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A Cyclooxygenase-2/Prostaglandin E2 Pathway Augments Activation-Induced Cytosine Deaminase Expression within Replicating Human B Cells

Hyunjoo Lee,*1 Joshua S. Trott,*1 Shabirul Haque,* Steven McCormick,† Nicholas Chiorazzi,‡§,‡ and Patricia K. A. Mongini*§

Within inflammatory environments, B cells encountering foreign or self-Ag can develop tertiary lymphoid tissue expressing activation-induced cytosine deaminase (AID). Recently, this DNA-modifying enzyme was detected in nonlymphoid cells within several inflamed tissues and strongly implicated in malignant transformation. This study examines whether a cyclooxygenase 2 (COX-2) pathway, often linked to inflammation, influences AID expression in activated B lymphocytes. In this paper, we report that dividing human B cells responding to surrogate C3d-coated Ag, IL-4, and BAFF express AID, as well as COX-2. A progressive increase in AID with each division was paralleled by a division-related increase in a COX-2–linked enzyme, microsomal PGE2 synthase-1, and the PGE2R, EP2. Cells with the greatest expression of AID expressed the highest levels of EP2. Although COX-2 inhibitors diminished both AID expression and IgG class switching, exogenous PGE2 and butaprost, a selective EP2 agonist, augmented AID mRNA/protein and increased the numbers of IgG+ progeny. Despite the latter, the proportion of IgG+ cells within progeny of PGE2-supplemented cultures, it appears more likely that PGE2 facilitates AID-dependent DNA double-strand breaks or to greater downstream switching. Rather, because phosphorylated ataxia telangiectasia mutated levels were increased in that block B cell cycle progression or promote activation-induced cell death, or both. Taken together, the results suggest that inhibitors diminished both AID expression and IgG class switching, exogenous PGE2 and butaprost, a selective EP2 agonist, augmented AID mRNA/protein and increased the numbers of IgG+ progeny. Despite the latter, the proportion of IgG+ cells within viable progeny generally declined with PGE2 supplementation. This was not due to PGE2-promoted differentiation to plasma cells or to greater downstream switching. Rather, because phosphorylated ataxia telangiectasia mutated levels were increased in progeny of PGE2-supplemented cultures, it appears more likely that PGE2 facilitates AID-dependent DNA double-strand breaks that block B cell cycle progression or promote activation-induced cell death, or both. Taken together, the results suggest that a PGE2 feed-forward mechanism for augmenting COX-2 pathway proteins promotes progressively increased levels of AID mRNA, protein, and function. The Journal of Immunology, 2010, 185: 5300–5314.

After its discovery as an inducible cyclooxygenase (COX) distinct from ubiquitously expressed COX-1, COX-2 was primarily considered a marker of inflammatory cells and inflamed tissues. More recent studies show that the enzyme has multiple functions, including promoting the growth of hematopoietic stem cells (1) and numerous malignancies (2). Importantly, exposure of quiescent human B cells to optimally stimulatory doses of BCR, CD40, or TLR ligands induces rapid upregulation of COX-2 mRNA and protein (3, 4). Within nontransformed human B cell clones generated by surrogate C3dg-coated Ag, IL-4, and BAFF (5), the latter cytokine upregulates COX-2 protein within replicating progeny (6). B cell-expressed COX-2 appears to be functionally relevant because pharmacologic inhibitors of the enzyme can ablate B cell Ab synthesis (7–9), as well as reduce the viability of progeny (6). This inhibition is reversible by exogenous PGE2—a downstream product of the COX-2 pathway (6). The latter recent findings showed that, during a replicative burst, normal human B cell clones mimic both malignant cells and hematopoietic stem cells in using a COX-2→PGE2 pathway for survival/growth.

Interestingly, mice deficient in COX-2 have significantly impaired IgG Ab responses to vaccination, whereas they exhibit normal or even heightened IgM responses (8, 9). Although diminished viability of replicating progeny may, in part, explain these findings, there is also evidence that COX-2 influences B cell Ig class-switch recombination. Supplementing mouse B cell cultures with PGE2 significantly augments LPS + IL-4–induced production of κ-germline transcripts (10), rearranged VDJ-ε transcripts (11), and secretion of IgG and IgE (10, 12, 13).

Our laboratory has been investigating whether COX-2/PGE2 might further influence class-switch recombination, and linked somatic mutation, through augmenting the expression and/or function of the enzyme critical for the latter events, activation-induced cytosine deaminase (AID). This possibility was heightened by recent reports that AID is atypically expressed within several nonlymphoid tissues characterized by significant inflammation and high COX-2 expression, for example, Helicobacter pylori-infected gastric epithelium, normal hepatocytes adjacent to hepatocarcinoma, and inflamed biliary tract epithelium (14–26). Importantly, in some of the above tissues, AID appeared to be functionally active and linked to mutations in the protooncogenes, p53 or c-Myc (16, 19, 20, 27). Although in B cells the Ig locus is
a preferred target for AID, the enzyme also can induce off-target, oncogenic mutations in certain actively transcribed, non-Ig genes, for example, p53, Bel-6, c-Myc, and PAX5 (28–31). This strong association of AID with the development of malignancy warrants a full understanding of the mechanisms for its regulation.

Both COX-2 and AID mRNA and protein are weakly, if at all, expressed in naive B cells, but induced by stimuli such as IL-4 + CD40-L, IL-4 + BAFF, or TLR ligands (3, 4, 6, 32–34). The NF-κB signaling pathway is common to the upregulation of both (35–40). An interesting, unexplained finding is that AID expression in B cells increases with successive cell divisions (33). This occurs in parallel with a division-related increase in Ig class-switched progeny, indicating that enzymes needed for the activation of AID by phosphorylation (41–43) are present within dividing lymphoblasts. Several attributes of COX-2 and downstream related molecules may be relevant to this increase in AID expression and function. First, COX-2 is expressed at higher levels in cycling B lymphoblasts than in noncycling blasts from the same activated cultures (6). Second, several COX-2–dependent prostanoids, including PGE2, are known to activate protein kinase A (PKA) (44), an enzyme with a critical role in phosphorlyating AID at Ser38 and mediating the latter’s activation (42). Third, EP2 and EP4, two PKA-activating receptors for PGE2 (45), are expressed on activated B cells and reported to function in augmenting IgG and IgE synthesis (46, 47). Finally, studies in nonlymphoid cells show that the COX-2 pathway is subject to significant positive feedback control; COX-2–driven PGE2 can promote the further upregulation of COX-2 (48–50), downstream PGE2 synthase (51), and EP2 (52). Thus, the progressive increase in AID levels within cycling B cells might reflect the influence of an increasingly functional COX-2/PGE2 pathway during B cell clonal expansion.

In this study, we investigate whether a COX-2 pathway influences the expression and function of AID within normal human B lymphocytes responding to a T cell–independent stimulus. Clonal expansion was induced by culturing quiescent CFSE-labeled, IgM+ human B2 cells with limiting C3dg-coated surrogate Ag (anti-IgM/anti-CD21/dextran) (53) and the innate immune system cytokines, IL-4 and BAFF. As discussed elsewhere (5, 6), these stimuli likely mimic those received by recirculating naive B cells encountering specific Ag (C3dg-coated microbes or apoptotic cells) within inflamed peripheral tissues. The latter sites are often characterized by the presence of IL-4 (or related IL-13)–producing cells (basophils, mast cells, NK-like T cells) and BAFF-producing cells (macrophages, dendritic cells, basophils, epithelial cells) (reviewed in Refs. 3, 5) (54, 55). We now report that, under such T cell–independent activation conditions, AID levels increase in a division-dependent manner. The increase in AID levels is temporally and functionally linked to not only COX-2, but the downstream PGE2 synthase, microsomal PGE2 synthase–1 (mPGES-1), and the EP2 receptor for PGE2. The findings suggest a mechanism whereby AID expression increases with division and have implications for the use of COX-2 pathway inhibitors in autoimmune disease and inflammatory conditions associated with oncogenesis.

**Materials and Methods**

**Reagents for culture**

The surrogate Ags used in this study were soluble mAb/dextran conjugates in which BCR ligand (anti-IgM mAb) was physically linked to high m.w. dextran by either stable thioester bonds (56) or a biotin–streptavidin linkage. In experiments with human B cells, a previously described high-affinity anti-IgM/anti-CD21/dextran conjugate (5, 52) modeled CD3-bound Ag (BCR-CD21-L). The latter was used, together with recombinant human BAFF and/or recombinant human IL-4, exactly as described (5, 6). PGE2 and butaprost, a synthetic analog of PGE2 (Cayman Chemical, Ann Arbor, MI) were prepared at 2–4 μM in ethanol. The specific COX-2 inhibitors, celecoxib (Celebrex, provided by Pharmacia/Pfizer, Chicago, IL), CAY10404, SC-58125, and NS-398 (Cayman Chemical) were prepared at 25–50 μM in DMSO. These stock solutions were stored at −70°C and diluted immediately before use.

**Purification of human and mouse B2 cell populations**

Human B cells were derived from tonsils, obtained by elective tonsillectomy at New York Eye and Ear Infirmary (New York, NY) or North Shore University Hospital (Manhasset, NY), and from spleens, obtained from National Disease Research Interchange and Cooperative Human Tissue Network, removed because of trauma, or during pancreatic surgery not involving malignancy. All were deidentified and used according to Institutional Review Board guidelines. High-density, IgM+ and IgD+ follicular (B2) cells were isolated through a multistep process described in previous publications by our laboratory (5, 6).

**Culture conditions**

B cells were cultured in a highly enriched RPMI 1640-based medium, as described previously (5, 6), and generally prelabeled with 1 μM CFSE (Invitrogen, Carlsbad, CA) (5, 53, 56). Preincubation of IgM and/or IgG prelabeled cells at a density determined to be limiting for PGE2, 103 cells/200 μl culture in 96-well plates (or for lysate preparations, 106 cells/ml culture in 24-well plates). Pulses of PGE2, butaprost, or COX-2 inhibitors were generally added on days 2 and 4, with culture harvest on day 5 or 6. Analysis of cell viability and recovery involved pulsing CFSE-labeled cultures with a known number of standardization beads (CountBright Absolute Counting Beads; Invitrogen) before harvest, fixation in 2% paraformaldehyde, and analysis by flow cytometry (FACScan or FACS-Calibur; BD Biosciences, San Jose, CA). Viable and apoptotic cells and standardization beads were distinguished by differences in forward and side scatter after prior gating to remove debris. The absolute yield of viable cells within each division subset was determined from the number of gated cells collected and the ratio of the standardization beads collected relative to beads added (5, 6).

**Cell surface staining for IgM, IgG, IgA, and IgE**

Membrane Ig isotypes were quantified using previously described methods (5). Cells were stained with a mixture of Alexa Fluor 647-mouse anti-pan human IgG mAb (or labeled IgG1 control) plus PE–anti-human IgM (Southern Biotechnology Associates, Birmingham, AL) or PE-Fab2 anti-human IgG mAb (or labeled IgG1 control) plus PE-Cy5–anti-human IgM (BD Pharmingen, San Diego, CA) exactly as described (5, 6). For IgA staining, human AID mAb (EK2 5G9; Cell Signaling Technology, Beverly, MA) or PE-labeled anti-IgG1 (Southern Biotechnology Associates) or PE–anti-human IgG (CatTag Laboratories, Burlingame, CA or BD Pharmingen).

**Intracellular staining for mPGES-1, AID, PGE2Rs (EP1–EP4), and phosphorylated ataxia telangiectasia mutated**

CFSE-labeled cultures were harvested into cold PBS, centrifuged, and fixed. For detection of mPGES-1 and EP1–EP4, cells were fixed and permeabilized with either BD Fix-Perm (BD Biosciences) or protocols described previously (5, 6). For detection of phosphorylated ataxia telangiectasia mutated (phospho-ATM), BD Phosflow Fix Buffer was used, using supplier’s protocols (BD Biosciences). For AID staining, harvested cells were first fixed in 2% EM-grade formaldehyde for 30 min, followed by exposure to ice-cold 100% methanol for a minimum of 2 h on ice or for 20°C overnight) to enhance detection of nuclear protein. Cells were then washed, re suspended in a previously described intracellular staining buffer containing 0.1% saponin + 30% human AB serum (5) for 15 min at room temperature to reduce nonspecific binding, and subsequently stained using rat anti-human AID mAb (EK2 5G9; Cell Signaling Technology, Beverly, MA) or a rat IgG2b isotype control (KLI/G2b-1; Southern Biotechnology Associates) at a final concentration of 0.8 μg/ml (35 min at room temperature), followed by a labeled detection Ab, PE–Cy5–goat Fab2 anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). In some experiments, mouse anti-AID mAb (ZA-001; Invitrogen), or mouse IgG1 control, was used for staining, followed by a labeled anti-mouse IgG Ab. Staining with non-AID–specific Abs was done with BD Perm-Wash, or alternatively in the case of phospho-ATM, BD Phosflow Perm Buffer III (BD Biosciences). mPGES-1 was detected with a rabbit anti-human mPGES-1 IgG Ab (5.3 μg/ml; Cayman Chemical) followed by PE–goat Fab(ab)2; anti-rabbit IgG (Southern Biotechnology Associates). EP1, EP2, EP3, and EP4 were detected with affinity-purified, receptor-specific rabbit IgG Abs.
(Cayman Chemical) with or without excess blocking peptide (Cayman Chemical), followed by PE-goat F(ab’2)-anti-rabbit IgG. Staining for phospho-ATM was performed with PE-mouse anti-phospho-ATM (Ser1981; Millipore clone 10H11-E12, 2.5 μg/ml; Millipore, Bedford, MA) or a PE-IgG1 isotype control. In all experiments, the levels of FL2 or FL3 staining (Δ mean fluorescence intensity [ΔMFI]) for a specific intracellular protein was assessed as a function of level of CSF fluorescence (FL1)—that is, division. In some cases, results from replicate experiments were pooled by setting corresponding values; the ΔMFI value for a given subset of stained cells is expressed as a percentage of the ΔMFI value of another subset of cells in the same experiment. In statistical comparisons of expressed protein within two different treatment groups, data were generally analyzed by a paired two-tailed Student t test, unless otherwise indicated.

**Immunoblotting experiments to assess AID**

Lysate preparation, protein quantification, gel electrophoresis, and Western blotting were performed as previously described (5). AID was detected with rat anti-AID mAb (EK2 5G9, 1:1000 dilution; Cell Signaling Technology) or with a murine anti-AID mAb (ZAA01, 1:250 dilution; Invitrogen) followed by an HRP-secondary anti-IgG Ab and ECL (with Pico- or Femto-detection reagent; Pierce, Rockford, IL). Blots were stripped and analyzed for actin as a loading control.

**Analysis of AID mRNA**

Total RNA extraction and qualitative RT-PCR. Total RNA was extracted from cultured human normal B cells (1–5 million) using Mini-prep Qiagen kit (Qiagen, Gaithersburg, MD), and cDNA synthesis was performed using Oligo dT primers from Invitrogen kit (Invitrogen). PCR was carried out using Ampli Taq Gold (Applied Biosystems, Roche, NJ). The full-length AID was amplified using forward and reverse primers 5’-AGGCCAGGAAGACACTCCTGGACACCC-3’ and 5’-GTGACATCTCCTGGAAGTTGC-3’. Respectively, PCRs were run under the following cycling conditions: 95°C for 30 s, 58°C for 60 s, and 72°C for 30 s for 35 cycles in a total reaction volume of 30 μl. PCR products were resolved in 1.5% agarose gel prepared in Tris-borate-EDTA buffer. The gel was imaged with the Bio-Rad gel documentation system (Bio-Rad, Hercules, CA).

**Quantitative PCR.** AID primers (forward: 5’-GACCTTGGTTACTCTTG-CAATAAGA-3’; reverse: 5’-AGGTCCTACGGCTGATGTAG-3’) for quantitative PCR (qPCR) were obtained from human universal probe library of Roche Applied Science (Indianapolis, IN). These primers recognize all splice forms of AID except AID E3-E4 (57). qPCR was executed in triplicates or quadruplicates using cDNA as template with Eurogentec 2× master mixes (AnaSpec, Fremont, CA). qPCR parameters were as follows, as per Eurogentec recommendations: initial uracil-N-glycosylase activation at 50°C for 2 min; HotGoldStar activation (and uracil-N-glycosylase inactivation) at 95°C for 10 min; followed by 45 cycles, each at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension). Amplification was extended to 45 cycles to reveal the plateau of maximal amplification was extended to 45 cycles to reveal the plateau of maximal

**Results**

**Levels of AID protein and the PGE2 synthase, mPGES-1, are upregulated within progeny of BCR-triggered, innate immune system-driven human B cell clones**

Although Ig class switching and Ig somatic mutation are often considered T cell-dependent events, several studies show that AID-dependent alterations in the Ig locus can occur in response to T cell-independent Ags (58, 59). In this study, we wished to determine whether AID mRNA and protein are upregulated in human B2 cells responding to T cell-independent stimulation by low-dose BCR:CD21-L + IL-4 and BAFF, physiologically relevant molecules that upregulate COX-2 within cycling lymphoblasts (6). Fig. 1A (lane 2) and 1B demonstrates that AID mRNA is significantly increased within day 5 cultures exposed to these stimuli, as well as in a human B cell line (CL-01) representing activated, germinal center cells (lane 3) (60). AID protein is furthermore evidenced under the previous T cell-independent activation conditions both by Western blotting (Fig. 1C) and by flow cytometry of cells stained intracellularly for AID (Fig. 1D, 1E). Although AID is minimally detected in lysates of cultures receiving signals from only BCR:CD21-L + IL-4, this is in large part a function of the diminished yield of viable progeny in the absence of BAFF (6). When viable-gated cells from these latter cultures are analyzed by flow cytometry, significant levels of AID are evident in the cycling subpopulation, albeit at levels slightly less than in similarly gated cells from cultures also containing BAFF. Under both activation conditions, AID is generally expressed most highly within the most extensively replicated lymphoblasts (representative experiments in Figs. 1D, 1E, 2A, 2B). Taken together, these findings indicate that, similarly to what we have observed with COX-2 (6), AID is preferentially expressed within cycling B cells and is most increased in B cell clones receiving ancillary signals from BAFF.

Interestingly, intracellular levels of the PGE2 synthase, mPGES-1, also increase with division (Fig. 2). The latter is one of three known PGE2 synthases that convert COX-2–generated PGH2 into PGE2; its expression and function have been strongly linked with COX-2 expression in other cell lineages (61, 62). Coinduction of COX-2 and mPGES-1 is likely responsible for the increased levels of PGE2 found in supernatants of cultures activated by BCR: CD21-L + IL-4 + BAFF (6). Although maximally expressed within dividing lymphoblasts, mPGES-1 was also evident within the activated nondividing population. The latter was not always the case with AID (Fig. 2A; data not shown).

The contemporaneous upregulation of COX-2, mPGES-1, and AID during B cell replication raised the possibility of a functional relation between AID and COX-2/mPGES-1. It was unlikely that a COX-2→PGE2 pathway was obligatory for AID expression, given that COX-2– and mPGES-1–deficient mice produce IgG Ab on immunization, albeit at a significantly reduced titer (8, 63). Nevertheless, it remained quite possible that a COX-2→PGE2 pathway exerted a modulatory role, as a cellular rheostat, to regulate AID levels or function, or both. The remainder of this study has sought to examine this hypothesis.

**Effect of COX-2 inhibitors on AID protein and mRNA expression within replicating human B lymphoblasts**

To test whether COX-2 function influenced AID expression, we examined the effects of selective pharmacologic inhibitors of COX-2. These studies generally used the most selective COX-2 inhibitor known, CAY10404 (64, 65), but additional COX-2 inhibitors such as celecoxib, SC58125, and NS398 were also used. Each was evaluated, together with DMSO vehicle control, for potential to reduce AID protein within activated human B cell clones. The doses used reduce the synthesis of PGE2 within activated lymphocyte cultures to below threshold levels (6, 65). The flow cytometric data in Fig. 3A show that viability-gated lymphoblasts, within cultures pulsed with CAY10404, contain substantially less AID than similarly gated cells from control cultures. The pooled data from several such analyses executed with CAY10404 and other COX-2 inhibitors (Fig. 3B) indicate that this decrease is statistically significant (p < 0.04).

The effects of CAY10404 COX-2 inhibitor were additionally examined on a pregerminal center B cell line (CL-01). Fig. 3C shows that CAY10404 suppresses the constitutive levels of AID in these transformed cells within 3 h of exposure. The rapidity of this decline is consistent with the known short half-life of AID mRNA (66) and suggests that a COX-2 pathway may influence AID mRNA transcription or stability. Fig. 3D–F further demonstrates...
that CAY10404 suppresses constitutive levels of AID protein within CL-01 cells. AID protein and mRNA expression were never fully eliminated by COX-2 inhibitors, possibly reflecting COX-2–independent mechanisms of triggering AID expression. Nevertheless, inhibitor instability, over the prolonged culture period needed for cycling, may also contribute. Alternatively, lymphoblasts within the large clumps typical of these activated cultures may have limited access to inhibitor. Finally, it is possible that the “constitutive” COX, COX-1, may compensate for the reduced function of COX-2. This possibility is heightened by the recent finding that COX-1 expression is upregulated by certain Ags and can, under these conditions, have significant effects on the formation of germinal centers and IgG Ab (67).

Effect of exogenous PGE2 supplementation on AID protein and mRNA expression within replicating human B lymphoblasts

In earlier studies, our laboratory showed that COX-2 inhibitors, which ablated the baseline levels of ∼10–50 nM PGE2 within activated B cell cultures, significantly reduced the Mcl-1 expression and viability of progeny (6). Furthermore, under conditions of limiting B cell density (∼10⁵ cells/well), supplementary PGE2 up to ∼500 nM augmented Mcl-1 levels, as well as the viability of highly divided progeny (6). At greater PGE2 concentrations, viability was diminished (6). In this study, we sought to determine whether exogenous PGE2 pulses could further increase stimulus-induced AID expression within activated human B cell cultures (Fig. 4).

These experiments (Fig. 4A, 4B) uniformly indicate that AID is upregulated within BCR:CD21-L + IL-4 ± BAFF-stimulated cultures receiving PGE2 pulses during clonal expansion (i.e., days 2 and 4). On average, supplementing the cultures with physiologically relevant doses of PGE2 (20–100 nM) augmented AID levels ∼2-fold within viable cells in stimulated cultures containing BAFF. The proportional increase was slightly greater in activated cultures without BAFF. The level of AID (dark line) or control (light line) fluorescence within the gated divided and undivided CFSE-labeled populations is shown in histogram form. AID was not detected in B cell cultures stimulated with low-dose BCR:CD21-L alone ± BAFF, by either flow cytometry or immunoblotting of lysates (data not shown).
mRNA transcription and/or stability, because significantly greater levels of AID mRNA were seen in cultures pulsed with PGE2 on day 2 + day 4 (Fig. 4E). These findings of PGE2-augmented AID are consistent with the diminished levels of AID mRNA and protein by the inhibition of an enzyme, COX-2, considered rate-limiting for PGE2 synthesis (48).

To eliminate the possibility that PGE2 might function in increasing B cell AID expression through modulating the function of a minor contaminating cell population, we subjected a population of routinely purified B2 cells to additional purification by sorting for low forward light scatter cells that coexpressed high levels of CD19 and CD22. Sorted and nonsorted B cell preparations were stimulated, with and without additional PGE2 pulses, and evaluated for AID expression. Exogenous PGE2 was found to increase AID levels in both populations (Supplemental Fig. 1). Thus, the AID-augmenting effects of exogenous PGE2 do not involve another cell type.

**FIGURE 2.** AID and the inducible PGE2 synthase, mPGES-1, both progressively increase as B cells undergo clonal expansion. A, CFSE-labeled cultures, stimulated with BCR:CD21-L + IL-4 + BAFF, were stained intracellularly for AID, mPGES-1 (dark lines), or the appropriate negative controls (light dotted lines). Histograms of AID or mPGES-1 fluorescence are shown for each division subset (cells gated for incrementally diminishing CFSE fluorescence). Values indicate the AID MFI (geometric MFI of AID or mPGES-1 above control background). The relative expression of AID and mPGES-1 within each division subset, in similarly activated cultures, is indicated for six and three experiments with AID and mPGES-1, respectively. Within each experiment, ΔMFI values were computed as percentage of the maximal expression observed. The plots represent the mean ± SEM of these standardized values. *p value indicating that the level of AID or mPGES-1 is significantly different from that noted within nondividing lymphoblasts within the same cultures (Student t test).

B cells are known to express each of the four classes of PGE2Rs, that is, intracellular Ca2+-augmenting EP1, cAMP-augmenting EP2 and EP4, and cAMP-suppressing EP3 (50). In an effort to define the PGE2R(s) responsible for positively modulating AID expression, we evaluated the expression of each as a function of division. The latter experiments were based on the premise that replicating cells with a greater sensitivity to the AID-augmenting effects of PGE2 would express greater levels of the relevant receptor. Specific Abs to EP1, EP2, EP3, and EP4 (46) were used for detection, in the presence or absence of the appropriate blocking peptide. An example of EP2 staining is shown in Fig. 5B. Staining for EP1, EP3, and EP4 with or without inhibitory peptide is shown in Supplemental Fig. 2. To facilitate the pooling of data from multiple such experiments, we expressed specific fluorescence of a given PGE2R within each divided cell subset as a percentage of that noted in undivided cells within the same cultures.

These experiments showed that EP2 was the PGE2R routinely upregulated with increasing division (Fig. 5A). The EP4 receptor also appeared to increase with division, although the latter increase was of only borderline significance. EP1 and EP3 were increased in all progeny, as compared with the undivided population, but their expression did not significantly change as cells progressed from one to four divisions.

To evaluate whether EP2 and AID were increased in a concordant manner within individual cells, we monitored the expression of these molecules in CFSE-labeled cultures by three-color flow cytometry. The dot blot in Fig. 5C (right) shows that 86% of the AID-expressing lymphoblasts coexpress EP2 and, furthermore, that there appears to be a direct relation between levels of AID and levels of EP2 in the coexpressing cells. Although nearly all AID+ cells coexpress EP2, a subpopulation of EP2+ cells negative for AID is consistently noted (four of four experiments). Figure 5D further demonstrates that within each division subgroup, cells with relatively high EP2 expression express high levels of AID, whereas cells with low EP2 expression exhibit low AID. Interestingly, within the infrequent undivided cells with high EP2, the levels of AID approximate those within extensively divided lymphoblasts expressing low EP2. The latter suggests that, although high EP2 expression is typically associated with division, EP2 may play a significant role in regulating AID expression, independently of division status.

To further validate the importance of EP2 in promoting PGE2-mediated upregulation of AID, we examined the modulatory effects of butaprost, a synthetic analog of PGE2 that is highly selective for the EP2 receptor (68). This agonist, which binds to EP2 with lower affinity than does PGE2, consistently upregulated AID protein at doses of 500–2000 nM (Fig. 5E, 5F). The greater dose of butaprost versus PGE2 required for increasing AID is consistent with reported EC50 values for human EP2 receptor signaling by each, that is, 5800 nM and 43 nM, respectively (44). Similar to findings with PGE2, B cells pulsed on days 2 and 4 with butaprost exhibited upregulated AID mRNA (data not shown). Thus, taken together, several findings strongly suggest that PGE2-augmented AID expression proceeds via an EP2-mediated signaling pathway.

**Effect of COX-2 inhibitor on the frequency of IgG+ switched progeny**

An increase in AID protein does not necessarily translate into heightened AID activity (69), because the function of the enzyme is highly controlled by its state of phosphorylation (70), among other factors. Given that a probe for readily detecting activated,
phosphorylated AID is not currently available, we sought to examine whether the COX-2/PGE2 pathway affected AID activity by assessing a downstream effect, that is, Ig class switching. Although most of the progeny from human B2 cells (IgM+, IgD+) stimulated by limiting BCR:CD21-L, IL-4, and BAFF exhibit high levels of membrane IgM (5), Ig class switching to IgG is routinely evident when sufficient numbers of progeny are evaluated by flow cytometry at days 5–7 of culture (Fig. 6A,6B). These findings are fully consistent with evidence of AID upregulation within the dividing lymphoblasts and past reports that T cell-independent Ags such as trinitrophenol-Ficoll can induce Ig class switching in murine B cells (71).

If COX-2 function contributed to AID expression, function, or both, one would anticipate that COX-2 inhibitors would reduce the above T cell-independent IgG switching process. In support of the above contention, when activated B cell cultures were pulsed with COX-2 inhibitors that ablate lymphocyte PGE2 production (6, 65), a substantially reduced yield of IgG+ class-switched cells was noted within the culture’s viable-gated cells (Fig. 6C). Complicating interpretations of these experiments is the fact that the proviability effects of PGE2 are preferentially observed in replicating blasts with two or more divisions (6). To better discriminate whether the consequences of inhibiting COX-2 represented a lesser frequency of the population most susceptible to class-switch recombination or a direct effect on the switching process, we also evaluated the inhibitors’ effect on the proportional representation of IgG+ cells within the viable blasts of two or more divisions. Evidence that the proportional representation of IgG+ cells within the latter subset was diminished by COX-2 inhibitor would suggest that IgG switching was affected independently of viability.

The data in Fig. 6C and 6D show that the proportional representation of IgG+ cells within viability-gated progeny does indeed decline in cultures exposed to COX-2 inhibitors. This is, at least in part, reversed when cultures are concomitantly pulsed with COX-2 inhibitor and exogenous PGE2 (Fig. 6E). Taken together, this pharmacologic approach shows that COX-2 promotes IgG class switching within BCR-triggered, innate immune system-driven B cell clones. Similar conclusions were reached in other studies with distinctly activated mouse and human B cell cultures with or without inhibitors of COX-2 (3, 8, 9).

**FIGURE 3.** COX-2 inhibitors diminish B cell AID expression in activated normal human B cells and a pregerminal center B cell line. A, CFSE-labeled cultures, stimulated as in Fig. 2, were pulsed with a highly selective COX-2 inhibitor, CAY10404 (50 or 25 μM) or DMSO vehicle control, as indicated. On day 5, cells were harvested and assessed for AID expression within the viable-gated subpopulation. Values represent the Δ MFI of anti-AID staining (dark lines) above the isotype control (light dotted lines) for each division-gated cell subpopulation. B, Pooled data from nine experiments in which several COX-2 inhibitors were individually tested as above. These include CAY10404, SC58125, NS398, and celecoxib (range of 25–200 μM). AID expression values were standardized as described in Fig. 2 and shown as mean ± SEM. *p values of significance; one-tailed Student t test, nonpaired. C–E, AID expression in the pregerminal center B cell line, CL-01, is suppressed by 16-h incubation with CAY10404 COX-2 inhibitor. C, AID mRNA in the CL-01 pregerminal center B cell line is suppressed by CAY10404 (25 μM) within 3 h of exposure. mRNA was isolated at various intervals after culture with COX-2 inhibitor or DMSO vehicle control. AID was quantified by qPCR, and results are expressed as fold change compared with respective cultures with vehicle (results shown representative of two performed experiments). D, AID-specific fluorescence (right dark line) versus IgG control fluorescence (left line) in viable-gated cells from cultures treated as indicated. E, AID-specific fluorescence after 16 h culture with varying concentrations of CAY10404. F, Western blotting of AID (top) and actin (bottom) in 16-h lysates from CL-01 cultures incubated with varying doses of CAY10404 or DMSO vehicle control.
Effect of exogenous PGE\(_2\) on the frequency of IgG\(^+\) switched progeny

In Fig. 7, we further examine whether supplementary doses of PGE\(_2\), which significantly augment AID expression in replicating B lymphoblasts, can increase IgG-switching to more than baseline levels. Fig. 7A shows the effects of a range of PGE\(_2\) concentrations on 1) absolute yield of viable lymphoblasts with three or more divisions (top left plot with gray circles) and 2) absolute yield of viable IgG\(^+\) blasts with three or more divisions (bottom left plot with black triangles) in three pooled experiments. The yields from each experiment were calculated as a percentage of the maximal AID expression observed in respective cultures without the exogenous PGE\(_2\) pulse. Shown are the mean ± SEM of these standardized values from multiple experiments. The \(p\) values are from Student nonpaired, one-tailed \(t\) test.

Dose–response analysis of exogenous PGE\(_2\) effects on intracellular AID within replicating blasts in cultures stimulated with BCR:CD21-L + IL-4 + BAFF (pool of four independent experiments). Asterisks indicate values significantly different from AID expression in each respective division in cells without PGE\(_2\) pulse (\(p < 0.05\)).

Western blots of AID within culture lysates of stimulated cultures plus or minus exogenous PGE\(_2\) (50 nM on day 2 [d2] + day 4 [d4]). mRNA was assessed by real-time qPCR (q-PCR) as in Fig. 1. Values shown represent the fold difference (\(\Delta\)) of \(\Delta\) CT values obtained in activated cultures receiving no exogenous PGE\(_2\) pulse. The mean ± SEM for three separate experiments is shown (\(p\) value shows that values for PGE\(_2\)-pulsed cultures are significantly different from values for nonpulsed cultures).
evidenced with doses as low as 0.1 nM (Fig. 7A, top left). The majority of these are IgM+ (data not shown). These cultures also exhibit a significant increase in the absolute yield of viable IgG+ lymphoblasts (Fig. 7A, bottom left).

As in the experiments with COX-2 inhibitor, it is important to assess how exogenous PGE2 affects the proportional representation of IgG+ cells within viable, highly divided progeny. Evidence in support of the latter would suggest that PGE2 affects the IgG switch independently of its effects on viability. The values for percentage of viable gated progeny undergoing an IgG switch are shown in standardized form in Fig. 7A (right panels). Somewhat surprisingly, in two of the three experiments, exogenous PGE2 at doses of 100–1000 nM decreased the proportion of viable progeny expressing membrane IgG. In the one experiment in which...
IgA was examined, cultures receiving PGE2 also showed a minor decline in the proportion of viable progeny expressing the latter. In all experiments, the proportion of highly divided blasts with IgMlow-neg expression was significantly decreased on PGE2 supplementation. In multiple more experiments, the proportion of IgG+, IgA+, and IgMlow-neg viable progeny in cultures supplemented (or not) with a single dose of PGE2 (100 nM) was evaluated. These data are shown in Fig. 7B. Similar to earlier results with the full PGE2 dose range, Supplementary PGE2 reduced the frequency of class-switched cells (changes were either statistically significant \( p = 0.03 \) for IgMlow-neg) or borderline significant \( p = 0.06 \) for IgG+ and 0.20 for IgA+).

A trend of potential importance emerges on plotting the ratio of percentage IgG+ cells in PGE2-pulsed versus PGE2-nonpulsed cultures as a function of the baseline expression of IgG+ blasts in cultures within the categories listed. Both the yield of viable, highly divided progeny and the proportional yield of IgG+ cells within the latter subset are reduced in COX-2 inhibitor-pulsed cultures. D. Pooled results from four experiments in which COX-2 inhibitor was pulsed into BCR:CD21-L + IL4 + BAFF-activated cultures; CAY10404 (25 \( \mu \)M) was pulsed on days 2 and 4 (two experiments); at days 0, 3, and 5 (one experiment); and at day 3 (one experiment). Cultures harvested on days 6–7. E. Exogenous PGE2 can reverse the effect of COX-2 inhibitor on yield of switched progeny. B cell cultures, activated as above, were pulsed on days 2 and 4 with CAY10404 (30 \( \mu \)M) or vehicle control \( \pm \) PGE2 (50 nM). Absolute cell yield was determined by adding a known number of standardization beads before harvesting, staining, and FACS analysis. Bar graph (left) shows the absolute yield of IgG+ cells with two to five divisions in cultures receiving the indicated day 2–4 treatments. Bar graph (right) shows the proportion of IgG+ cells within the recovered viable cells with two to five divisions. Values shown represent the percentage inhibition of each parameter, on adding COX-2 inhibitor (data representative of two experiments).

IgA was examined, cultures receiving PGE2 also showed a minor decline in the proportion of viable progeny expressing the latter. In all experiments, the proportion of highly divided blasts with IgMlow-neg expression was significantly decreased on PGE2 supplementation. In multiple more experiments, the proportion of IgG+, IgA+, and IgMlow-neg viable progeny in cultures supplemented (or not) with a single dose of PGE2 (100 nM) was evaluated. These data are shown in Fig. 7B. Similar to earlier results with the full PGE2 dose range, Supplementary PGE2 reduced the frequency of class-switched cells (changes were either statistically significant \( p = 0.03 \) for IgMlow-neg) or borderline significant \( p = 0.06 \) for IgG+ and 0.20 for IgA+).

A trend of potential importance emerges on plotting the ratio of percentage IgG+ cells in PGE2-pulsed versus PGE2-nonpulsed cultures as a function of the baseline expression of IgG+ blasts in nonpulsed cultures (Fig. 7C). This comparison reveals an apparent inverse relation between the class switch-augmenting effects of PGE2 and the baseline incidence of IgG+ cells within activated cultures. Although a larger sample size is needed for confirmation, it appears that cultures with significant AID activity (as reflected by IgG+ cells) are more prone to PGE2-mediated suppression of IgG+ responses. Conversely, cultures with less AID activity are more likely to manifest an increase in the percentage of IgG+ cells on PGE2 supplementation.

Two possibilities were considered, tested, and excluded for the PGE2-mediated decline in the proportion of divided IgG+ lymphoblasts. First, sequential switching to downstream isotypes unlikely explains the decline. The frequency of IgA+ cells did not increase, and in many cases was reduced, by PGE2 supplementation (Fig. 7A, 7B). Furthermore, surface IgE was assessed in three experiments and found to be negligible (data not shown). Second, the PGE2-mediated decline in the proportion IgG+ cells is not due to plasma cell differentiation. The pooled data presented in Fig. 7D clearly show that Ab secretion is reduced, rather than augmented, by supplementing activated cultures with PGE2. Within each experiment, suppressed supernatant Ab levels were noted.
both for IgM and IgG. When evaluated, CD138+ (syndecan 1) plasma cells were not evident, either in the presence or absence of exogenous PGE2 (5) (data not shown). Thus, plasma cell formation is not evident under these activation conditions and the minor amount of Ab produced, likely from CD138+ plasmablasts, is reduced rather than augmented by the addition of exogenous PGE2. A suppressive effect of PGE2 on Ab secretion has been previously noted under some, but not all, activation conditions (72, 73).

**PGE2 upregulates levels of phospho-ATM (Ser$^{1981}$) in cycling human B cells**

A further possibility for the reduced proportion of class-switched progeny in PGE2-supplemented cultures is that the augmented expression/function of AID leads to excessive DNA damage and ensuing cell death. Although, as noted earlier, PGE2 can have prosurvival effects on replicating lymphoblasts, these prosurvival effects may predominate in blasts with more limited AID activity. As one means of testing the hypothesis that increased AID leads to DNA damage-induced death, we examined whether PGE2-pulsed cultures show greater levels of phospho-ATM protein kinase. ATM is an enzyme that rapidly becomes activated, after DNA double-strand breaks, by autophosphorylation at Ser$^{1981}$ (74). The kinase’s activation leads to stabilization of p53 and other damage-response proteins that either induce cell cycle arrest and repair or, alternatively, apoptosis (74).

Expression of phospho-ATM was examined by subjecting PGE2-pulsed and nonpulsed CFSE-labeled cultures to intracellular staining with a flow-validated specific mAb for phospho-ATM at Ser$^{1981}$. As seen by the data in Fig. 8A and 8B, cells from both BCR:CD21-L + IL-4–activated cultures and BCR:CD21-L + IL-4 + BAFF-activated cultures exhibit notable baseline expression of phospho-ATM. Maximal levels were achieved in the divided B cell subsets, particularly in BAFF-containing cultures. Exposure to relatively high concentrations of exogenous PGE2 (100–1000 nM) consistently increased phospho-ATM levels, under both culture conditions. Within cultures activated by BCR:CD21-L + IL-4 (no BAFF), smaller doses of pulsed PGE2 had a notable effect. Interestingly, PGE2 augmentation of phospho-ATM preferentially occurred within the dividing blasts. Undivided cells were little, if at all, affected by exposure to exogenous PGE2.
We suspect that the increases in phospho-ATM after PGE2 pulsing primarily reflect a greater level of DNA double-strand breaks, although ATM can also be activated as a result of replication-associated nicking of DNA, as well as other perturbations (74). This interpretation is consistent with this study’s findings that exogenous PGE2 significantly augments the expression of AID, an enzyme known to promote the formation of DNA double-strand breaks. It is also consistent with recent evidence that AID contributes to DNA damage-dependent death of activated mouse B cells (75).

A schematic illustrating a possible mechanism whereby the COX-2/PGE2 axis augments AID levels within dividing B lymphoblasts is presented in Fig. 9.

**Discussion**

This study presents evidence that B cell expression of AID, an enzyme critical for the formation of high-affinity, isotype-switched memory and the source of occasional oncogenic mutations, is both temporally and functionally linked to molecules of the COX-2 → PGE2 pathway. Evidence for a temporal link comes from finding that quiescent human B2 cells, on receiving stimuli found within inflamed peripheral tissues, undergo clonal expansion characterized by the coordinate and progressive upregulation of AID and several COX-2 pathway proteins. These include: 1) COX-2, which converts arachidonic acid to PGH2 (6); 2) mPGES-1, an inducible PGE2 synthase using PGH2 as a substrate (62); and 3) the EP2 receptor for PGE2 (68). The presence of a PGE2 synthase was previously inferred from recent evidence that activated B cells produce significant amounts of PGE2 (3, 6). Nevertheless, three such synthases have been identified, cPGES, mPGES-1, and mPGES-2 (62), and until now it was unclear which was expressed in activated B cells. Although this study does not exclude a role for cPGES and mPGES-2, the fact that mPGES-1 and COX-2 are closely linked in multiple cell lineages (62) strongly suggests that mPGES-1 has the major role in PGE2 production by activated B cells. Previous studies have reported EP2 receptor expression in both murine and human B lymphocytes and, furthermore, have linked it to IgE production (46). We believe it is significant that, during the first few cycles of a replicative burst, B cells upregulate two inducible enzymes for autocrine PGE2 synthesis, that is, COX-2 and mPGES-1, and this functionally important PGE2R. As is shown by this study, such a division-linked, autochthonous pathway appears to ensure that AID reaches sufficiently high levels for activity as the burst of replication progresses (Fig. 9 schematic).

The mechanism(s) responsible for the concordant upregulation of COX-2, mPGES-1, EP2, and AID within dividing cells, and in particular the nearly synchronous upregulation of EP2 and AID within progressive divisions, will require further study. Nevertheless, we consider it pertinent that COX-2, mPGES-1, and EP2 are each subject to a feed-forward process by PGE2 in other cell lineages. Thus, PGE2 synthesized from inducible COX-2 and mPGES-1 initiates signals that further increase mRNA/protein levels of COX-2, mPGES-1, and EP2 (48–52). In support of such a feed-forward process in B cells is preliminary evidence that pulsing cultures with PGE2 does augment COX-2 and EP2 mRNA and/or protein. Furthermore, COX-2 inhibitor suppresses B cell mPGES-1 and EP2 expression (P. Mongini, H. Lee, and S. Haque, unpublished observations). It is reasonable to speculate that AID may be subject to the same PGE2-mediated mechanism(s) for amplification of COX-2 pathway proteins, leading to a progressive increase of not only the latter, but also AID, with each successive division (Fig. 9). This might
COX-2 and COX-1) (80). Moreover, these findings may provide an additional mechanism for the augmented switching to IgG and IgE in LPS + IL-4–stimulated mouse B cell cultures supplemented with PGE2 (10, 11, 81) and the reduced isotype switching previously noted in immunized COX-2–deficient and mPGES-1–deficient mice (8, 9, 63).

Although it influences AID levels, COX-2 is not essential for AID expression and function. This is indicated by the presence of IgG Ab, albeit at lower levels, in immunized COX-2–deficient mice (8, 9) and by the inability of COX-2 inhibitors to fully abrogate AID mRNA and protein expression in this study. Nevertheless, it remains unclear whether PGE2, or a related prostanoid acting through similar G-protein–coupled receptors, might play a critical role. Importantly, a recent study has demonstrated that the IgG Ab response to Borrelia Ag is significantly impaired in mice deficient in COX-1 (67). The latter PGH2–producing COX has been generally considered constitutive and noninducible in most body cells. Nevertheless, mouse B cells exposed to Borrelia exhibited a significant increase in COX-1 mRNA and protein, as well as COX-1–dependent downstream prostanooids (67). A possibility is that both COX-2 and COX-1 participate in generating the rate-limiting PGH2 substrate needed for downstream regulation of AID expression/activity by arachidonic acid-derived prostanooids. Further studies are needed to clarify these issues.

The precise mechanism whereby a PGE2–induced EP2 signaling pathway augments AID mRNA and protein levels is unknown, but might be speculated. Two intracellular enzymes known to be downstream of PGE2→EP2 signaling, PKA (82) and p38 MAPK (83–86), have a prior demonstrated role in promoting AID mRNA expression. Thus, PKA and downstream CREB were reported to promote IL-4–driven, STAT6–dependent AID mRNA expression. This, PKA and downstream CREB were reported to promote IL-4–driven, STAT6–dependent AID mRNA expression. However, p38 MAPK was found necessary for BAFF synergy with IL-4 in promoting AID mRNA synthesis in human B cells (88). Interestingly, p38 MAPK can affect the activation, function, and/or expression of NFκB, STAT6, and E2A (36, 83, 86, 89–93)—three molecules with important roles in regulating AID expression within B cells (86, 89). Consistent with a role for PKA and p38 MAPK in PGE2–augmented AID expression, inhibitors of PKA or p38 MAPK blocked PGE2–augmented AID protein in our activated B cell cultures (P. Mongini, unpublished results). In addition to augmenting transcription factor availability/function, it remains possible that PGE2–induced pathways might also function in stabilizing AID protein. AID is highly susceptible to proteosomal degradation (95), and PGE2 can dampen the ubiquitination of at least some cellular proteins, for example, survivin and likely Mcl-1 (96, 97).

During the progress of this study, we unexpectedly discovered that, although the absolute yield of viable IgG+ progeny increased after exogenous PGE2 pulsing, the proportional representation of mIgG+ cells within the viable highly divided population declined. This occurred in a manner directly related to PGE2 dose. In investigating the basis for this phenomenon, two possible mechanisms were excluded: PGE2–promoted switching to downstream isotypes and PGE2–promoted plasma cell differentiation. A third possibility, that PGE2–augmented AID expression/function caused excessive DNA damage and AID-dependent activation-induced cell death, appears more likely. Consistent with this point, cells exposed to exogenous PGE2 pulses manifest greater levels of phospho-ATM (Ser1981)—an indicator of DNA double-strand breaks. Furthermore, significantly less activation-induced death of progeny (5) was noted in AID-deficient mouse B cells, as compared with wild type mouse B cells, after stimulation with BCR-L (anti-IgM/dextran) + IL-4 ≥ BAFF (H. Lee and P. Mongini, unpublished results).
Finally, in another recent study, AID was demonstrated to contribute to the in vivo and in vitro activation-induced death of activated mouse B cell clones, via a DNA damage-induced mechanism (75). Thus, although further mechanisms might also contribute to the diminished proportion of IgG2a cells within the progeny of PGE2-pulsed cultures, loss of cells with excessive AID expression/activity is likely to be in part responsible.

The fact that PGE2 can initiate signals that support the viability of replicating human B cells (6) might appear to contradict the possibility that PGE2 promotes AID-dependent, activation-induced death. Nevertheless, this would not be the first example that the same signal inducer can elicit positive or negative outcomes during a B cell immune response. PGE2 is intrinsically more complex than most stimuli, given B cell expression of four PGE2Rs, each with distinct downstream properties. Furthermore, emerging studies in other lineages indicate that at least some of these receptors can be compartmentalized to both the cytoplasmic and nuclear membranes, and together can modulate levels of several critical intracellular mediators: cAMP, Ca2+, Akt, p38, and ERK MAPK. It is not unreasonable to suspect that PGE2-promoted survival pathways in replicating B lymphoblasts may have evolved in an effort to counteract the deleterious effects of replication-associated stresses, including AID-induced DNA damage.

This study’s major finding that a COX-2/PGE2 pathway contributes to increased AID expression in B cells has significant implications for therapy. Although it provides further support for the contention of Phipps and colleagues (8, 9) that COX-2 inhibitors are part responsible.

loss of cells with excessive AID expression/activity is likely to be in part responsible.

References
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