Constitutive Activation and Environmental Chemical Induction of the Aryl Hydrocarbon Receptor/Transcription Factor in Activated Human B Lymphocytes

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ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates immunosuppression induced by a variety of ubiquitous environmental pollutants, including polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dioxins. Although the normal physiological role for the AhR in the absence of environmental chemicals is uncertain, recent studies suggest its contribution to cell growth and apoptosis. Because B cells seem to be directly affected by AhR ligands in animal models, it was postulated that the AhR is predominantly expressed in activated human B cells and that it may contribute to cell growth regulation. To begin to address these issues and to extend detailed analyses of AhR function to a human system, AhR expression in resting and activated human B cells was studied. In addition, the response of activated B cells to an environmental AhR ligand was investigated to provide insight into a possible physiological role for the AhR. Resting peripheral human B cells expressed little or no AhR. However, activation with CpG or CD40 ligand profoundly up-regulated AhR mRNA and protein. AhR nuclear translocation, constitutive DNA binding, and induction of an AhR-regulated gene, *CYP1A1*, in stimulated B cells in the absence of exogenous ligands suggested constitutive AhR activation. Cell division was not required for AhR up-regulation. Treatment of AhR-expressing B cells with a prototypic environmental AhR ligand, benzo[a]pyrene, significantly suppressed cell growth. These data help explain the sensitivity of B cells to environmental AhR ligands and strongly suggest that the AhR plays an important function within the human B cell compartment.

The aryl hydrocarbon receptor (AhR) is a cytoplasmic protein that belongs to the Per-ARNT-Sim family of transcription factors. Per-ARNT-Sim family members (e.g., hypoxia inducing factor-1α, clock, and MOP3) perform critical cellular functions, including the regulation of hypoxic responses, circadian cycle, and neurogenesis (Zhong et al., 1999; Bunger et al., 2000; Liu et al., 2003). These observations, and the high level to which the AhR has been conserved throughout evolution, suggest that the AhR similarly has an important physiological function.

The AhR has been studied primarily for its responsiveness to environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, and planar polychlorinated biphenyls. Many of these chemicals, such as benzo[a]pyrene (B[a]P) and 2,3,7,8-tetrachlorodibenzo-p-dioxin, are immunotoxic and carcinogenic (Kerkvliet et al., 2002; Laiosa et al., 2003). Activation of the AhR and transcription of AhR-regulated genes typically are required for the manifestation of environmental chemical-induced immunotoxicity and carcinogenicity (Allan et al., 2003).

Inactive AhR is complexed in the cytoplasm with heat shock protein 90, an immunophilin-like accessory molecule (XAP/ARA9) (Perdew, 1991), and p23 (Shetty et al., 2003), which collectively influence AhR ligand binding, cellular localization, and transcriptional activity. Upon ligand binding the receptor translocates to the nucleus where it associates with another cofactor termed ARNT. The activated AhR:ARNT complex binds specific gene promoter sites (aromatic hydrocarbon response elements; AhREs),

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; B[a]P, benzo[a]pyrene; ARNT, AhR nuclear translocator; AhRE, AhR response element; B[e]P, benzo[e]pyrene; PBMC, peripheral blood mononuclear cell; FITC, fluorescein isothiocyanate; rIL, recombinant interleukin; MHC, major histocompatibility complex; mAb, monoclonal antibody; RT, reverse transcription; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; TBE, Tris borate-EDTA; IL, interleukin; LPS, lipopolysaccharide.

resulting in the modulation of gene transcription (Sulentic et al., 2000). AhREs are present in proto-oncogenes, cytokine genes, at least one death-promoting gene (*Bax*), and genes encoding PAH-metabolizing enzymes (e.g., *CYP1A1*) (Masten and Shiverick, 1995; Lai et al., 1996; Matikainen et al., 2001).

The identity of physiologically relevant endogenous ligands remains uncertain. Indeed, the endogenous function of the AhR in the absence of environmental pollutants has only begun to be elucidated. Several studies indicate that the AhR influences cell cycle. For example, the AhR is highly expressed in rapidly growing tumors and immortalized cell lines (Trombino et al., 2000). Ectopic AhR expression increases epithelial cell growth (Shimba et al., 2002), whereas its down-regulation with *AhR* antisense cDNA or transforming growth factor-β slows murine hepatoma and human lung carcinoma cell growth, respectively (Ma and Whitlock, 1996; Dohr and Abel, 1997).

The AhR also has been associated with growth repression. *AhR* disruption with small interfering RNAs increases, whereas AhR hyperactivation decreases breast cancer cell growth (Abdelrahim et al., 2003). Transfection of rat hepatoma cells with AhR delays cell cycle progression (Weiss et al., 1996). It is noteworthy that the ability of the AhR to up- or down-regulate proliferation is cell type-specific (Abdelrahim et al., 2003), a situation that probably reflects context-dependent differential recruitment of coactivators or corepressors.

Together, these studies suggest important physiological functions for the AhR in several nonlymphoid cells. Likewise, the AhR is likely to play an important role in the immune system, potentially through growth regulation. At least one strain of $AhR^{-/-}$ mice exhibits deficiencies in the accumulation of mature splenic lymphocytes (Fernandez-Salguero et al., 1995) and aberrations in bone marrow B cell development (Thurmond et al., 2000). AhR transgenic mice have increased numbers of mature bone marrow B cells and decreased numbers of peritoneal B-1 cells (Andersson et al., 2003). Environmental AhR ligands suppress a variety of B cell-mediated responses (Burchiel et al., 1993; Wood et al., 1993; Sulentic et al., 2000). It is interesting that activated B cells seem to be more sensitive than high-density, resting B cells (Tucker et al., 1986; Wood et al., 1993; Crawford et al., 1997).

Despite the likelihood that the AhR plays an important role in normal B cell physiology and is a key mediator of AhR ligand-mediated B cell toxicity, little is known about the factors regulating AhR expression and function in primary lymphocytes in general, or in human B cells in specific. To bridge this gap, we evaluated AhR expression and activity in resting and activated primary human B cells. Two physiologically relevant B cell stimuli were used. Bacterial CpG, a TLR-9 ligand, was used to model B cell activation during innate immune responses. Polyvalent CD40 ligand (CD40L) was used to mimic B cell stimulation by activated CD40L$^+$ T helper cells during adaptive immune responses. Furthermore, the effects of an environmental AhR ligand, B[*a*]P, on activated human B cells was assessed to determine whether activated lymphocytes are susceptible to this prototypic PAH and to test the hypothesis that AhR engagement with ligand will alter cell growth.

Materials and Methods

Chemicals and Reagents. B[*a*]P and its congener benzo-[*e*]pyrene (B[*e*]P) (Sigma-Aldrich, St. Louis, MO) were dissolved in dimethyl sulfoxide (Sigma-Aldrich). Stock solutions of each were made in ethanol.

B Cell Preparation and Culture. CD40L-transfected L cells (American Type Culture Collection, Manassas, VA) were maintained at 37°C in 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 μg/ml Plasmocin (Invivogen, San Diego, CA), and hypoxanthine thymidine. These cells were periodically screened and selected for high CD40L expression by flow cytometry. All culture reagents were obtained from Cellgro (Mediatech, Herndon, VA), unless indicated otherwise. PBMCs were prepared from individual healthy donor blood packs (New York Biologics, Inc., New Jersey, NY) by centrifugation of buffy coats through Ficoll (Amersham Biosciences Inc., Uppsala, Sweden). PBMCs were then depleted of T cells by sheep red blood cell (MP Biomedicals, Aurora, OH) rosetting for 1 h on ice in RPMI 1640 medium. After a second centrifugation through Ficoll, cells were stained with FITC-labeled CD20-specific antibody and purified by fluorescence activated cell sorting (MoFlo; DakoCytomation California Inc., Carpinteria, CA). Approximately 10^7 B cells (>99% CD20$^+$) were recovered per donor. Control "resting" cells were obtained by culturing for 24 h in Iscove's medium (Invitrogen, Carlsbad, CA) supplemented with 5% human AB serum (MP Biomedicals, 50 μg/ml human transferrin (Invitrogen), 0.5% human serum albumin (Aventis Behring, Kanakakee, IL), 5 μg/ml human insulin (Sigma-Aldrich), and 25 μg/ml Plasmocin. For short-term experiments, purified B cells were cultured either on confluent monolayers of irradiated (3000 rads from a ^{137}Cs γ-cell irradiator; Gammacell-40; Nordion International, Ontario, Canada) CD40L-transfected L cells in media containing 50 ng/ml human rIL-4 (Research Diagnostics, Flanders, NJ) or in media containing 6 μg/ml PS2006 CpG (Oligos Etc. Inc.,Wilsonville, OR). Cells were harvested after 24 h for analysis of surface markers (MHC I, MHC II, B7-1, or B7-2) and for AhR expression. For longer term experiments, approximately 10^7 Ficoll-enriched PMBCs were cultured on irradiated CD40L-transfected L cell monolayers in the presence of rIL-4 as described above and 0.55 μM cyclosporin A (Sigma-Aldrich) for 1 to 2 weeks with transfer of the expanding B cell population to fresh, irradiated CD40L-transfected L cell monolayers every 3 to 4 days. After 1 to 2 weeks in culture, approximately 10^7 PBMCs proliferated to yield several hundred million activated B cells (>97% CD19$^+$).

Flow Cytometry and Antibodies. Phenotypic analyses of B cells were performed using fluorescent monoclonal antibodies directed against CD20, CD80 (B7-2), CD86 (B7-2), human leukocyte antigen class I, and human leukocyte antigen class II (BD Biosciences PharMingen, Chicago, IL). Nonspecific mAb binding was blocked by incubating cells for 10 min in phosphate-buffered saline containing 5% fetal bovine serum, 1% sodium azide, and 0.01 mg/ml normal mouse IgG (Caltag Laboratories, Burlingame, CA). Cells were then labeled with mAb or isotypic control antibodies according to the manufacturer's instructions. After one wash, cells were fixed in phosphate-buffered saline containing 3.7% paraformaldehyde and analyzed in a BD Biosciences FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA). Cell viability was determined by trypan blue and propidium iodide exclusion by light microscopy and flow cytometry, respectively, as reported previously (Allan et al., 2003).

Semiquantitative RT-PCR. Total RNA was prepared from B cell pellets by using RNA STAT-60 (Tel-Test Inc., Friendswood, TX). Total RNA (2 μg) was reverse-transcribed as described previously (Allan et al., 2003). All enzymes were obtained from Invitrogen. PCR was conducted using 2 μl of cDNA, 0.2 μM each of specific primers (Integrated DNA Technologies Inc., Coralville, IA), and 5 units of Platinum *Taq* DNA polymerase according to the manufacturer's instructions. The PCR primer sequences and number of cycles used

were as follows: *AhR* 5′-CTGGCAATGAATTTCCAAGGGAGG-3′/5′-CTTTCTCCAGTCTTAATCATGCG-3′ (31 cycles), *CYP1A1* 5′-TTCATCCCTATTCTTCGCTAC-3′/5′-TCCATCAGCATCTAT-GCC-3′ (32–36 cycles), or *β-actin* 5′-GTCGTCGACAACGGCTC-CGGCATGTG-3′/5′-CATTGTAGAAGGTGTGGTGCCAGATC-3′ (26 cycles). The optimal number of amplification cycles was determined for each primer set to be within the exponential portion of the PCR curve. Amplifications were performed in a programmed thermocycler (Barnstead/Thermolyne, Dubuque, IA), and then 5 μl of each product was separated on an agarose gel and visualized with ethidium bromide. Images were captured by digital photography (Kodak transilluminator and Kodak DC290 digital camera). Relative band intensities were determined with the Kodak Digital Sciences ID program. *AhR* and *CYP1A1* band intensities were normalized to the corresponding *β-actin* band intensities.

Western Blotting. Total cell lysates were prepared from B cells by incubating washed cell pellets for 10 min in lysis buffer (50 mM KHPO$_4$, pH 7.4, and 5 mM DTT) and 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich) on ice. After sonication (3 × 15-s pulses) and a 10-min centrifugation, protein concentrations of total cell lysates were quantified using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Nuclear and cytoplasmic fractions were prepared from CD40L-activated B cells using the Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Equal amounts of protein (typically 10–40 μg of total protein) were boiled for 5 min in 1× SDS-PAGE sample buffer (50 mM Tris buffer, pH 6.8, 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 1% β-mercapto-ethanol) before SDS-PAGE electrophoresis through a 6.8% polyacryl-amide gel and overnight transfer onto a nitrocellulose membrane (Bio-Rad). After transfer, membranes were blocked with 5% skim milk powder in 1× Tris-buffered saline plus 0.05% Tween 20. The primary antibody was polyclonal rabbit anti-human AhR antibody (Santa Cruz Biotechnology, Inc.), and the secondary antibody was horseradish peroxidase-linked rabbit Ig-specific antibody (Pierce Chemical, Rockford, IL). Bands were detected using enhanced chemiluminescence substrate (Sigma-Aldrich) and exposure to X-ray film (Fuji, Tokyo, Japan). The membranes were stripped (Chemicon International, Temecula, CA) and reblotted with β-actin-specific mAb (Sigma-Aldrich). Lamins A/C- (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) and α-tubulin-specific (Oncogene Science, Boston, MA) mAbs were used to examine purity of each cell fraction. Protein loading was visualized by staining an identical SDS-PAGE gel, run in parallel, with Coomassie Blue. Band densities were quantified with Molecular Dynamics PhosphorImager (Amersham Biosciences Inc.) using ImageQuant software (Amersham Biosciences Inc.).

EMSA. Complementary oligonucleotides containing the human *CYP1A1* AhRE sequence (5′-TCC GGT CCT TCT <u>CAC GCA</u> ACG CCT GGG-3′ and 5′-CCC AGG CGT <u>TGC GTG</u> AGA AGG ACC GGA-3′) were used (the core AhR binding site is underlined). DNA was end-labeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ-^{32}P]ATP and was purified using a Centrispin-20 column (Princeton Separations, Adelphia, NJ). Nuclear extract protein (5 μg), prepared from naive sorted B cells or from CD40L-activated B cells after 1 to 2 weeks of culture, and labeled probe (~0.5 ng; 50,000 cpm) were incubated in buffer [final reaction conditions: 20 mM HEPES, pH 8.0, 150 mM sodium chloride, 0.2 mM EDTA, 5 mM DTT, 0.1% bovine serum albumin, 2.5 mM MgCl$_2$, 5% glycerol, and 2 μg of poly(dI-dC)]. The specificity of the shifted bands was determined by including 200× excess unlabeled double stranded competitor oligonucleotides containing the consensus *CYP1A1* AhRE site or a mutated AhRE site (5′-TCC GGT CCT TCT *CAA T*CA *A*CG CCT GGG-3′/5′-CCC AGG CG*T* T*GA T*TG AGA AGG ACC GGA-3′ (mutated bases in italics). These were added to the reaction mixtures immediately before addition of labeled probe. The identities of the shifted complexes were determined by adding 4 to 6 μg of normal rabbit Ig, ARNT-specific, or AhR-specific polyclonal antibodies (Santa Cruz Biotechnologies, Inc.) to the reaction mixtures for 20

min. Labeled probe was added, and the mixture was incubated at room temperature for 30 min. Nondenaturing 5% polyacrylamide gels in 0.5× TBE buffer (44 mM Tris base, pH 8.3, 44 mM boric acid, and 0.8 mM EDTA) and 5% glycerol were prerun at 200 V for 30 min. Mixtures were then electrophoresed at 200 V for 1.5 h in 0.5× TBE. In EMSA supershift experiments using AhR-specific antibodies, nuclear extracts were combined with Tris buffer (final concentration 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, and 5% glycerol), the reactions were electrophoresed through nondenaturing 4% acryl-amide gels in 1× TGE buffer (containing 50 mM Tris, pH 7.5, 0.38 M glycine, and 2 mM EDTA). The gels were dried and exposed to film.

Proliferation Assays. After 1 week of culture on CD40L-express-ing 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). In experiments where anti-CD40 antibody was used instead of CD40L-transfected cells, activated B cells were transferred to plates precoated with either anti-CD40 mAB (Ancell, Bayport, MN) or with an isotype control antibody, MOPC21 (MP Biomedicals). CD40L-activated cells were treated for 21 h with either vehicle or the indicated concentrations of B[*a*]P or B[*e*]P. [^{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 Inc., Gaithersburg, MD), and radionucleotide incorporation was measured using a liquid scintillation counter (PerkinElmer Wallac, Turku, Finland). For each donor, B cell treatments were performed in triplicate. The means of the triplicate radioactivity counts per minute (cpm) were used to obtain an average for each indicated data point.

Statistical Analysis. Student's paired *t* test, one-factor analyses of variance, and linear regression analyses (correlation Z test) were used to analyze the data using Statview (SAS Institute, Cary, NC). For analyses of variance, the Dunnett's multiple comparisons test was used to determine significant differences.

Results

AhR Expression in Resting and Activated Human B Cells. Many studies have demonstrated that the AhR is highly expressed in transformed cells, including human B and T cell lines. The relative sensitivity of nontransformed B cells to AhR ligands suggests that the AhR is expressed in these normal cells as well. However, few of these studies have evaluated AhR expression in human lymphoid organs, and none have evaluated the possibility that AhR levels and activity vary with the extent of human lymphocyte activation.

To address these issues, primary peripheral human B cells were activated with either CpG, to mimic B cell activation during an innate immune response, or with CD40 ligand (CD40L), to model B cell activation by antigen-specific CD40L$^+$ T helper cells. IL-4 was added routinely to help maintain cell viability. For short-term studies, B cells were highly enriched by fluorescence-activated cell sorting (>99% CD20$^+$ B cells). Sorted B cells were activated for 24 h with a CpG oligodeoxynucleotide sequence (PS2006) selected for its strong stimulation of human B cells, or with multivalent CD40L, provided by a monolayer of fibroblasts stably transfected with human CD40L. Controls consisted of sorted B cells "rested" for 24 h by culture in the absence of either CpG or CD40L.

rIL-4 alone had little or no effect on the expression of several immunologically relevant activation markers, including MHC I, MHC II, B7-1 (data not shown), and B7-2 (Fig. 1A). In contrast, activation with CpG or CD40L for 24 h in the presence or absence of rIL-4 significantly increased ex-
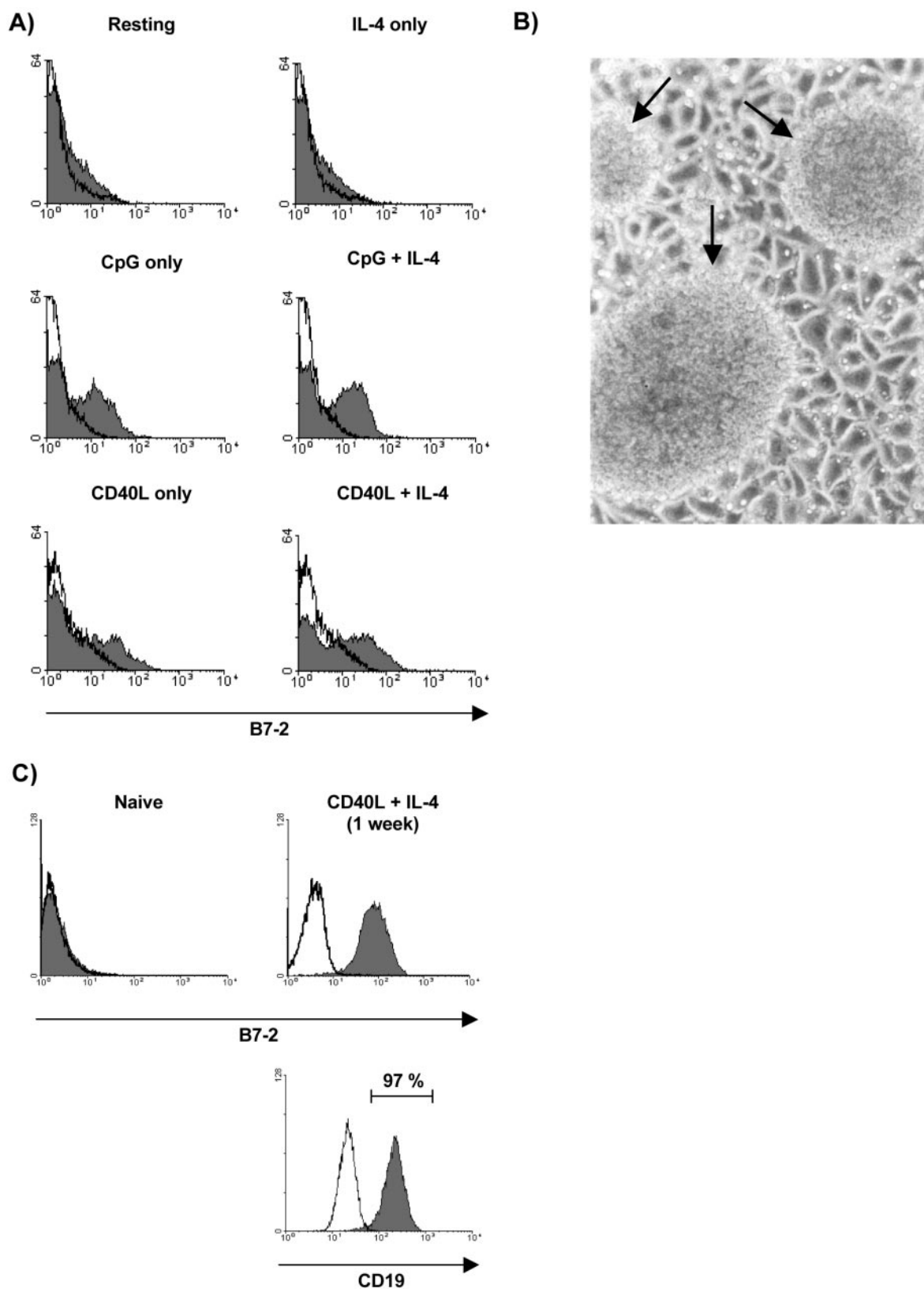
Fig. 1. Activation of human B cells with CD40L or CpG. A, CD20^{+} B cells were purified by fluorescence activated cell sorting from human PBMCs and cultured for 24 h in media alone (resting) or media containing 50 U/ml rIL4, 6 μg/ml CpG, CpG + rIL-4, CD40L (in the form of irradiated CD40L-transfected L cell monolayers), or CD40L + rIL-4. B cells were harvested and stained with FITC-conjugated B7-2-specific antibody and analyzed by flow cytometry. Data from one representative experiment (five total) are shown. B, PBMCs were cultured on CD40L-transfected L cells + rIL-4 for 1 to 3 weeks. Clusters of human B cells are visualized as large clumps (arrows). C, freshly sorted B cells (naive) and B cells activated for 1 week on CD40L-transfected cells + rIL-4 were stained with FITC-conjugated B7-2- or CD19-specific antibody (gray fill) or with isotype control antibody (no fill). Data from one representative experiment (eight total) are shown.

pression of all of these markers (e.g., see Fig. 1A for B7-2 expression).

We also took advantage of our previous observation that B cell populations can be rapidly expanded by culturing for several weeks on CD40L-expressing fibroblasts in the presence of rIL-4. These cells, ≥97% of which express the CD19 B cell marker, grow in large clusters (Fig. 1B) and express high levels of MHC I, MHC II, B7-1 (not shown), and B7-2 (Fig. 1C).

To quantify AhR expression, whole cell lysates from resting, CpG- or CD40L-activated B cells were generated and analyzed by AhR-specific Western immunoblotting. Freshly isolated "naive" B cells, B cells rested for 24 h, or B cells cultured for 24 h in IL-4 alone expressed little or no AhR protein (Fig. 2A). In contrast, B cells activated for 24 h with CD40L or CpG expressed high levels of AhR (Fig. 2A). Furthermore, activated B cells maintained in culture on CD40L-transfected cells in the presence of IL-4 for a week retained high levels of AhR compared with starting naive cells (Fig. 2A, donor 3).

To determine whether this up-regulation of AhR protein reflected increases in *AhR* mRNA, semiquantitative *AhR*-specific and, as a control, *β-actin*-specific RT-PCR assays

Fig. 2. B cell activation induces AhR protein and mRNA expression. A, sorted (naive) B cells were cultured for 24 h with media (resting), CpG, CpG + rIL-4, rIL-4 alone, or CD40L + rIL-4 (donors 1 and 2) or were cultured for 1 week on CD40L-transfected L cells in the presence of rIL-4 (donor 3). AhR levels were assayed by immunoblotting with AhR- and β-actin-specific antibodies. Blots obtained from three representative donors are shown. B, sorted B cells were cultured for 24 h in media alone (resting), rIL-4, CpG + rIL-4 or for 1 week on monolayers of CD40L-transfected L cells + rIL-4. RNA was extracted and analyzed by RT-PCR with *AhR*- and *β-actin*-specific primers. Data from one representative experiment (eight total) are shown.

were performed with RNA extracts from resting and activated B cells. Resting B cells and B cells cultured for 24 h in rIL-4 expressed low levels of *AhR* mRNA (Fig. 2B). rIL-4 alone seemed to occasionally increase *AhR* mRNA levels in some donors, although this apparent increase never reached statistical significance (Fig. 6). In contrast, B cells activated with CpG or CD40L expressed significantly higher levels of *AhR* mRNA (Fig. 2B). These data are consistent with the hypothesis that AhR up-regulation during B cell activation is mediated, at least in part, by an increase in steady-state *AhR* mRNA levels.

CD40L- or CpG-mediated B cell activation results in both cellular differentiation (e.g., expression of activation markers) and proliferation. If AhR up-regulation in these activated B cells is a consequence of cellular proliferation, then it would be expected that blockade of proliferation in cells activated by CpG or CD40L, for example by radiation treatment, would prevent AhR up-regulation. To test this possibility, purified B cells were irradiated (500–750 rads) and stimulated for 24 h with CpG plus rIL-4 or CD40L plus rIL-4. Cells were then phenotyped for activation markers, analyzed for proliferation, and assayed for AhR levels by Western immunoblotting.

No significant differences in cell viability were observed between irradiated and control cell populations 24 h after CpG or CD40L treatment (not shown). As expected, irradiated cells stimulated with either CpG or CD40L were able to differentiate, as assessed by up-regulation of B7-2 (Fig. 3A). However, little or no proliferation, as assessed by [³H]thymidine incorporation, was evident after irradiation (Fig. 3B). In spite of this block in proliferation, CpG- or CD40L-activated B cells expressed elevated levels of AhR comparable with that of activated, nonirradiated cells (Fig. 3C). These data demonstrate that the intracellular signaling cascade that is initiated by either CpG or CD40L and results in AhR up-regulation is independent of cellular proliferation.

AhR Localization and DNA Binding. Up-regulation of the AhR in activated B cells suggests that these cells may be targets of environmental AhR ligands. However, the demonstration of higher protein levels per se did not address the question of whether the AhR is performing some intrinsic biological function in activated B cells. This possibility would be supported if the AhR were found to constitutively localize to the nucleus and to bind DNA in the absence of exogenous ligands. Therefore, AhR nuclear localization and binding to a consensus AhRE were evaluated.

Human B cell populations were expanded for 1 to 2 weeks by culture on CD40L-transfected L cells in the presence of rIL-4. Nuclear and cytoplasmic protein extracts were prepared and analyzed for AhR protein levels by Western immunoblotting. Consistent with previous experiments using whole cell extracts (Fig. 2), significant levels of AhR protein were detected in the cytoplasm of CD40L-activated B cells (Fig. 4, lanes marked C). Significantly, AhR was also present in nuclear extracts (lanes marked N). Among the five donors examined, 37 ± 8% of the total cellular AhR, as determined by the ratio of nuclear AhR to total cell AhR band densities, was contained in the nuclear fraction. To ensure that the nuclear extracts were not contaminated with cytoplasmic proteins or vice versa, nuclear and cytoplasmic extracts were also analyzed for the presence of the nuclear proteins lamins A/C and the cytoplasmic protein tubulin. As seen in Fig. 4,
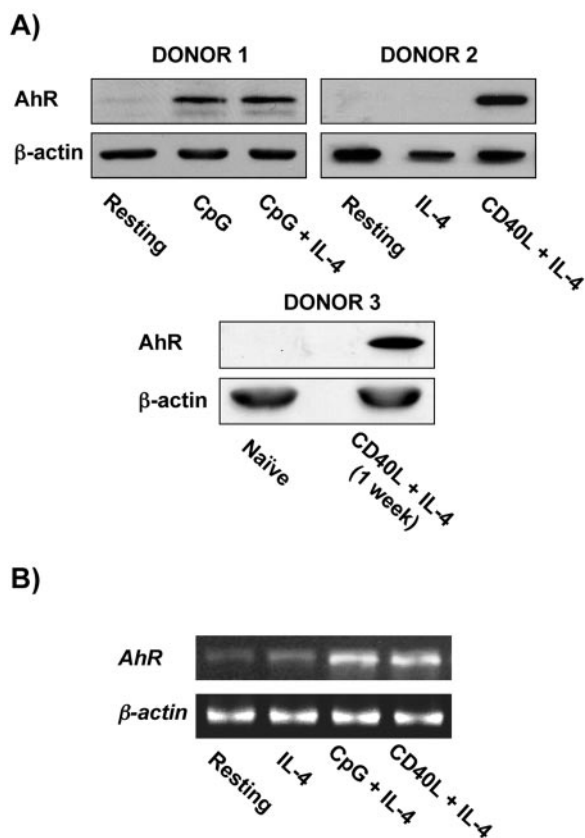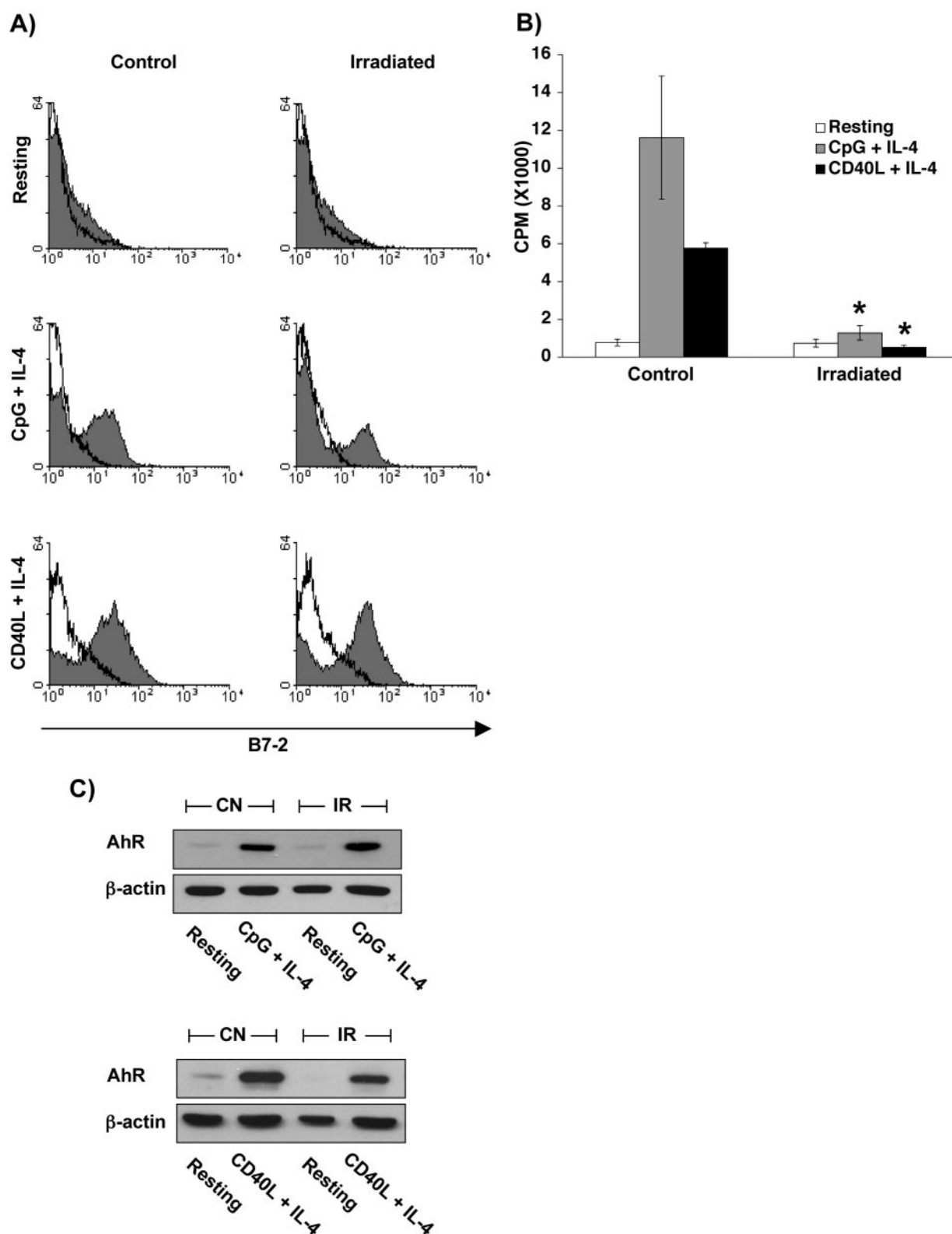
Fig. 3. AhR up-regulation in activated B cells in the absence of proliferation. Sorted naive B cells were γ-irradiated (500–750 rads) and cultured for 24 h with CD40L + rIL-4 or CpG + rIL-4. A, irradiated and control B cells were stained with FITC-labeled B7-2-specific antibodies (gray) or isotype control antibody (no fill). B, [^{3}H]Thymidine was added to cultures of irradiated and control B cells, and incorporation was assayed 18 h later. [^{3}H]Thymidine incorporation from a minimum of three experiments is expressed as cpm $\pm$ S.E. An asterisk indicates a significant decrease in [^{3}H]thymidine incorporation ($p < 0.05$) compared with corresponding unirradiated controls. C, proteins from irradiated and control B cells cultured for 24 h with CpG + rIL-4 or with CD40L-transfected L cells + rIL-4 were extracted and analyzed by Western immunoblotting with AhR- and β-actin-specific antibodies. Representative blots from a total of three experiments are shown.

nuclear and cytoplasmic extracts were free of cross-contamination.

EMSAs were performed with nuclear extracts from freshly isolated naive B cells and from B cells that were expanded for 1 week on CD40L-transfected L cells to determine whether constitutively nuclear AhR is capable of binding DNA at the AhRE consensus site. The probe used for these studies was derived from the promoter of a prototypic AhR-regulated gene, *CYP1A1*. Specific binding to the AhRE probe was not detected when using extracts from naive B cells (Fig. 5A). In contrast, extracts from CD40L-activated B cells consistently yielded a pair of AhRE-binding bands (Fig. 5A, arrows), formation of which was specifically inhibited with unlabeled AhRE but not with an AhRE mutant containing a double base pair mutation within the core sequence (mutAhRE). This result is reminiscent of that previously reported with guinea pig liver extracts (Swanson et al., 1993). In addition, both complexes could be completely supershifted with polyclonal ARNT-specific antibody. The appearance of two AhRE-binding complexes, containing ARNT, is similar to results obtained with extracts containing constitutively active AhR from B cell tumors (Masten and Shiverick, 1996). Also consistent with observations reported in B cell lines, the nuclear AhR complex in primary activated B cells was partially supershifted with an AhR-specific antibody (Fig. 5C). These data demonstrate constitutive AhR-DNA binding in CD40L-activated human B cells.

CYP1A1 Transactivation in Activated B Cells. Nuclear localization and DNA binding of the AhR in activated B cells, in the absence of exogenous ligands, supports the hypothesis that the AhR is constitutively active in these cells. If the AhR is indeed transcriptionally active in activated B cells, it would be predicted that levels of endogenous *CYP1A1* mRNA would be similarly up-regulated. To test this prediction, *CYP1A1* mRNA expression was compared by semiquan-
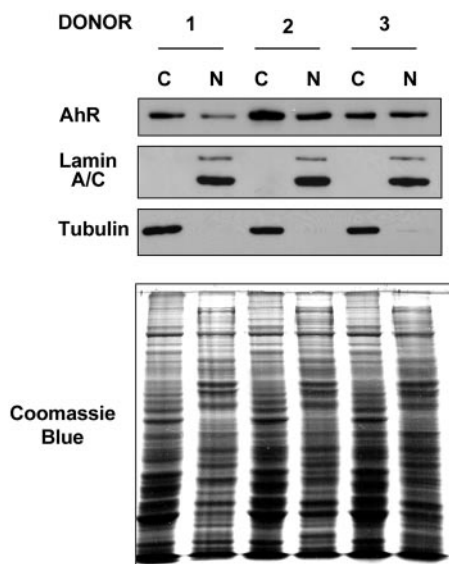

Fig. 4. AhR nuclear localization in CD40L-activated B cells. Cytoplasmic (C) and nuclear (N) cell extracts prepared from B cells grown for 1 week on CD40L-transfected L cell monolayers + rIL-4 were analyzed by Western immunoblotting with AhR-specific antibody (top) and by Coomassie Blue staining (bottom) after SDS-PAGE. Blots were stripped and lamin A/C- and α-tubulin-specific antibodies were used to examine purity of cell fractions. Representative data from three donors (five donors total) are shown.
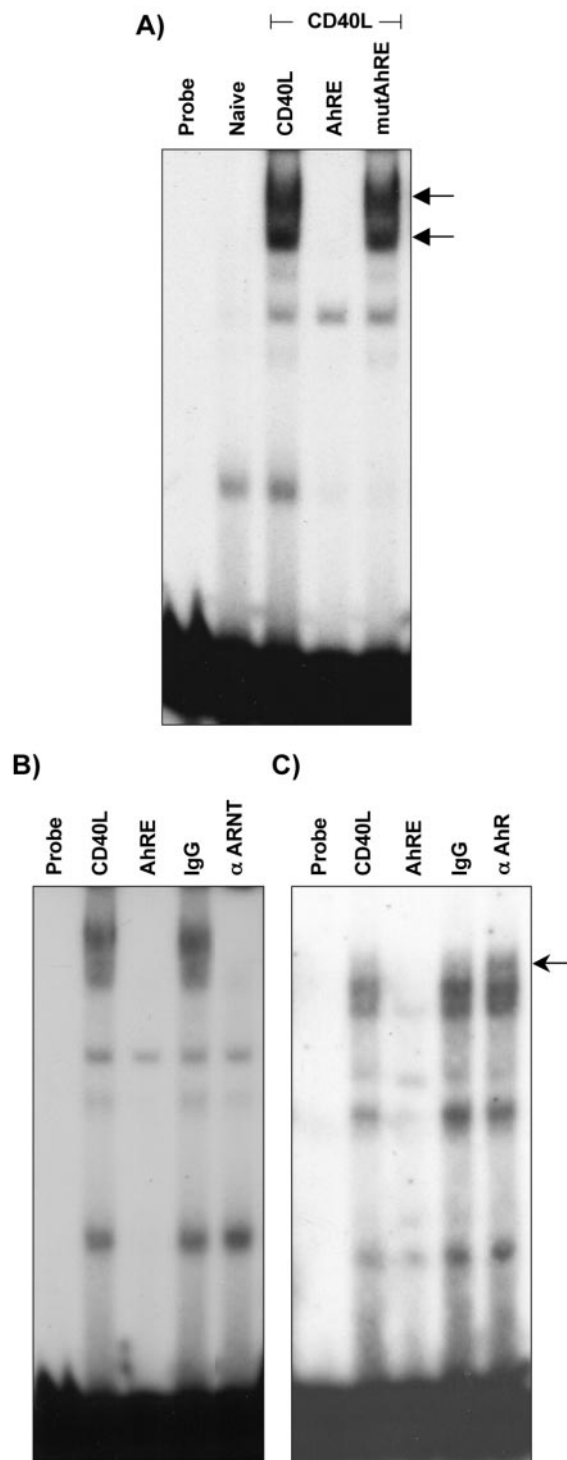

Fig. 5. AhR-AhRE binding in CD40L-activated B cells. Nuclear extracts from sorted naive B cells and from B cells grown for 1 week on CD40L-transfected L cells + rIL-4 were analyzed by EMSA for ^{32}P-labeled *CYP1A1* AhRE oligonucleotide binding. A, competition analyses were performed by including 200× excess unlabeled probe (AhRE) or mutant probe (mutAhRE) containing a two nucleotide change in the AhR consensus binding site. Arrows indicate specific AhR-DNA complexes. B, supershift analyses were performed by adding normal rabbit IgG or ARNT-specific antibody to reaction mixtures before electrophoresis (5% acrylamide TBE gel). C, supershift analyses were performed by adding normal rabbit IgG or AhR-specific antibody to reaction mixtures before electrophoresis (4% acrylamide TGE gel). The arrow indicates the supershifted AhR complex. Data from one representative experiment (from at least three total) are shown.

titative RT-PCR in purified resting B cells and in B cells activated for 24 h with CpG or CD40L, as in previous experiments. No *CYP1A1* mRNA was detected in freshly isolated B cells (not shown) or in B cells cultured for 24 h in media alone (Fig. 6, A and B, resting). In some donors, culture with rIL-4 seemed to increase *AhR* and *CYP1A1* expression (Fig. 6A, donor 2), although this trend did not reach statistical significance when pooling data obtained with eight donors (Fig. 6B). As in previous experiments, activation with CpG or CD40L in the presence of rIL-4 significantly increased *AhR* expression. It is noteworthy that linear regression analysis indicates a significant correlation between *AhR* and *CYP1A1* levels (Z test; $p < 0.05$). Although other factors may contribute to this up-regulation of *CYP1A1* mRNA, the correlation between AhR levels and *CYP1A1* up-regulation and the find-

ing that the AhR constitutively binds the *CYP1A1* promoter are consistent with the hypothesis that the AhR in activated B cells constitutively transactivates the *CYP1A1* gene.

Effect of AhR Ligand on CYP1A1 Expression in Activated B Cells. The experiments outlined above have established that *CYP1A1* is expressed in activated B cells in the absence of exogenous AhR ligands. This result raises the issue of whether constitutively active AhR in primary human B cells is able to respond to exogenous ligands, presumably to augment *CYP1A1* mRNA up-regulation. Indeed, studies in transformed human lymphocyte lines expressing high AhR levels indicate that treatment with AhR ligands may not necessarily induce *CYP1A1* mRNA (Masten and Shiverick, 1996). To resolve this issue in primary human B cells, we assayed for *CYP1A1* induction in CD40L-activated B cells dosed for 18 h with vehicle or 10^{-6} M B[*a*]P. For these studies, the number of amplification cycles was reduced to visualize high B[*a*]P-induced *CYP1A1* mRNA levels (Fig. 2B). B[*a*]P significantly augmented *CYP1A1* expression in CD40L-activated cells (Fig. 7), indicating that the AhR is indeed responsive to exogenous ligands despite its high level of apparent constitutive activity.

AhR Engagement with Exogenous Ligand Alters Proliferation of Activated B Cells. Up-regulation of the AhR through B cell activation and its engagement with ex-
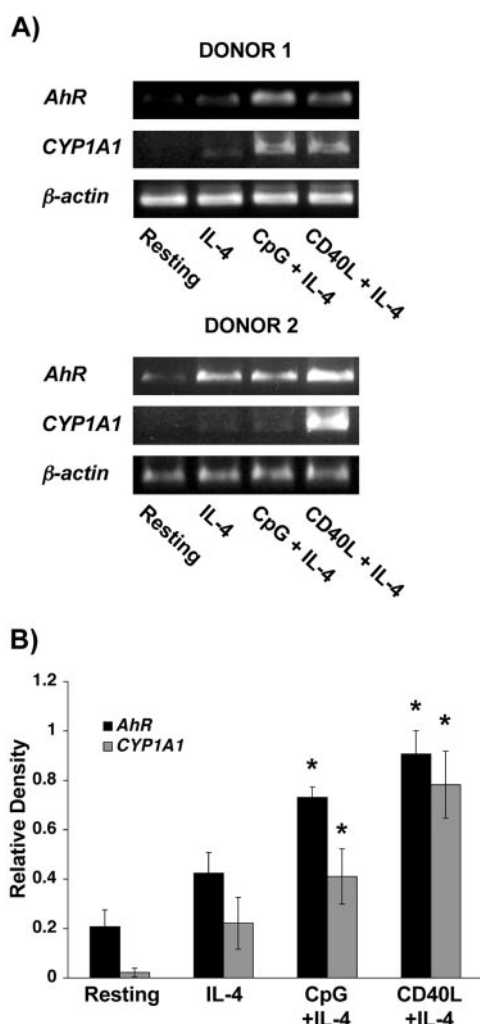

Fig. 6. B cell *AhR* and *CYP1A1* expression is induced upon stimulation with CpG or CD40L. Sorted B cells were cultured for 24 h in media alone (resting), rIL-4, CpG, CpG + rIL-4, or for 1 to 2 weeks on monolayers of CD40L-transfected L cells + rIL-4. RNA was extracted and analyzed by RT-PCR with *AhR*-, *CYP1A1*-, and *β-actin*-specific primers. A, data from two representative donors (seven total) are presented. B, *β-Actin* signals were used to normalize *AhR* and *CYP1A1* band densities. Results are presented as means ± S.E. from at least seven donors per treatment. An asterisk indicates a significant increase in band densities relative to the corresponding resting B cell control ($p < 0.05$; Dunnett's). Normalized *AhR* and *CYP1A1* levels did not increase significantly when B cells from eight donors were cultured with rIL-4 alone ($p > 0.07$). Linear regression analysis indicates a significant correlation between *AhR* and *CYP1A1* levels (Z test; $p < 0.05$).
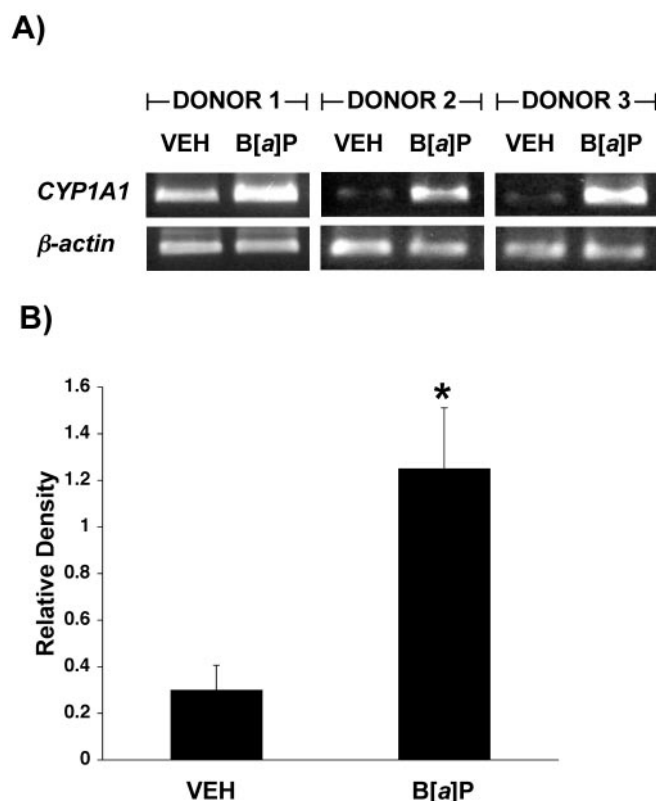

Fig. 7. B[*a*]P, an environmental AhR ligand, augments *CYP1A1* expression in CD40L-activated B cells. B cells were cultured for 1 to 2 weeks on CD40L-transfected L cells + rIL-4. Cells then were treated with vehicle (final volume 0.1%) or 10^{-6} M B[*a*]P for 18 h. Cells were harvested, RNA extracted and *CYP1A1*- and *β-actin*-specific RT-PCR performed. A, data from three representative donors (five total) are shown. B, *CYP1A1* band densities were normalized to *β-actin* band densities. Representative data from a total of five donors are presented as mean ± S.E. An asterisk indicates a significant increase in *CYP1A1* mRNA compared with vehicle controls ($p < 0.05$; Dunnett's).

ogenous ligand both induce nuclear localization and *CYP1A1* induction. Therefore, it was postulated that engagement of the AhR with exogenous ligands would provide an insight into the function of constitutively active AhR.

B cells grown for 1 week on a monolayer of CD40L-transfected L cells with rIL-4 were harvested and replated on a monolayer of irradiated CD40L-transfected cells in 96-well plates. Cells then were treated with vehicle, 10^{-5}–10^{-9} M B[*a*]P, or similar doses of B[*e*]P, a B[*a*]P congener that binds poorly to the AhR. [³H]Thymidine was added after 21 h, and cells were cultured for another 18 h before harvest and analysis of [³H]thymidine incorporation.

Proliferation of CD40L-activated B cells was significantly reduced at B[*a*]P doses as low as 10^{-7} M B[*a*]P (Fig. 8A; $p < 0.001$). In contrast, B[*e*]P did not affect proliferation significantly, even at the highest dose tested (10^{-5} M). Because it was formally possible that B[*a*]P was affecting the monolayer of L cells on which B cells depend for CD40L stimulation, these experiments were repeated in culture wells coated with soluble CD40L as a substitute for CD40L-transfected L cells. The stimulation provided by plate-bound CD40L (Fig. 8B) was approximately equivalent to that provided by transfected L cell monolayers and similar results were obtained; i.e., B[*a*]P significantly suppressed B cell growth. In addition, 10^{-5} M B[*a*]P significantly inhibited the growth of these B cells in the absence of additional CD40L signaling (Fig. 8B). These results demonstrate that activated B cells are directly targeted by an environmental AhR ligand, and they support the hypothesis that the AhR expressed in activated human B cells is capable of regulating cell growth.

Discussion

The present study was designed to determine whether, and under what circumstances, the AhR is expressed in primary human B cells, to address the possibility that environmental AhR ligands target activated B cells, and to begin to accumulate data that would implicate a physiological role for this receptor/transcription factor in B cells. Because of previous studies that suggest that B cells are immediate targets of immunotoxic AhR ligands, and reports in which high AhR levels were noted in tumors or rapidly growing cell lines, we focused primarily on the possibility that the AhR is upregulated as part of a response to B cell activation. Therefore, systems were established that model B cell stimulation during innate and adaptive immune responses. The ability to maintain and expand normal human B cells in culture by stimulating them with multivalent CD40L in the presence of rIL-4 greatly facilitated these studies.

Initial experiments indicated that little or no AhR protein or mRNA was expressed in resting peripheral B cells. However, 24-h activation with either CpG or CD40L significantly increased AhR levels. B cells grown for longer periods (>2 weeks) on CD40L-transfected L cells plus rIL-4 retained high AhR levels comparable with those seen in human tumor cell lines (not shown). A parallel increase in steady-state *AhR* mRNA levels indicated that AhR induction is probably mediated at least at the transcriptional level, although increased AhR mRNA or protein stability cannot be ruled out (Chen et al., 1997; Meyer et al., 2000; Song and Pollenz, 2002). In and of itself, this profound change in AhR expression suggests that the AhR may perform an important function during normal human B cell stimulation. At the very least, it is likely that high AhR levels expressed in activated B cells make these cells sensitive targets of environmental ligands. Induction of *CYP1A1* in CpG- or CD40L-activated B cells (Fig. 7) and inhibition of cell growth (Fig. 8) after B[*a*]P treatment are consistent with this conclusion (see below).

As noted previously, AhR levels are generally associated with rapidly growing tumors (Masten and Shiverick, 1995, 1996; Singh et al., 1996; Trombino et al., 2000). However, it was not known whether the high AhR levels seen in rapidly dividing cells is the cause or the consequence of accelerated cell growth. Consistent with tumor cell studies, AhR upregulation correlated with increased growth of normal CpG-
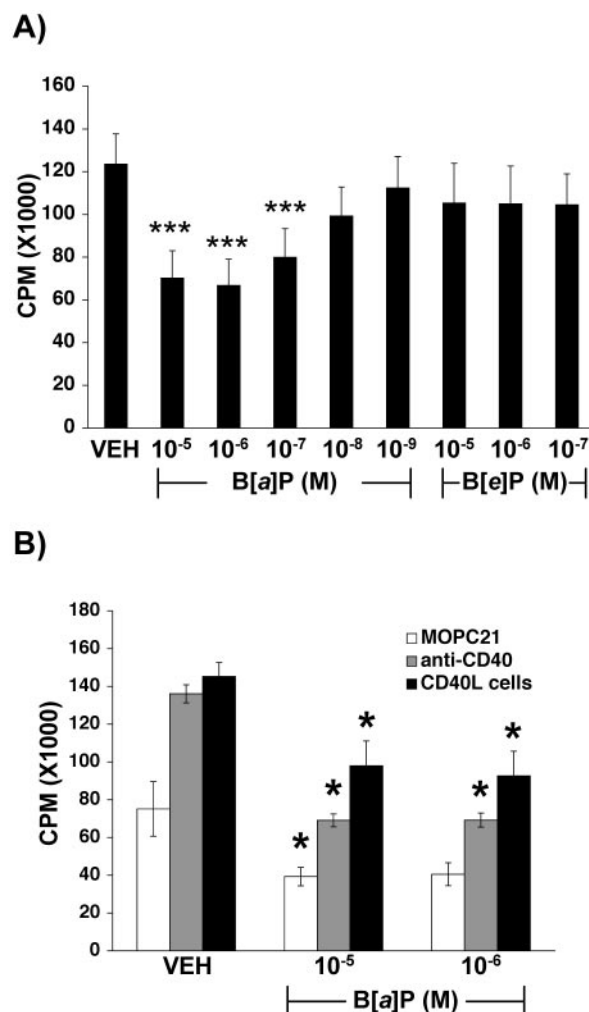

Fig. 8. B[*a*]P directly suppresses proliferation of CD40L-activated B cells. A, B cells were cultured on CD40L-transfected L cells + rIL-4 for 1 week and transferred to fresh monolayers of CD40L-transfected cells. Vehicle, 10^{-5}–10^{-9} M B[*a*]P, or 10^{-5}–10^{-7} M B[*e*]P was added to triplicate wells and cells were cultured for an additional 21 h. ³[H]Thymidine was added and incorporation assayed 18 h later. The means of the triplicate counts were then averaged for each indicated data point. Data are presented as mean ± S.E. from at least six donors. The triple asterisk indicates a significant decrease in [³H]thymidine incorporation compared with vehicle controls ($p < 0.001$). B, B cells were prepared and treated as described above with the exception that, after culture for 1 week on CD40L-transfected L cells, B cells were transferred to wells coated with CD40L-specific antibody or with an isotype control, MOPC-21. Data are presented as mean ± S.E. from four donors. An asterisk indicates a significant decrease in [³H]thymidine incorporation compared with vehicle controls ($p < 0.05$).

or CD40L-activated human B cells. However, cell growth per se was not required for AhR up-regulation because irradiation before activation inhibited cell growth but did not block induction of AhR or a costimulatory molecule, B7-2. These results indicate that the signal transduction pathways required for AhR up-regulation in normal cells can be dissociated from those required for entry into cell cycle. Moreover, they suggest that signaling requirements for induction of proteins important for antigen presentation (i.e., B7-2) overlap with those required for AhR induction. Because both CD40L and CpG/TLR-9 signaling pathways are well characterized, this hypothesis is readily testable.

CpG or CD40L activation resulted in AhR nuclear localization. Because the AhR resides in the cytoplasm in most nontreated, nontransformed cells (Song and Pollenz, 2002), this result suggests some level of constitutive AhR activation. A similar conclusion was reached when nuclear AhR was detected in situ in primary rat mammary tumors, human adult T cell leukemias, or untreated human myeloma, breast carcinoma, cervical carcinoma, or Epstein-Barr-transformed lymphocyte cell lines (Masten and Shiverick, 1995, 1996; Singh et al., 1996; Trombino et al., 2000). Thus, the phenotype of nuclear AhR localization, like that of increased total AhR protein, in activated, nontransformed primary B cells resembles that of neoplastic cells.

Because AhR signaling is primarily attributed to AhR transcriptional activity, the presence of the AhR in the nucleus of activated B cells further suggests that the AhR modulates gene expression in the absence of exogenous ligands. However, the mere presence of nuclear AhR does not prove constitutive DNA binding or target gene transactivation. For example, murine T cells activated with CD3-specific antibody and treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin express nuclear AhR, which does not bind consensus AhREs or transactivate *CYP1A1* (Lawrence et al., 1996). Furthermore, nuclear AhR translocation can be effected in the absence of exogenous ligands by treatment with geldanamycin without an increase in transcriptional activity (Song and Pollenz, 2002). Nevertheless, we found that nuclear AhR from cells stimulated with CD40L in the absence of exogenous ligands readily bound a consensus AhRE probe (Fig. 5). It is interesting that EMSAs revealed two major bands of ARNT- and AhR-containing complexes that specifically bound to the AhRE probe. This result is similar to that reported with human myeloma and Epstein-Barr-transformed lymphocyte cell lines where distinct nuclear DNA-binding forms were identified (Masten and Shiverick, 1996). It is noteworthy that this additional band was not seen in transformed human hepatic or mammary carcinoma lines, suggesting that the presence of multiple AhRE-binding complexes is a characteristic primarily of either transformed or activated normal B cells (Masten and Shiverick, 1996).

We also noted a significant increase in *CYP1A1* mRNA in CpG- and CD40L-activated B cells (Fig. 6). As seen in AhR-transgenic mice (Andersson et al., 2003), an increase in this AhR-regulated gene correlated with an increase in AhR levels. Likewise, B[*a*]P hydroxylase (i.e., CYP1A1) activity correlates with human lymphocyte and monocyte activation states (Whitlock et al., 1972). These results suggest that the active AhR in stimulated B cells constitutively enforces *CYP1A1* transcription. Regardless of whether the induction of *CYP1A1* is caused by constitutive AhR transactivation

and/or to the influences of other transcriptional regulators, the likely outcome of CYP1A1 induction after B cell stimulation is increased susceptibility to metabolizable AhR ligands like B[*a*]P and related PAHs. The ability of B[*a*]P, and presumably other AhR ligands, to further up-regulate *CYP1A1* in activated B cells (Fig. 7) provides additional support for the hypothesis that activated B cells, in particular, are sensitive targets of PAH immunotoxicity.

Treatment of CD40L-activated B cells with B[*a*]P revealed one level on which environmental PAHs may affect B cell responses (i.e., direct suppression of B cell growth) (Fig. 8). Furthermore, and perhaps more importantly, it suggests that the AhR is at least capable of regulating growth of activated B cells. Three general mechanisms may be responsible for AhR-ligand-mediated growth suppression: 1) If constitutively active AhR enhances cell growth, as suggested by some studies with transformed cells (Fernandez-Salguero et al., 1995; Ma and Whitlock, 1996; Dohr and Abel, 1997; Shimba et al., 2002), then its engagement by an environmental ligand could divert the intracellular signaling pathway away from a growth pathway toward a CYP1A1-dependent metabolism pathway. 2) If up-regulation of constitutively active AhR is an attempt by the cell to limit growth of activated B cells, as suggested by studies with human carcinoma cell lines (Weiss et al., 1996; Abdelrahim et al., 2003), then exogenous AhR ligands may enhance AhR signaling and thereby increase growth inhibition. 3) Either constitutive or PAH-inducible, AhR-dependent increases in CYP1A1 could result in increased production of PAH metabolites that either alter AhR signaling, by virtue of their ability to bind and activate the AhR (Mann et al., 1999), or damage DNA, thereby inducing the activation of cell cycle inhibitors. Should this last mechanism be invoked, the level of DNA damage would have to be sufficient to induce growth inhibition but not severe enough to induce death because apoptosis of activated B cells was not observed even at the highest B[*a*]P doses. In either case, the ability of PAH metabolites to suppress CD40L-activated human B cell growth would be consistent with previous animal studies in which PAH metabolites were shown to suppress antigen-specific T and B cell responses or to compromise lymphocyte development at doses lower than those required to induce immunosuppression with the respective parent compounds (Mann et al., 1999).

It is important to note that the current study differs significantly from those in which mitogens (LPS or phorbol 12-myristate 13-acetate plus ionophore) were used to activate murine B cells and to modulate AhR expression (Crawford et al., 1997; Marcus et al., 1998). In addition to evaluating, for the first time, AhR up-regulation and function in activated primary human B cells, AhR induction in the present system was effected through specific receptors CD40 and TLR9. Signal transduction through these receptors is distinct from the signals activated nonspecifically by phorbol 12-myristate 13-acetate plus ionophore or by LPS via CD14 and TLR4. Indeed, unlike murine B cells, human B cells do not respond significantly to LPS. Furthermore, the current studies demonstrate that CD40L- or CpG-induced AhR up-regulation is not dependent on cell proliferation and is retained over long periods.

Finally, these studies are consistent with the hypothesis that the AhR is up-regulated after physiologically relevant

stimulation and that the ensuing constitutively active AhR probably participates in cell growth regulation. Proof that the AhR regulates B cell growth in the absence of environmental chemicals awaits manipulation of AhR levels in CD40L-activated human B cells through molecular techniques.

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