity even in the absence of environmental chemicals [34] and since CYP1A1 biotransforms PAH but not TCDD into biologically active intermediates. Therefore, we sought to confirm the predicted hyperinduction of CYP1A1 activity following B[*a*]P exposure and its putative role in B[*a*]P-mediated growth inhibition. As expected, both AhR ligands, TCDD and B[*a*]P significantly increased the level of CYP1A1 enzyme (ethoxyresorufin–*o*-deethylase/EROD) activity over the modest levels seen in vehicle-treated, CD40L-activated B cells (Fig. 3) indicating that the ability of B[*a*]P but not TCDD to inhibit growth is not due to differential CYP1A1 induction.

A CYP1A1 inhibitor, 1-(1-propynyl)pyrene (1-PP) was then used to determine if CYP1A1 activity is critical to B[*a*]P-mediated growth inhibition. This inhibitor is relatively specific for CYP1A1 with an IC_{50} for CYP1A1 that is more than 10-fold lower than that for CYP1A2 and 30-fold lower than for CYP1B1 [36]. Furthermore, it has been shown to inhibit basal and AhR ligand-induced CYP1A1 enzyme activity in several cell lines [37]. EROD assays were performed to confirm that CYP1A1 enzyme activity in human B cells was in fact abolished by 1-PP

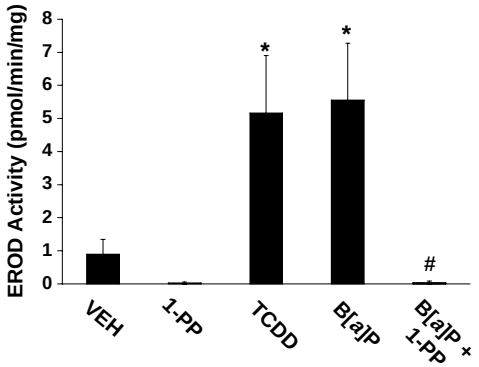

Fig. 3. 1-PP blocks CYP1A1 (EROD) activity. CD40L-activated B cells were plated into wells coated with CD40-specific antibody. Cells then were treated with vehicle, 10^{-5} M 1-PP, 10^{-9} M TCDD, 10^{-6} M B[*a*]P, or 10^{-6} M B[*a*]P plus 10^{-5} M 1-PP in duplicate wells for 24 h. Total cell lysates were prepared from harvested cells. Lysate EROD activity was determined in the presence of NADPH and 7-ethoxyresorufin. Plates were read in a fluorometer every 2 min for 20 min and absorbance was compared to a standard curve of resorufin. Protein concentrations were determined for each sample and used to normalize EROD activity to obtain pmol/min/mg. The data are presented as means ± SE from four individual donors. An asterisk (∗) indicates a significant increase in EROD activity as compared with vehicle-treated cells ($p < 0.05$; Tukey/Kramer). A pound sign (#) indicates a significant decrease in EROD activity as compared with B[*a*]P-treated cells.

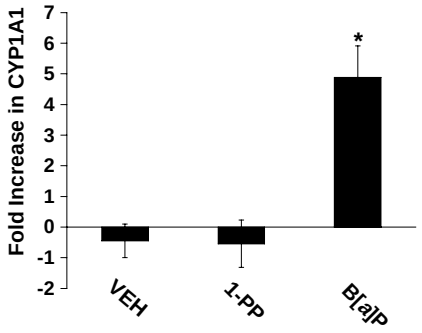

Fig. 4. 1-PP does not induce AhR-activation. CD40L-activated B cells were either left untreated or were treated with vehicle, 10^{-5} M 1-PP, or 10^{-6} M B[*a*]P for 16 h. RNA was extracted and analyzed by relative quantitative real-time PCR with *CYP1A1*- and *β-actin*-specific primers. Threshold cycle fluorescence (*Ct* values) was obtained for each primer. For each treatment, the average of triplicate determinations of *CYP1A1 Ct* values was normalized to average *Ct* values obtained with *β-actin*-specific primers. Fold differences were determined by comparing the normalized *Ct* values of untreated samples to each corresponding treated sample. The data are presented as means ± SE from six donors. An asterisk (∗) indicates a significant increase in normalized *CYP1A1* expression as compared with vehicle-treated samples ($p < 0.05$; Tukey/Kramer).

treatment. CD40L-activated B cells were plated in wells coated with a CD40-specific antibody and treated with vehicle, 10^{-5} M 1-PP, 10^{-6} M B[*a*]P, or with B[*a*]P plus 10^{-5} 1-PP. These studies were performed in the absence of CD40L-transfected L cells to eliminate the possible contribution of CYP1A1 from the "feeder" L cells to the EROD assay. 1-PP tended to lower the aforementioned background levels of CYP1A1 activity, although this decrease did not reach statistical significance (Fig. 3). As expected, B[*a*]P induced significant levels of EROD activity, indicative of CYP1A1 induction. Significantly, co-treatment with 10^{-5} M 1-PP completely abolished B[*a*]P-induced CYP1A1 enzyme activity.

1-PP has been shown to be a low affinity AhR agonist at high concentrations [37]. In order to confirm that the concentration of 1-PP used to inhibit CYP1A1 enzyme activity in these studies was not at the same time inducing AhR activation, AhR transcriptional activity (*CYP1A1* mRNA induction) was assessed in the presence of 1-PP. *CYP1A1* expression was measured by quantitative real-time PCR following a 16 h treatment of CD40L-activated B cells with vehicle, 10^{-6} M B[*a*]P, or 10^{-5} M 1-PP. As expected, B[*a*]P significantly increased *CYP1A1* mRNA expression (Fig. 4). However, 10^{-5} M 1-PP failed to up-regulate *CYP1A1* mRNA, indicating that 1-PP does not induce AhR activation in human B cells under these conditions.

To determine whether blocking CYP1A1-dependent metabolite formation protects B cells from B[*a*]P-induced growth inhibition, CD40L-activated B cells were pre-treated for one hour with 10^{-5} M 1-PP. Cells then were dosed with vehicle (DMSO) or 10^{-5}–10^{-7} M B[*a*]P and proliferation assays were performed as in previous experiments.

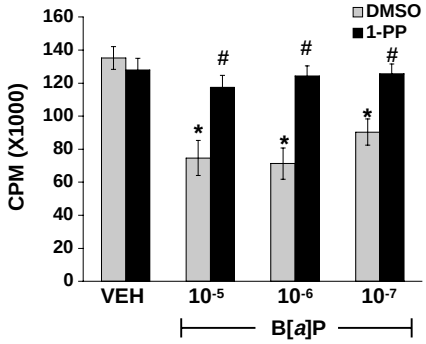

Fig. 5. 1-PP protects B cells from B[*a*]P-induced suppression of proliferation. CD40L-activated B cells were cultured on a monolayer of CD40L-transfected cells treated with vehicle (VEH) or 10^{-5} M 1-PP for one hour. Cells were then dosed with vehicle or 10^{-5}–10^{-7} M B[*a*]P and proliferation assays performed as in Fig. 1. The means of the triplicate counts from each donor were averaged for each indicated data point. Data are presented as means ± SE from at least 10 donors. An asterisk (∗) indicates a significant decrease in [^{3}H]thymidine uptake as compared with vehicle-treated cells ($p < 0.05$; Tukey/Kramer). A pound sign (#) indicates significant protection by 1-PP at the corresponding B[*a*]P dose.

Consistent with previous experiments, treatment with 10^{-5}–10^{-7} M B[*a*]P significantly suppressed proliferation of activated B cells (Fig. 5). In contrast, cells pre-treated with 1-PP were completely protected from B[*a*]P-induced growth inhibition, indicating that CYP1A1 plays an essential role in B[*a*]P-mediated inhibition of B cell proliferation.

CYP1A1 inhibition protects B cells from a proximal B[a]P metabolite but not from the terminal reactive metabolite, B[a]P-7,8-dihydrodiol-9,10-epoxide

The finding that 1-PP blocks CYP1A1 enzyme activity and rescues B cells from B[*a*]P-induced growth suppression

suggests that PAH metabolism is essential for this immunosuppressive effect. B[*a*]P is metabolized by CYP1A1 and an epoxide hydrolase (EH) into B[*a*]P-7,8-dihydrodiol and B[*a*]P-4,5-dihydrodiol (Fig. 6A). The 4,5-dihydrodiol is not further metabolized. However, subsequent oxidation of the 7,8-dihydrodiol, again by CYP1A1, leads to the formation of the highly reactive B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE). If proximal B[*a*]P diols, such as B[*a*]P-7,8-dihydrodiol, are the effector molecules in the suppression of B cell proliferation, then it would be predicted that growth inhibition would be observed following addition of the respective diol metabolite and that this growth arrest would not be inhibited by 1-PP. However, if growth inhibition is dependent upon further metabolism of a diol into the terminal, reactive diol epoxide, then 1-PP should rescue B cells from the putative inhibitory effects of B[*a*]P diols, but not from the effects of the terminal metabolite, BPDE. In order to test these possibilities, both diols and BPDE were tested alongside B[*a*]P for their ability to inhibit B cell proliferation in the absence or presence of 1-PP.

Again, B[*a*]P was shown to inhibit CD40L-activated human B cell growth in a dose-dependent fashion (Fig. 6B, shaded bars). Like B[*a*]P, the B[*a*]P-7,8-diol metabolite was a potent inhibitor of B cell proliferation at doses of 10^{-6} and 10^{-7} M (Fig. 6C). Pre-treatment with 1-PP ablated growth inhibition mediated by B[*a*]P and B[*a*]P-7,8-diol (Figs. 6B and C, solid bars). Thus, metabolism of B[*a*]P-7,8-diol by CYP1A1 is likely to be essential for B[*a*]P-7,8-diol-dependent growth inhibition.

The terminal epoxide, BPDE, also inhibited B cell proliferation (Fig. 6D). However, unlike 7,8-dihydrodiol, which is metabolized into BPDE by CYP1A1, 1-PP was unable to rescue cells from BPDE-induced growth suppression (Fig. 6D). In contrast, B[*a*]P-4,5-dihydrodiol, which is not metabolized into BPDE, failed to suppress B cell proliferation either in the presence or absence of 1-PP (Fig. 6E). These data demonstrate that B[*a*]P- and B[*a*]P-7,8-dihydrodiol-mediated growth inhibition of AhR^{high}, activated human B cells is likely mediated by the terminal metabolite BPDE, and that this effect is a result of AhR-regulated, B cell cytochrome P450-dependent biotransformation of B[*a*]P into a proximal diol and ultimately into BPDE.

Discussion

In previous studies examining the expression of AhR in primary human B cells, we found that resting B cells

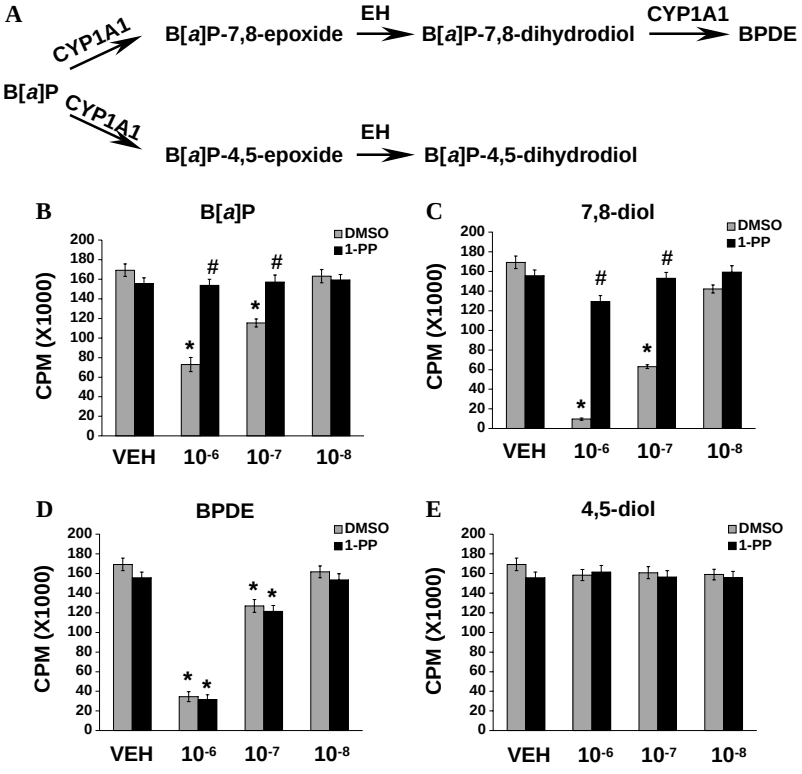

Fig. 6. 1-PP protects B cells from a proximal B[*a*]P metabolite but not from the terminal reactive metabolite B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE). (A) A simplified scheme of CYP1A1- and epoxide hydrolase (EH)-dependent B[*a*]P metabolism. (B–D) CD40L-activated B cells were transferred to fresh monolayers of CD40L-activated L cells supplemented with rIL-4, and treated in triplicate wells for 21 h with vehicle (VEH), B[*a*]P (B), B[*a*]P-7,8-dihydrodiol (7,8-diol) (C), B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE) (D), or B[*a*]P-4,5-dihydrodiol (4,5-diol) (E). [^{3}H]thymidine was added and cells were cultured for an additional 18 h. The means of the triplicate counts from each donor were averaged for each indicated data point. Data are presented as means ± SE obtained from at least 10 donors. An asterisk (*) indicates a significant decrease in [^{3}H]thymidine uptake as compared with vehicle-treated cells ($p < 0.05$; Tukey/Kramer). A pound sign (#) indicates significant protection by 1-PP. For ease of presentation, the average values obtained for vehicle-treated cells are repeated in each graph.

expressed little or no AhR. However, activation with CpG or CD40 ligand, surrogates for stimuli invoked during innate and adaptive immune responses, respectively, profoundly up-regulated AhR mRNA and protein [34]. These findings support the hypothesis that activated B cells are susceptible targets of immunosuppressive AhR ligands by virtue of their high AhR expression and the induction of AhR-regulated genes. Towards understanding the impact of AhR ligand exposure on human B cell function, we evaluated the effect of B[*a*]P, a prototypic environmental PAH and AhR ligand, on B cell growth.

Using two assays for growth, [^{3}H]thymidine incorporation and CFSE labeling, we demonstrated that B[*a*]P (Figs. 1, 5, and 6) and DMBA (data not shown) significantly suppress activated human B cell proliferation at doses as low as 10^{-7} M. These results contrast with those obtained in a report on the effects of PAH on primary human B cell growth [38]. In that study it was shown that 10^{-4} M DMBA was required for growth inhibition of purified tonsillar or splenic B cells. The significantly higher dose requirement seen in that study could reflect the nature of the B cell stimulus, a T cell superantigen that requires the presence of T cells to induce low levels of B cell proliferation. Furthermore, the requirement for irradiated T cells in the B cell proliferation assay makes it difficult to separate the effects of DMBA on B cells from those on T cells. In support of this argument, it was shown that DMBA profoundly inhibits T cell activation that in turn is likely to reduce the level of B cell proliferation [38].

The low PAH dose shown to be effective in the present system also was significantly lower than that required to induce apoptosis in transformed human B cells [33]. Indeed, neither B[*a*]P nor DMBA induced apoptosis or suppressed proliferation in our activated primary B cell populations (data not shown). These results underscore the limits of transformed cell line models and the importance of using non-transformed, primary B cell populations for the evaluation of putative environmental immunosuppressants.

The finding that B[*a*]P suppresses B cell proliferation by approximately 50%, rather than by 100% (Fig. 1A), may reflect either the complete inhibition of proliferation in approximately half of the activated B cells, or a partial delay in progression through cell cycle in most, if not all cells. The observation that a small portion of CFSE-stained cells exposed to B[*a*]P for three days progressed through five or six divisions (Fig. 1B) suggests that a sub-population of B cells is relatively insensitive to B[*a*]P. It is not yet known if this putative subset has failed to up-regulate AhR or CYP1A1 following CD40L activation or if it possesses some AhR-independent property which enables it to bypass the growth inhibitory signal initiated by B[*a*]P. In the former case, it would be predicted that the remaining B cells would be less likely to sustain CYP1A1-dependent, PAH epoxide-mediated mutations. In contrast, the latter possibility would predict that PAH exposure selects out cells that are relatively resistant to growth-inhibitory signals. This outcome, together with the ability of PAH epoxides to induce mutations, could increase the likelihood of neoplastic transformation in the activated B cells of PAH-exposed individuals.

In order to determine if AhR activation and CYP1A1 induction are sufficient for growth suppression, the effect of TCDD, a high affinity, relatively non-metabolizable AhR ligand on primary B cell growth was evaluated. Interestingly, B cell proliferation was unaffected by TCDD despite the ability of TCDD to up-regulate CYP1A1 mRNA transcription (data not shown) and enzyme activity (Fig. 3). Therefore, neither AhR activation nor CYP1A1 induction is sufficient for suppression of B cell growth. The inability of TCDD to affect B cell growth in the current system may be contrasted with its ability to suppress antibody secretion by transformed B cells [39]. This suggests that different AhR ligands may affect humoral immunity at different levels.

One major difference between the two AhR ligands, TCDD and B[*a*]P, is the extent to which they can be metabolized by CYP1A1. We directly tested our hypothesis that growth inhibition is mediated by AhR-regulated CYP1A1 and the resulting reactive B[*a*]P metabolites by determining the ability of 1-PP to block growth inhibition mediated by the parent compound and its CYP1A1-generated metabolites. Indeed, 1-PP completely protected CD40L-activated B cells from B[*a*]P- and B[*a*]P-7,8-dihydrodiol-induced growth suppression. However, 1-PP had no effect on growth inhibition mediated by BPDE, the terminal reactive B[*a*]P metabolite. These data, together with the ability of low (10^{-7} M) BPDE doses to suppress growth, strongly suggest that BPDE is the effector of B[*a*]P-mediated growth inhibition. Furthermore, they suggest that activated B cells, presumably those responding to antigenic stimuli, are particularly sensitive to B[*a*]P, by virtue of their increased CYP1A1 expression and consequent ability to readily metabolize B[*a*]P into BPDE. In this regard, it is interesting to note that BPDE is likely to be the effector molecule for both B[*a*]P-induced mutagenesis and immunosuppression, at least at the level of the B cell compartment.

Although the mechanism through which BPDE inhibits B cell growth is still under investigation, it is possible that mutagenesis and immunosuppression are mediated by a common pathway, i.e., BPDE-DNA adduct formation and DNA damage. It is well established that genotoxic stress induces growth inhibition in part by up-regulation of p53 and a resulting decrease in cell cyclin activity [40]. Indeed, B[*a*]P induces p53 expression in CD40L-activated B cells within 24 h (data not shown). Regardless of the molecular mechanism, the demonstration that a prototypic environmental chemical, such as B[*a*]P, profoundly inhibits activated human B cell growth has important implications for normal B cell physiology. B cell growth is critical for the expansion of antigen-specific B cells during clonal selection [41], for the differentiation of B cells into antibody-secreting plasma cells [42,43], and for the development of memory B cells [44,45]. Thus, exposure of activated B cells

to PAH may inhibit the humoral immune response at multiple levels of B cell activity.

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