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Activated Human B Lymphocytes Express Cyclooxygenase-2 and Cyclooxygenase Inhibitors Attenuate Antibody Production[1]

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammatory diseases and target cyclooxygenases 1 and 2 (Cox-1, Cox-2) that are responsible for PG production. Newer Cox-2-selective drugs have been heavily prescribed to quench inflammation. Little is known about whether or not these drugs influence human B lymphocytes and their ability to produce Ab. We report herein that activated human B cells not only highly express Cox-2 and produce PGs, but that the NSAID indomethacin and Cox-2-selective drugs profoundly inhibit the ability of human B cells to produce IgG and IgM in vitro. Human blood B cells highly express Cox-2 mRNA and protein and produce PGs after activation with CD40L, pansorbin, or CD40L plus BCR engagement. Cox-2 is also highly expressed by human tonsil B cells, as shown by immunohistochemistry. Cox-inhibiting drugs modestly affect purified B cell proliferation but profoundly reduce Ab production. The ability of whole blood to produce IgM and IgG following stimulation is also strongly inhibited. In support that Cox-2 plays a seminal role in B lymphocyte Ab production, Cox-2 knockout mice have 64% less IgM and 35% less IgG than normal littermate controls. These findings support that NSAIDs and the new Cox-2-selective drugs have an unsuspected target, the B cell, and attenuate Ab production in humans. Use of NSAIDs may therefore influence autoantibody production in autoimmune diseases and may dampen humoral immunity in response to antigenic challenge/vaccination. ***The Journal of Immunology,*** **2005, 174: 2619–2626.**

Prostaglandins are critical mediators of inflammation that affect both humoral and cell-mediated immune responses. For example, PGE_2 enhances Ab production and promotes type 2 immune responses (1, 2). PGE_2 has been shown to directly promote Ig class switching in B cells acting through the EP2 and EP4 PGE_2 receptors (3). Although it has been well established that PGs derived from accessory cells, such as macrophages and fibroblasts, signal in a paracrine manner to B cells, what has not been known was whether human B cells could synthesize PGs and thus signal in an autocrine manner. The recent finding that activated T cells express cyclooxygenase-2 (Cox-2)[3] (4), an inducible enzyme that catalyzes a series of reactions to generate PGs, led us to hypothesize that human B cells express Cox-2 and therefore synthesize PGs upon activation. Indeed, this hypothesis is supported by our previous findings that proinflammatory signals increase Cox-2 expression and PG production in a mouse biphenotypic B/macrophage cell type (5).

PG endoperoxide synthase, also referred to as Cox, catalyze a Cox (bis-oxygenase) reaction in which arachidonic acid is converted to PGG_2, and a subsequent peroxidase reaction in which PGG_2 undergoes a two-electron reduction to PGH_2, the common precursor to all prostanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxane. Two forms of Cox are known: a constitutive type (Cox-1), present in most cells and tissues, supporting prostanoid synthesis required for tissue homeostasis, and an inducible isoenzyme (Cox-2) expressed in response to cytokines, growth factors, and stressors such as hypoxia and free radicals (6). Cox-2 is the predominant isoform contributing to high levels of PGE_2 found in chronic inflammatory conditions. Interestingly, elevated Cox-2 levels have been reported in autoimmune diseases, such as systemic lupus erythematosus, where chronic inflammation persists at multiple sites in the body. This explains the clinical utility of highly selective Cox-2 inhibitors such as celecoxib (Celebrex) and rofecoxib (Vioxx) to reduce the pain associated with inflammation (7).

In the study reported herein, we demonstrate that human B cells strongly express Cox-2 mRNA and protein and produce PGs upon activation. In addition, nonsteroidal anti-inflammatory drugs (NSAIDs) (Cox-1/Cox-2 inhibitors) and the highly selective Cox-2 drugs markedly reduce Ab production. These studies suggest a novel autoregulatory pathway for B cell activation. In addition, such studies suggest that patients taking NSAIDs or selective Cox-2 inhibitors may have suboptimal Ab responses to vaccination.

Materials and Methods

Culture conditions and reagents

Normal human B lymphocytes were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 5×10^5 M 2-ME, 10 mM HEPES, 2 mM L-glutamine, and 50 μg/ml gentamicin. B cells were

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[3] Abbreviations used in this paper: Cox, cyclooxygenase; EIA, enzyme immunoassay; NSAID, nonsteroidal anti-inflammatory drug.

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stimulated with nothing or with recombinant human CD40L (8), pansorbin (*Staphylococcus aureus* Cowen I strain; Sigma-Aldrich), or rabbit anti-human F(ab′)$_2$ anti-IgM Ab (Jackson ImmunoResearch Laboratories). Arachidonic acid, (Nu-Chek Prep) dissolved in 100% ethanol, was diluted to working concentrations in culture medium.

Small molecule Cox inhibitors

Indomethacin, a Cox-1/Cox-2 inhibitor, was purchased from Sigma-Aldrich. SC-58125, a highly selective Cox-2 inhibitor, was purchased from Cayman Chemical. Experiments were also performed using NS-398, a highly selective Cox-2 inhibitor purchased from Cayman Chemical (data not shown). Results were very similar to that obtained with SC-58125. Stock concentrations (10 mM) of indomethacin dissolved in 95% ethanol were made fresh each day and diluted to working concentrations in culture medium. SC-58125 and NS-398 were dissolved in DMSO (10 mM stock stored at −20°C) and diluted to working concentrations in culture medium. Pharmacological doses of these drugs that have been previously shown to block PG production range from 5 to 40 μM (9, 10). Dose response experiments (1, 5, 10, 20, 40, and 80 μM) were conducted to determine the optimal dose for PG inhibition using the supplier's protocol for the PG screening kit (Cayman Chemical).

B lymphocyte preparation

Normal human B lymphocytes were isolated from peripheral blood obtained from healthy donors at the University of Rochester. Ethical permission for blood collection was obtained from the Research Subjects Review Board at the University of Rochester. The isolation of highly purified B lymphocytes has been previously described (11). In brief, buffy coats were separated, and samples were diluted with an equal volume of 1× PBS. PBMCs were separated by Ficoll-Paque (Amersham Biosciences AB) gradient centrifugation. Leukocyte layers were pooled to single tubes and washed with PBS. CD19 Dynabeads (Dynal Biotech) were added to the PBMCs and placed on an orbital shaker at 4°C for 30 min. Then CD19 Dynabead cell rosettes were captured on a magnet (Dynal Biotech). The bead cell rosettes were resuspended in RPMI 1640 medium and transferred to a 2-ml tube. Of the volume used for CD19 Dynabeads, 25% was the volume of Detachabeads added to the rosettes. Detachabead CD19 (Dynal Biotech) is a polyclonal anti-F(ab′)$_2$ to release cells from Dynabeads CD19. The Detachabeads with rosettes were placed on an orbital shaker for 1 h at 4°C. The CD19-positive cells were washed and counted. Viability was determined using the trypan blue exclusion method. B lymphocytes isolated in this manner are >98% surface CD19 positive and <2% CD3 or CD14 positive (as determined by flow cytometry).

Immunofluorescence

To determine the percentage of B lymphocytes expressing Cox-1 and Cox-2, whole blood or freshly isolated B cells were incubated in the presence or absence of CD40L plus 10 μg/ml anti-IgM Ab for 24 h. RBC were lysed using 1× FACS lysing solution (BD Biosciences) before staining with Abs. Whole blood or purified B lymphocytes were stained with mouse anti-human CD19 APC (BD Biosciences) in cold PBS buffer with sodium azide (0.02%) and BSA (0.3%) for 20 min at 20°C. Intracellular staining was performed with a fixation and permeabilization kit (Caltag Laboratories) using the supplier's protocol. Intracellular Cox-1 and Cox-2 proteins were detected by PE-labeled mouse anti-human Cox-1 Ab (Cayman Chemical) and FITC-labeled mouse anti-human Cox-2 Ab (Cayman Chemical). The percentage of CD19-positive B cells expressing Cox-1 and Cox-2 was determined by flow cytometric analysis using Cell Quest software on a FACSCalibur flow cytometer (BD Biosciences).

Immunocytochemistry

CD40L plus anti-IgM (5×10^4)-activated B cells were cytospun onto glass microscope slides and stained for surface CD19 and intracellular Cox-2 expression as described above for immunofluorescence. Images were visualized on an Olympus BX51 system microscope, and photographs were taken using SPOT camera with SPOT RT software (SPOT Diagnostic Instruments).

Immunohistochemistry

Paraffin-embedded tonsil tissue sections (4 μm) were obtained from Department of Pathology and Laboratory Medicine at the University of Rochester. Slides were processed for immunohistochemistry. In the primary Ab reaction step, slides were incubated with the monoclonal mouse anti-human CD20 Ab (Zymed Laboratories) or the polyclonal mouse anti-human Cox-2 Ab (Cayman Chemical). Multiple tonsil tissue sections were stained. For positive controls, sections of prostate tissue expressing the Cox-2 protein were included in each staining procedure. Images were visualized on an Olympus BX51 system microscope, and photographs were taken using SPOT camera with SPOT RT software.

Real time RT-PCR

Total RNA was extracted using Qiagen RNAeasy mini kit according to the supplier's protocol. RNA was measured on a Bio-Rad SmartSpec 3000. RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus RT primed by an oligo(dT) primer. PCRs for human Cox-1, Cox-2, and GAPDH were performed using Platinum *Taq*DNA polymerase (Invitrogen). Human Cox-1 sequences were 5′-TGGAGACAATCTGGAGC GTCA-3′ and 5′-GGAACTGGACACCGAACAG-3′. Human Cox-2 sequences were 5′-TCACAGGCTTCCATTGACCAG-3′ and 5′-CCGAG GCTTTTCTACCAGA-3′. Human GAPDH sequences were 5′-ACCAC AGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. Semiquantitative real time RT-PCR using CYBERGREEN master mix as the fluorescent DNA intercalating agent was analyzed using Bio-Rad Icycler Software. DNA products were electrophoresed on a 1.2% agarose gel. The identities of these products were confirmed by automated ABI sequencing.

SDS-PAGE/Western blotting

Total cellular protein was harvested from rigorously purified B lymphocytes immediately following isolation or after culture for 24 h. B cells were lysed in protein isolation buffer (1% IGEPAL, 150 mM sodium chloride, 50 mM Tris, 10% protease inhibitor mixture) and quantified by bicinchoninic acid protein assay kit (Pierce). Thirty micrograms of protein was fractionated by 7.5% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. After blocking in 10% nonfat powdered milk in 1× PBS/0.1% Tween 20, membranes were incubated with either a primary mouse anti-human Cox-2 (Cayman Chemical), mouse anti-human Cox-1 (Cayman Chemical), or a mouse anti-actin (Oncogene Research Products) Ab for 2 h at 20°C. After washing in PBST buffer, membranes were incubated with secondary goat anti-mouse-HRP conjugated Ab (Jackson ImmunoResearch Laboratories) for 1 h at 20°C. Bands were visualized with ECL (Amersham Biosciences) and autoradiography film according to the manufacturer's protocol.

Measurement of PG production

Triplicate samples of B lymphocyte cell culture supernatants were analyzed by highly specific competitive enzyme immunoassays (EIA) (Cayman Chemical) to determine the concentration of PGE_2 and $PGF_{2\alpha}$ after 36 h of incubation.

Proliferation assays

B lymphocytes were cultured in flat-bottom 96-well plates. Cells were incubated for 48 h to 5 days in triplicate at 4×10^5 cells/ml. Arachidonic acid (5 μM) was added to cells that were stimulated with CD40L plus anti-IgM Ab. Cells were treated with nonselective Cox-1/Cox-2 and highly selective Cox-2 inhibitors and compared with untreated (vehicle) cells as a control. Plates were harvested after 2, 3, 4, and 5 days, and incorporation of [^{3}H]thymidine was determined for a 24-h period by scintillation spectroscopy.

Mitochondrial membrane permeability

One million B lymphocytes were incubated in duplicate with CD40L plus anti-IgM Ab and treated with vehicle (DMSO or ethanol) or drug (up to 20 μM indomethacin or 20 μM SC-58125). After 48 h, 40 nM 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (3) (Molecular Probes) was diluted in culture medium and added to the cells for 15 min. Cells were harvested, washed in 1× PBS, and immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cells with intact mitochondrial membrane potential incorporate $DiOC_6$ into the mitochondria.

Ig production assays

Highly purified B lymphocytes (5×10^5 cells/ml) were cultured in RPMI 1640 (10% FBS) medium for 7 days in 96-well flat-bottom microtiter plates. After 7 days of treatment with activating agents and 5 μM arachidonic acid, in the presence and absence of Cox-1/Cox-2 inhibitors, the supernatants were harvested and tested for their concentrations of IgM and IgG by using human-specific ELISAs (Bethyl Laboratories). Whole blood (2 ml) was washed two times with 1× PBS and resuspended in 6 ml of IMDM (Invitrogen Life Technologies). Diluted blood (1 ml) was cultured for 7 days with or without activating agents in the presence or absence of Cox-1/Cox-2 inhibitors (12). Supernatants were harvested and tested for IgM and IgG levels as described above for highly purified B cells.

Ptgs2-knockout (Cox-2 deficient) mouse studies

Cox-2-deficient (B6.129P2-Ptgs2^{tm1Smi}) mice (8- to 12-wk-old) and their wild-type barrier colony controls were purchased from Taconic Farms. The Animal Care and Use Committee of the University of Rochester approved all mouse protocols. Mice were anesthetized with sodium pentobarbital (60 mg/kg). Eye bleeds were performed to harvest peripheral blood in heparin-coated hematocrit tubes. Whole blood was centrifuged, and plasma was isolated for analysis of Ig levels by mouse-specific ELISAs (Bethyl Laboratories). Mouse B lymphocytes were isolated as previously described (13). In brief, normal mouse splenic B lymphocytes were isolated from wild-type and Cox-2-deficient mice. Erythrocytes were lysed with ammonium chloride buffer, and the suspension was depleted of adherent cells by two successive rounds of adherence on Falcon tissue culture dishes (BD Biosciences) at 37°C for 2 h. Nonadherent cells were collected by rinsing the plates with warm medium. T cells were depleted with anti-T cell mixture consisting of Abs 30-H12 (anti-Thy 1.2), 3.155 (anti-CD8), and GK 1.5 (anti-CD4) and low toxicity baby rabbit complement (Cedarlane Laboratories) followed by incubation at 37°C. The cells were washed and counted, and viability was determined using the trypan blue exclusion method. B lymphocytes isolated in this manner are >97% surface B220 positive.

Results

Activation of human B lymphocytes up-regulates Cox-2 protein expression

The expression of Cox-1 and Cox-2 on healthy donor human B lymphocytes from whole blood (defined by their expression of CD19) was determined by a sensitive and rapid flow cytometric assay (14). We first used this technique to evaluate the expression of Cox-1 and Cox-2 in unstimulated whole human blood B cells. One hundred percent of the CD19$^+$ gated cells from freshly isolated whole blood expressed Cox-1, although <2% expressed Cox-2 (Fig. 1*A*). In contrast, after 24 h ex vivo stimulation with CD40L plus anti-IgM Ab at 37°C, 96% of the CD19$^+$ gated cells expressed Cox-2. The expression of Cox-1 was no different between the unstimulated and stimulated B lymphocytes.

Encouraged by the findings of constitutive Cox-1 and inducible Cox-2 expression on human B lymphocytes, we next studied Cox-2 expression on purified B cells. Freshly isolated B lymphocytes, as well as B lymphocytes stimulated in vitro for 24 h (with CD40L plus anti-IgM Ab), were stained for surface CD19 expression and for intracellular Cox-1 and Cox-2 expression (Fig. 1*B*). Density plot analysis indicates that there was no change in the percentage of Cox-1-positive cells before and after stimulation. In contrast, only 3% of freshly isolated B cells expressed Cox-2, whereas after 24 h of in vitro stimulation, >95% of activated B cells were Cox-2 positive (Fig. 1*B*). In Fig. 1*C*, cytospin preparations of highly purified activated B cells were stained for surface CD19 (red) and intracellular Cox-2 expression (green). Dual-color fluorescence microscopy was next used to confirm that activated B cells were in fact positive for Cox-2. CD19$^+$ B cells expressing Cox-2 are shown as red and green cells.

Since in vitro activated B cells expressed Cox-2, we next investigated whether there was a population of activated B cells that

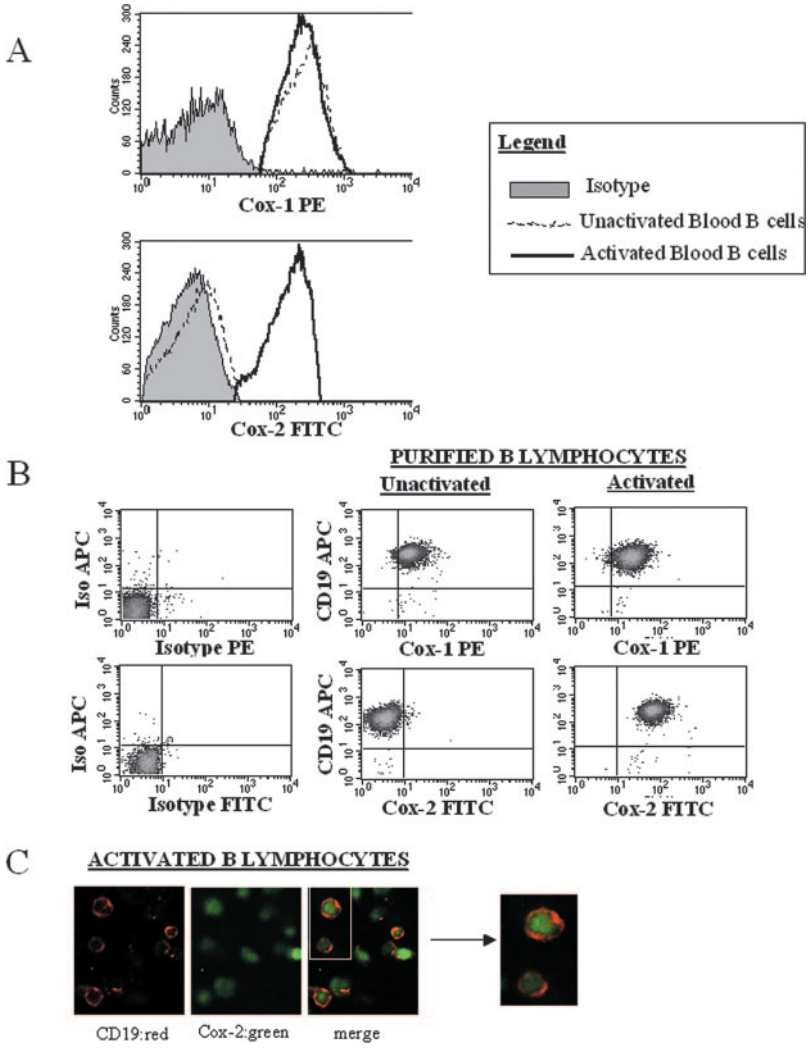

FIGURE 1. Human peripheral B lymphocytes express Cox-1 constitutively and can be induced to express Cox-2. *A*, Freshly isolated and activated whole blood was analyzed for intracellular Cox-1 and Cox-2 expression by flow cytometry. Histogram plot shows Cox-1 (*top*) and Cox-2 (*bottom*) expression by CD19$^+$-gated B cells in whole blood. The shaded area represents staining with isotype Ab. The dashed line indicates that CD19-gated B lymphocytes in fresh blood express Cox-1, and the solid line indicates that there is no change in the percentage of Cox-1-positive B cells following activation with CD40L plus 10 μg/ml anti-IgM Ab for 24 h. CD19-gated B cells (<2%) express Cox-2 in fresh blood (dashed line) whereas CD19-positive B lymphocytes (>95%) express Cox-2 following activation with CD40L plus 10 μg/ml anti-IgM Ab for 24 h (solid line). Similar results were obtained when the experiment was repeated with four different normal blood donors. *B*, Flow cytometry analysis of rigorously purified B cells shows that the percentage of CD19$^+$ B cells expressing Cox-1 protein was not changed with activation and that Cox-2 protein was induced by activated B cells. Greater than 95% of B cells expressed Cox-2 after activation with CD40L plus anti-IgM Ab. *C*, Immunofluorescence microscopy was used to visualize B cells that express Cox-2 after activation with CD40L plus anti-IgM Ab. The merged image shows coexpression of surface CD19 (red) and intracellular Cox-2 (green) on activated human B cells.

express Cox-2 in vivo. To assess this, histologically normal tonsil tissue (obtained from tonsillectomy samples) was stained with Abs against Cox-2 and CD20 (L-26 clone) in formalin-fixed, paraffin-embedded, sequential tissue sections. As Fig. 2 demonstrates, Cox-2 was coexpressed with CD20 on B lymphocytes.

Constitutive Cox-1 and inducible Cox-2 mRNA and protein levels from activated human B lymphocytes

To determine whether the increase in Cox-2 protein expression after B cell activation is due to an increase in Cox-2 mRNA, total RNA from highly purified human peripheral blood B cells was next analyzed by real time RT-PCR analysis. Quantitative analysis of Cox-1 and Cox-2 mRNA expression in human B lymphocytes was performed by normalization of cycle threshold values for the target genes to GAPDH mRNA levels. Fig. 3*A* (gel electrophoresis) first shows that steady-state Cox-1 mRNA levels were constitutively expressed and remained unchanged during B cell activation. These findings are consistent with the cellular maintenance functions of Cox-1. In contrast, Cox-2 mRNA was not detectable in unactivated B cells (*lane 1*). However, activation of B cells with CD40L and/or anti-IgM Ab (as single or combined treatments) resulted in the substantial induction of steady-state Cox-2 mRNA levels (lanes 2–4). A significant 4-fold induction of Cox-2 mRNA was seen as early as 4 h following treatment with stimuli (data not shown). After 8 h of activation, a 9-fold increase in Cox-2 mRNA compared with that of nonactivated B cells was seen as shown in Fig. 3*B*.

We next analyzed the expression pattern of Cox-1 and Cox-2 proteins from freshly isolated blood B cells by Western blot analysis. As shown in Fig. 3*C*, unactivated B cells expressed Cox-1 but not Cox-2. These results are consistent with the flow cytometry results for Cox-1/Cox-2 shown in Fig. 1*B*. Consistent with our quantitative PCR data, Cox-2 protein expression was highly up-regulated in B lymphocytes activated by pansorbin, CD40L, anti-IgM Ab, or by a combination of signals from CD40L plus anti-IgM Ab (Fig. 3*C*). Both Cox-1 and Cox-2 had the same mass (70 and 72 kDa, respectively), as the Cox-1 and Cox-2 enzyme from IL-1β-stimulated L828 human fibroblasts. β-Actin levels were used in densitometry analysis as a protein-loading control. Densitometry of Western blots shows a 25- to 30-fold relative increase in Cox-2 protein levels in activated B cells compared with that of untreated controls after 24 h (Fig. 3*D*). As expected, Cox-1 protein expression was unchanged following activation with B cell stimuli. Taken together with the data presented above, our findings demonstrate that B cell stimulation through either CD40 or the BCR transduces a signal(s) that up-regulates both Cox-2 mRNA and protein expression.

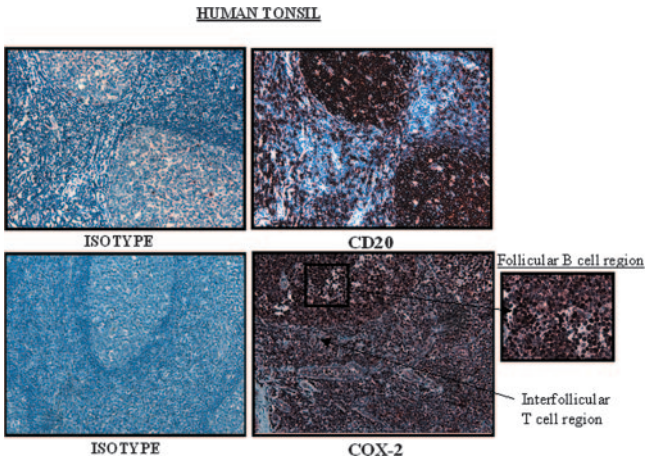

FIGURE 2. Cox-2 expression in activated B lymphocytes in vivo. Immunohistochemistry shows that $CD20^+$ B lymphocytes expressed Cox-2 in paraffin-embedded human tonsil tissue. The *arrows* highlight lymphocytes within the follicular B cell region and the interfollicular T cell region that express Cox-2. Multiple sections were analyzed. The figure shows one representative tonsil section.

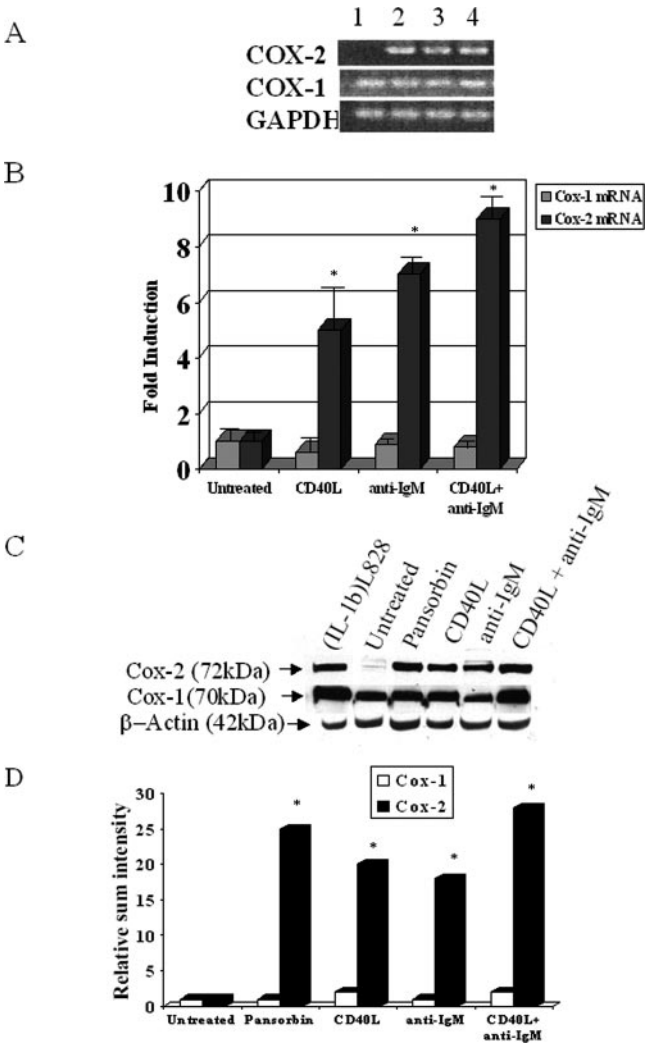

FIGURE 3. CD40 and BCR stimulation increase steady-state Cox-2 mRNA and protein levels, and Cox-1 levels remain unchanged. *A*, Highly purified human B lymphocytes isolated from peripheral blood were untreated (*lane 1*) or were treated for 8 h with CD40L (*lane 2*), 10 μg/ml anti-IgM Ab (*lane 3*), or a combination of CD40L plus anti-IgM (*lane 4*). B cell purity was >98%. Quantitative RT-PCR products were electrophoresed on a 1.2% agarose gel. No Cox-2 product was detected in unactivated B cells and was expressed with B cell stimuli. Cox-1 mRNA was constitutively expressed. *B*, Cox-1 and Cox-2 mRNA levels were measured by quantitative RT-PCR. Cycle threshold values were normalized to GAPDH, and fold induction was calculated over untreated control levels. *, Statistical significance by Student's *t* test ($p < 0.05$). *C*, Western blot shows immunoreactivity of Cox-1 and Cox-2 in B cells. *Lane 1* shows IL-1β stimulated L828 human fibroblasts as a strong positive control. β-Actin expression is shown as a protein-loading control. *D*, Densitometry of the Western blot shows that Cox-1 protein expression was not changed with activation, whereas there was a 25-fold increase in Cox-2 protein expression.

B lymphocytes synthesize PGE_2 and $PGF_{2\alpha}$, via Cox-2

Given that activated B cells express Cox-2 and that Cox-2 catalyzes a series of reactions resulting in PG synthesis, we next determined whether activated B cells have the capacity to produce and secrete the PGs, PGE_2 and $PGF_{2\alpha}$. Freshly isolated B cells were incubated with and without the nonspecific B cell activator pansorbin or with CD40L ± anti-IgM Ab for 36 h. The supernatants were then assayed for PGE_2 and $PGF_{2\alpha}$ using a specific EIA analysis. In Fig. 4*A*, PGE_2 levels in the medium were dramatically increased up to 10-fold following B cell treatment with pansorbin, CD40L, and/or anti-IgM Ab (single or dual stimulation). Similarly, $PGF_{2\alpha}$ levels were increased upon B cell activation, although not to the same extent as PGE_2. To determine whether or not PGE_2 and $PGF_{2\alpha}$ production was synthesized mainly via Cox-2, B lymphocytes were treated with either indomethacin, a nonselective Cox-1/Cox-2 inhibitor, or with SC-58125, a highly selective Cox-2 inhibitor (15). As shown in Fig. 4*B*, indomethacin blocks the production of nearly all the PGE_2 and $PGF_{2\alpha}$. Treatment with the Cox-2-selective inhibitor, SC-58125, resulted in ~85% decrease in PGE_2 and $PGF_{2\alpha}$ produced, indicating that the majority of PGE_2 and $PGF_{2\alpha}$ were produced by the inducible Cox-2 (Fig. 4*B*). The use of another selective Cox-2 inhibitor, NS-398, supports these findings as this drug also reduced PG synthesis by up to 85% of the PGs produced (data not shown). These data demonstrate that Cox-1/Cox-2 inhibitors significantly blunt PGE_2 and $PGF_{2\alpha}$ production seen after B cell activation.

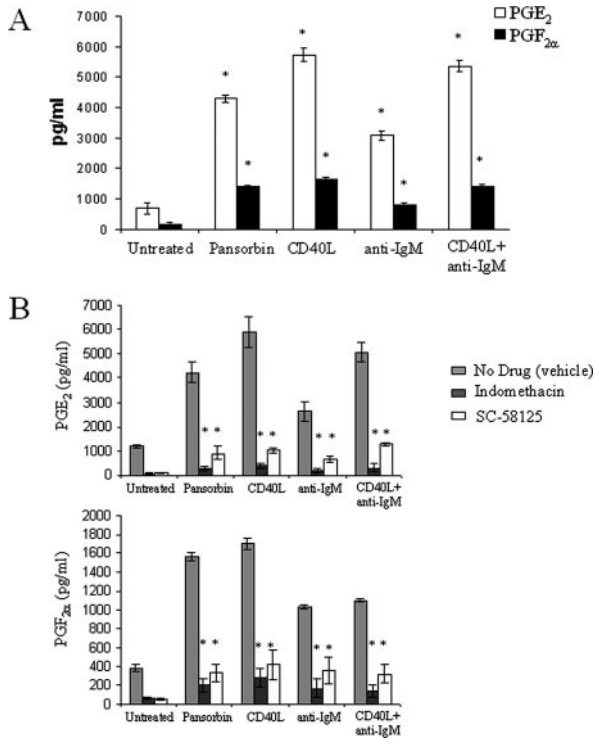

FIGURE 4. Activated human peripheral B lymphocytes produce PGE_2 and $PGF_{2\alpha}$. *A*, B cells stimulated with nothing, pansorbin (1:1000), CD40L, anti-IgM Ab (10 μg/ml), or a combination of CD40L plus anti-IgM Ab. PG levels were measured by EIA. *B*, Nonselective and highly selective Cox-2 inhibition decreased PGE_2 and $PGF_{2\alpha}$ synthesis by activated B cells. B cells were incubated with stimulus and 20 μM indomethacin or 20 μM SC-58125 for 36 h. Each bar represents the means ± SD ($n = 3$). Vehicle solvent was added as a negative control. The statistical significance of the reduction in PG synthesis by Cox inhibitors was determined by Student's *t* test ($p < 0.05$).

Cox-2 inhibition dramatically reduced IgM and IgG production by activated B lymphocytes

When B cells are activated, they differentiate into Ig-secreting plasma cells (16). Ig production by human B cells was assayed by measuring the amount of Ig present in culture supernatants of B cells stimulated by selected activators. As expected, when B lymphocytes were incubated with CD40L plus anti-IgM, 3300 ± 205 ng/ml and 2033 ± 328 ng/ml IgM and IgG, respectively, were released into the culture supernatants after 7 days. PGs were previously reported to enhance Ab production by promoting class switching in B cells (17). However, it has not been determined whether or not B cell-derived PGs promote Ab production.

Since we showed that human B cells expressed Cox-2 upon activation and produced PGs, we hypothesized that these PGs enhanced Ab production in an autocrine fashion. To test this, we evaluated Ab production after activation of purified B cells in the

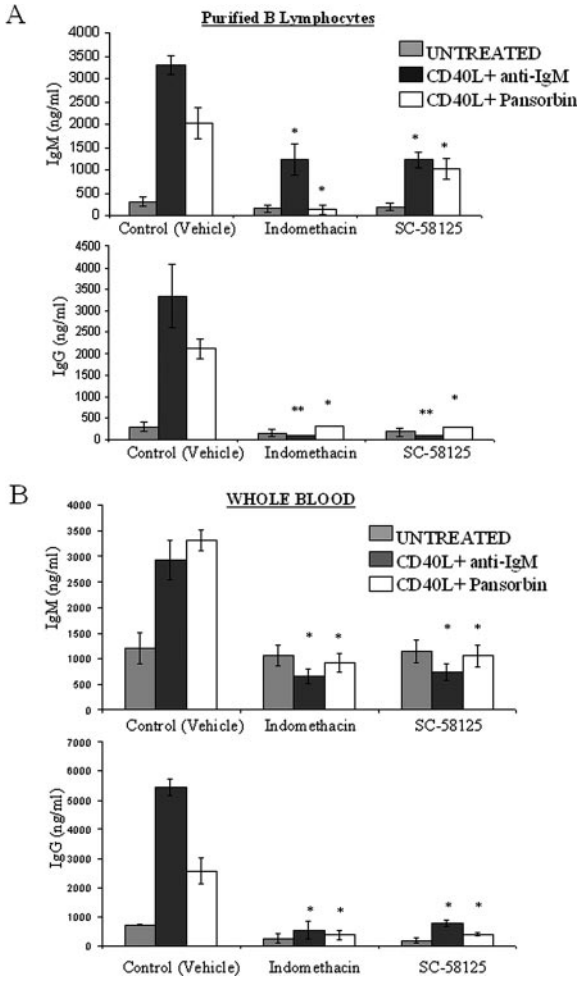

FIGURE 5. Cox-2 inhibition reduces Ig production by human B lymphocytes and whole blood. *A*, Purified B cells were stimulated with CD40L plus anti-IgM Ab or CD40L plus pansorbin for 7 days with 5 μM arachidonic acid. B lymphocytes were activated with stimuli in the presence and absence of 20 μM indomethacin or 20 μM SC-58125. Vehicle solvent was added as a negative control. Cox-2 inhibition significantly decreased both IgM and IgG levels. *B*, Whole blood, washed and cultured for 7 days in medium, was stimulated with or without B cell activators (as shown in *A*) in the presence and absence of 20 μM indomethacin or 20 μM SC-58125. Significant reductions in Ab production were measured following treatment with drugs for CD40L plus anti-IgM (or pansorbin)-stimulated cells. Each bar represents the means ± SD ($n = 3$). *, Statistical significance by Student's *t* test ($p < 0.05$).

presence of the Cox-1/Cox-2 inhibitor indomethacin (added to culture every other day for 7 days) or a highly selective Cox-2 inhibitor SC-58125 (added once at start of culture). The data in Fig. 5*A* demonstrate that in vitro activated B cells failed to optimally produce Ab when incubated with indomethacin or SC-58125, compared with vehicle-treated cells. A 50% reduction in IgM and a 95% reduction in IgG synthesis were measured following inhibition of Cox-1/Cox-2 in CD40L plus anti-IgM Ab-activated B cells, with similar results seen with CD40L plus pansorbin-stimulated cells. The selective Cox-2 inhibitor NS-398 reduced Ab production to the same extent as SC-58125 (data not shown). These compelling findings demonstrate that human B cell Cox-2 is an important autocrine signal for promoting optimal Ig production in vitro.

Given that Cox-1/Cox-2 inhibitors greatly reduced Ab production by purified B cells, we next investigated whether or not these drugs reduced Ab production by whole blood ex vivo. Whole human blood was washed two times to remove plasma and was then diluted in culture medium (see *Materials and Methods*). Ig production was measured after whole blood was activated and treated with or without Cox-1/Cox-2 inhibitors for 7 days. Fig. 5*B* shows that activation of whole blood with CD40L plus anti-IgM Ab (or pansorbin) increased IgM and IgG levels compared with that of unactivated blood. As anticipated from the data with purified B cells, a significant reduction in IgM (up to 80%) and in IgG (up to 90%) levels was seen following treatment with Cox-1/Cox-2 inhibitors. Herein, our findings reveal that B cell activation-induced Cox-2 activity and the subsequent increase in PG production are important immunoregulatory signals for optimal Ab production by purified B lymphocytes in vitro and by whole blood cells ex vivo.

Cox inhibitors modestly reduce B lymphocyte proliferation

Pharmacological concentrations of indomethacin and the selective Cox-2 inhibitors that inhibited PG synthesis (see Fig. 4*B*) and reduced Ab production (see Fig. 5) were used to test the ability of Cox inhibitors to affect B cell proliferation using a [^{3}H]thymidine incorporation assay. Cox inhibitors did not influence B cell proliferation in response to CD40L or anti-IgM Ab stimulation (data not shown). However, both the nonselective and selective Cox-2 inhibitors, indomethacin and SC-58125, modestly reduced proliferation of CD40L plus anti-IgM Ab stimulated B cells. Treatment with Cox inhibitors showed a significant 35% decrease in proliferation after 48 h and a 25% reduction after 4 and 5 days in culture as compared with vehicle treated cells (Fig. 6*A*). The reduction in proliferation following Cox-1/Cox-2 inhibition on days 2, 4, and 5 were also seen by MTT assay (data not shown).

We next wanted to determine whether or not the reduction in B cell proliferation (as shown by [^{3}H]thymidine incorporation and the MTT assay) was due to an increase in early apoptotic cells or cell death. Therefore, the $DiOC_6$ incorporation assay was used to measure the percentage of live and dead B cells following Cox-1/Cox-2 inhibition by flow cytometry. Early apoptotic and dead cells have a permeable mitochondrial membrane and do not incorporate $DiOC_6$. As shown in Fig. 6*B*, there was no significant change in the percentage of CD40L plus anti-IgM Ab-stimulated B cells that incorporate $DiOC_6$ following treatment with indomethacin or SC-58125 for 48 h. There was no change in the number of dying/dead cells after 3, 4, and 5 days of incubation with drug (data not

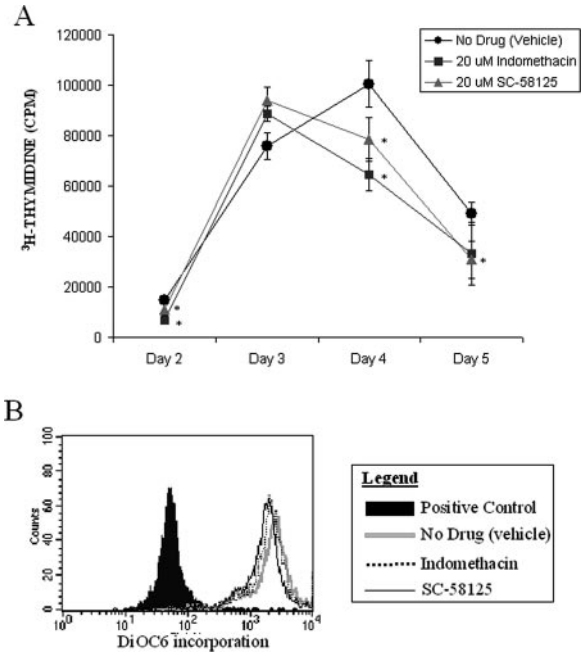

FIGURE 6. Cox-2 inhibition reduces B lymphocyte proliferation. *A*, B cells were activated with stimuli in the presence and absence of indomethacin or SC-58125 for 2, 3, 4, and 5 days. Cells were pulsed with [^{3}H]thymidine for 24 h in culture. Data are presented as cpm. *, Statistical significance by Student's *t* test ($p < 0.05$). *B*, Indomethacin (20 μM) or SC-58125 did not change $DiOC_6$ incorporation in CD40L plus anti-IgM-stimulated B cells as measured by flow cytometry. Live cells incorporate $DiOC_6$ whereas dead/dying cells incorporate less. Shaded area represents highly purified peripheral B cells that were serum starved for 48 h. These dead/dying cells were used as a positive control.

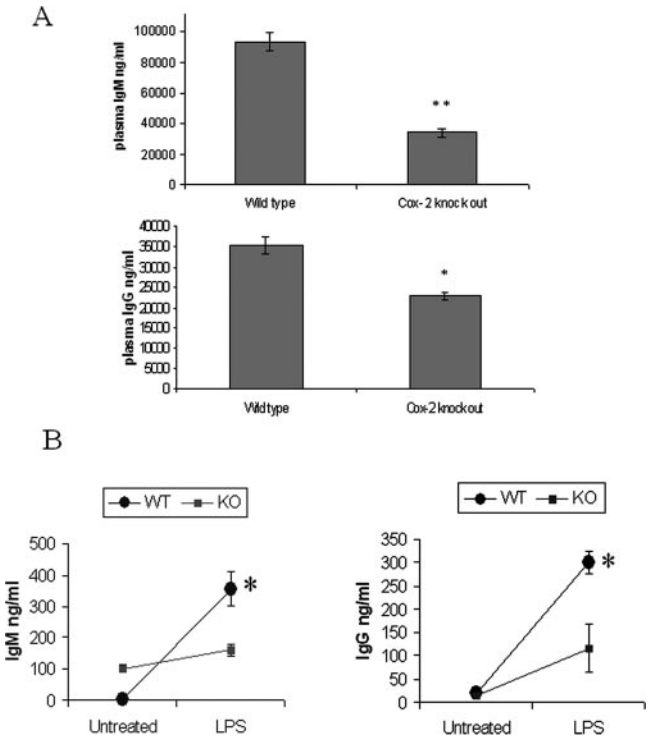

FIGURE 7. Cox-2-deficient mouse B lymphocytes produce significantly lower IgM and IgG compared with wild-type controls. *A*, Plasma isolated from five wild-type and five Cox-2-deficient mice was analyzed for IgM and IgG levels by mouse isotype-specific ELISA. Each bar represents the means ± SD ($n = 5$). *B*, B cells isolated from wild-type and Cox-2-deficient mouse spleen were untreated or stimulated in vitro for 7 days with LPS (10 μg/ml). *, Statistical significance $p < 0.05$ or **, $p < 0.01$ (unpaired Student's *t* test) comparing Ig production from wild-type and Cox-2-deficient mice.

shown). Thus, indomethacin and SC-58125 reduced day 4 proliferation of CD40L plus anti-IgM Ab-stimulated B lymphocytes, but did not induce apoptosis or B cell death. These data reveal that the Cox inhibitory drugs did not elicit mitochondrial membrane permeability, a hallmark of apoptosis, and suggest that the modest reduction in proliferation may account for some, but not all of the dramatically reduced IgM and IgG levels (Fig. 5).

Reduced IgM and IgG levels in peripheral blood of Cox-2-deficient mice

We next determined whether or not B cell Cox-2 activity was important for Ab production in vivo by comparing peripheral blood plasma Ig levels in Cox-2-deficient mice with that of normal littermate controls. Peripheral blood was harvested in heparinized hematocrit tubes (see *Materials and Methods*). Cox-2-deficient mice ($n = 5$) had significantly lower levels of IgM ($p < 0.01$) and IgG ($p < 0.05$) in their plasma compared with that of control mice ($n = 5$) exposed to the same diet and environment. As shown in Fig. 7*B*, we determined that the reduction in plasma Ab levels in Cox-2-deficient mice was likely due to a defect in the B lymphocyte. In support of this, in vitro LPS-stimulated B cells from Cox-2-deficient mouse spleen produced ~75% less IgM and ~60% less IgG compared with wild-type control. It has been previously reported that elevated Cox-1 levels may compensate for the loss of Cox-2 in Cox-2-deficient mice compared with wild-type controls (18). Our findings suggest that elevated Cox-1 activity may account for the slightly elevated IgM levels produced by the unstimulated Cox-2 knockout B cells after 7 days in culture. However, the in vitro LPS-stimulated Cox-2 knockout B cells show a marked reduction in IgM levels compared with wild-type controls, which supports our hypothesis that the increase in Cox-2 activity by activated B lymphocytes is required for optimal Ab production. These in vivo and in vitro findings using a Cox-2-deficient model provide strong support for the human B cell data, which demonstrate that Cox-2 is required for optimal Ab production.

Discussion

The CD40-CD40L interaction and BCR cross-linking signal a series of complex events essential for B cell activation, proliferation, and differentiation to Ab-producing cells (16, 19). Our findings that activated B cells express Cox-2, secrete PGE_2 and $PGF_{2\alpha}$, and produce Ig in a PG-dependent fashion supports a novel mechanism through which CD40-CD40L interaction and BCR cross-linking activate normal human B cells. The results presented here also demonstrate that the Cox-2 isoform is preeminent over Cox-1 for the stimulated production of PG products by activated human B lymphocytes. Understanding the mechanisms involved in regulating Cox activity in B lymphocytes has clinical significance because pharmacological agents that inhibit Cox enzyme activity are commonly used for acute and chronic inflammatory conditions (15). We report for the first time that there are direct effects of Cox-1/Cox-2 inhibition, as well as selective Cox-2 inhibition, on B lymphocytes. Activated B cells have not been considered primary targets for NSAIDs and Cox-2-selective inhibitors because normal B cells were not previously known to express Cox-2 or to produce PGs.

Following exposure to CD40L plus anti-IgM Ab (or pansorbin) for 7 days, B lymphocytes become activated, proliferate, and differentiate, thus increasing their production of IgM and IgG (20). Our findings of drastically reduced IgM and IgG levels following incubation with indomethacin or the selective Cox-2 inhibitor SC-58125 supports the concept that the B cell Cox-2-derived products are required for optimal Ab production. A few studies have begun to address whether or not Cox inhibitory drugs influence humoral immune responses in animal models. For example, a study using DBA/1J mice demonstrated that in vivo administration of indomethacin, a nonselective Cox-1/Cox-2 inhibitor, following OVA immunization, reduced OVA-specific IgG Ab production and splenocyte proliferation (21). Comparable results were obtained in a *Mycobacterium butyricum*-induced adjuvant arthritis rat model, which showed a significant reduction in plasma anti-*Mycobacterium* IgG levels following in vivo administration of two different selective Cox-2 inhibitors (22). These findings suggested an important role for Cox-2-derived products in promoting a humoral immune response. Finally, an in vitro study using pokeweed mitogen-stimulated human PBMCs demonstrated a significant reduction in IgM and IgG production following treatment with the Cox-1/Cox-2 inhibitor indomethacin (23). These studies support the concept that Cox-1/Cox-2-derived products are important signals for Ab production in humans. However, the report of decreased Ig levels following indomethacin treatment of stimulated PBMCs did not investigate whether there were direct effects of these drugs on B cells. Rather, these reports suggested an indirect effect on B cells as a result of reduced macrophage or T cell-derived factors (23). We conclude that the decrease in Ig production is not solely due to decreases in macrophage and T lymphocyte-associated Cox activity but rather due to reduced PG synthesis by B cells. We have observed up to a 15% reduction in syndecan-1 (CD138) expression in B cells activated with CD40L plus anti-IgM Ab and treated with Cox-2 inhibitors (data not shown). We interpret these data as supporting the hypothesis that Cox-1/Cox-2 inhibitors reduce B cell Ab production, in part, by attenuating B cell differentiation to plasma cells.

PGE_2 modulates many aspects of inflammation and immune responses including inhibition of IL-2 synthesis by T cells, inhibition of IL-12 production in macrophages and dendritic cells, and increased vascular permeability and vasodilation (24–26). Our laboratory previously reported that PGE_2 enhances CD40L and IL-4-induced mouse B lymphocyte production of IgG1 and IgE (27). However, it is not clear at present whether these same PG-induced class-switch mechanisms occur in human B cells. Our findings suggest that human B cell-derived PG products signal by an autocrine mechanism to enhance B cell proliferation and Ab production. Further investigation of the mechanisms by which B cell Cox-2-derived products modulate human B cell effector functions is necessary. Based on our results of dramatically reduced IgG levels following Cox-2 inhibition, we speculate that most cells failed to undergo class switching required for IgG synthesis. Our data suggest that the widespread use of NSAIDs and Cox-2-selective drugs may alter B cell function and the immune response in ways that are not yet fully appreciated.

Emerging data show that the growth-promoting properties of Cox-2 in normal physiological responses is exploited in malignancy and autoimmune disorders. Wun et al. (28, 29) recently reported elevated Cox-2 expression in human B cell lymphomas. Cox-2 was also implicated as playing a role in extranodal marginal cell lymphomas of mucosa-associated lymphoid tissue (30). These low-grade B cell malignancies are thought to arise in a background of chronic B cell activation, although development has been linked to several chronic bacterial and/or microorganism infections (31). We speculate that the increased Cox-2 levels seen in B cell lymphomas were a result of inducible Cox-2 expression by normal activated B cells before their conversion to a malignant state. However, the mechanisms by which excess eicosanoid production by elevated Cox-2 promotes malignancy are unknown. Interestingly, anti-apoptotic functions of Cox-2 were recently reported in human

lupus T cells as Cox-2 inhibitors were reported to reduce cell viability. These drugs were also capable of suppressing IgG autoantibody production by mixed lymphocyte cultures in mouse lupus models, whereas Cox-2-deficient mice were incapable of developing autoimmune arthritis (32, 33). Our compelling finding of reduced Ab production by Cox-2-deficient mice and following selective Cox-2 inhibition of human B cells suggests that these agents may be suppressing autoantibody production in lupus models via direct effects on B cells. Thus, it will be important to further evaluate these drugs as potential therapeutic agents to control abnormal B lymphocyte proliferation seen in non-Hodgkin lymphoma as well as the abnormal Ab production seen in autoimmune diseases.

The findings reported herein also have important implications for the use of Cox-1/Cox-2 inhibitory drugs following vaccinations, where the goal is to promote a humoral immune response. Although these drugs are commonly used to alleviate the pain associated with injection of the vaccine, our findings suggest that there may be an adverse effect on Ab production and/or the immune response following secondary exposure. Because this report is the first to show Cox-2 activity in human B lymphocytes and to demonstrate the important role of these enzymes in promoting Ab production, additional studies are warranted to study the effects of NSAIDs and selective Cox-2 inhibitors on human B lymphocyte effector functions in vivo.

Acknowledgments

We thank Pat Bourne for assistance with the human tonsil tissue staining.

Disclosures

The authors have no financial conflict of interest.

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CORRECTIONS

Roderich E. Schwarz and John C. Hiserodt. The Expression and Functional Involvement of Laminin-Like Molecules in Non-MHC Restricted Cytotoxicity by Human Leu-19$^+$/CD3$^-$ Natural Killer Lymphocytes. *The Journal of Immunology*, 1988, 141: 3318–3323.

The Journal received the following letter from Roderich E. Schwarz requesting correction of this article, which was published in the November 15, 1988 issue:

To the editor:

This is a formal request for a correction to a paper which appeared in *The Journal of Immunology* in November 1988 (R. E. Schwarz and J. C. Hiserodt), in response to a recommendation by the Office of Research Integrity (ORI) of the Public Health Service, Department of Health and Human Services. In 1994, the ORI had performed an investigation against the senior author of the manuscript titled "The expression and functional involvement of laminin-like molecules in non-MHC restricted cytotoxicity by human Leu-19$^+$/CD3$^-$ natural killer lymphocytes," the summary of which has been tagged to the PubMed reference of this article (http://grants1.nih.gov/grants/guide/notice-files/not94-105.html). In this ORI report, a requirement to correct the journal article, namely the indication that Fig. 2 of the article cannot be relied upon, was issued. Such recommendation remains sensible today, albeit 16 years after the original publication, as the investigation apparently failed to identify documented experimental data upon which the figure had been generated, and is hereby formally requested. However, the general ability to inhibit human adherent lymphokine-activated killer (A-LAK) cell cytotoxicity by F(ab′)$_2$ of anti-laminin Ab, as stated in the legend of Fig. 2, should not be questioned. A figure, which is based on actual experimental data and reflects the inhibitory effect, is therefore added to this correction request, to replace the original Fig. 2.

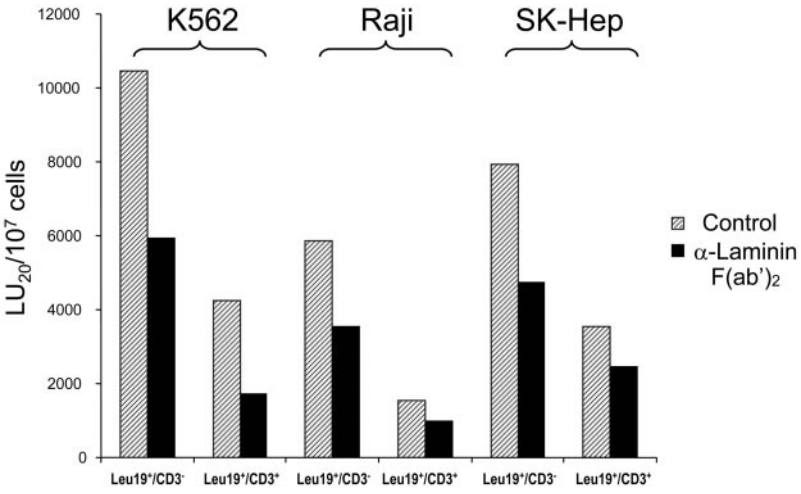

FIGURE 2. Inhibition of cytotoxicity by two different human A-LAK cell populations through F(ab′)$_2$ of affinity-purified anti-laminin Ab. Sorted Leu-19$^+$/CD3$^-$ and Leu-19$^+$/CD3$^+$ A-LAK cell populations were mixed with Cr-labeled target cells in the continued presence of 150 μg/ml Ab for 4 h, and the resulting lytic activity was calculated as specified in *Materials and Methods*.

0022-1767/05/$02.00

Sambasiva P. Rao, Kalpit A. Vora, and Tim Manser. Differential Expression of the Inhibitory IgG Fc Receptor FcγRIIB on Germinal Center Cells: Implications for Selection of High-Affinity B Cells. *The Journal of Immunology*, 2002, 169: 1859–1868.

The Journal received the following letter requesting correction of this article, which was published in the August 15, 2002 issue:

The authors would like to alert the scientific community to the fact that we have been unable to reproduce one of the findings presented in this manuscript. In Fig. 5*B* of this manuscript we showed the results of flow cytometric studies designed to measure the levels of surface expression of FcγRIIB on splenic germinal center (GC) B cells (defined as $B220^+$, IgD^-, $GL7^+$) as compared with splenic non-GC B cells (defined as $B220^+$, IgD^+, $GL7^-$) using the anti-FcγRIIB mAb K9.361. These cells were isolated from C57BL/6 mice that had been immunized i.p. 8 days earlier with 3×10^8 sheep RBC (SRBC) per mouse. Fig. 5*B* illustrated ~5-fold lower levels of K9.361 staining on GC B cells as compared with non-GC B cells. In Fig. 6, we presented the results of the semiquantitation of FcγRIIB mRNA levels, via RT-PCR and in gel hybridization, in these two populations of B cells that had been purified by FACS. This figure indicated ~6-fold lower levels of FcγRIIB mRNA in GC, as compared with non-GC B cells.

In multiple recent experiments designed to extend these published studies, neither the reduced levels of K9.361 surface staining of $B220^+$, IgD^-, $GL7^+$ splenic B cells detected by flow cytometry or the reduced levels of FcγRIIB mRNA in such cells isolated by FACS 8 days after i.p. immunization of C57BL/6 mice with SRBCs (evaluated via real-time RT-PCR) have been observed.

In several other figures in the above-referenced manuscript, the results of immunohistological analysis of FcγRIIB expression in the GCs of SRBC immunized C57BL/6 mice were illustrated and interpreted to corroborate the results of the studies presented in Figs. 5 and 6. Due to the relative insensitivity of immunohistology as compared with flow cytometry, whether GC B cells stained 5- to 6-fold less intensely with anti-FcγRIIB mAbs as compared with non-GC B cells could not have been unequivocally determined using the former approach. Nonetheless, our previous interpretations of these immunohistological data with regard to levels of FcγRIIB on GC B cells appear to have been incorrect. In addition, arguments we forwarded in *Discussion* based on the conclusion that GC B cells express lower levels of FcγRIIB than non-GC B cells may no longer hold merit.

We currently can provide no compelling explanation for why our previously published results on the expression levels of FcγRIIB on GC B cells and the results of our more recent studies differ, but are actively investigating several possibilities. We should hasten to point out that our failure to reproduce the results presented in Figs. 5*B* and 6 does not influence the validity of any of the data or conclusions presented in the above-referenced manuscript regarding the expression and function of FcγRIIB on follicular dendritic cells.

Per O. Anderson, Anette Sundstedt, Zihni Yazici, Sophie Minaee, Richard Woolf, Kirsty Nicolson, Nathaniel Whitley, Li Li, Suling Li, David C. Wraith, and Ping Wang. IL-2 Overcomes the Unresponsiveness but Fails to Reverse the Regulatory Function of Antigen-Induced T Regulatory Cells. *The Journal of Immunology*, 2005, 174: 310–319.

The fifth author's name, Emma J. O'Neill, was inadvertently omitted. The correct list of authors and affiliations is shown below.

Per O. Anderson,*† Anette Sundstedt,§ Zihni Yazici,§ Sophie Minaee,§ Emma J. O'Neill,§ Richard Woolf,§ Kirsty Nicolson,§ Nathaniel Whitley,§ Li Li,*† Suling Li,†‡ David C. Wraith,§ and Ping Wang*†

*Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, London, United Kingdom; †Tumor Immunology, Lund University, Lund, Sweden; ‡Department of Biological Sciences, Brunel University, Uxbridge, London, United Kingdom; and §Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, Bristol, United Kingdom

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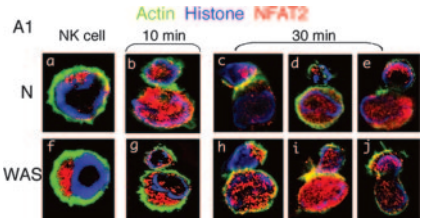
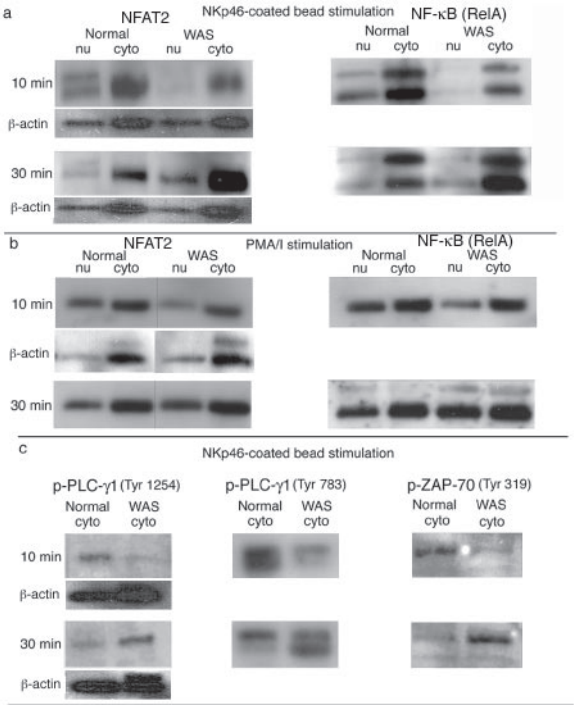
Winifred Huang, Hans D. Ochs, Bo Dupont, and Yatin M. Vyas. The Wiskott-Aldrich Syndrome Protein Regulates Nuclear Translocation of NFAT2 and NF-κB (RelA) Independently of Its Role in Filamentous Actin Polymerization and Actin Cytoskeletal Rearrangement. *The Journal of Immunology*, 2005, 174: 2602–2611.

In *Results*, Fig. 2*A1* was incorrectly published in black and white. The error has been corrected in the online version, which now differs from the print version as originally published. The correct color figure is shown below.



Also in *Results*, Fig. 3 fails to mention the headings "NFAT2" and "NF-κB (RelA)" on both panels *a* and *b*. The legend to this figure is written with the presumption that the headings are present on each of the two panels. The correct figure is shown below.



Elizabeth P. Ryan, Stephen J. Pollack, Thomas I. Murant, Steven H. Bernstein, Raymond E. Felgar, and Richard P. Phipps. Activated Human B Lymphocytes Express Cyclooxygenase-2 and Cyclooxygenase Inhibitors Attenuate Antibody Production. *The Journal of Immunology*, 2005, 174: 2619–2626.

The second author's last name is misspelled. The correct name is Stephen J. Pollock.

In This Issue. *The Journal of Immunology*, 2005, 174: 3135–3136.

In the last summary of *In This Issue* titled "*Helicobacter pylori*-neutrophil interactions," an error was made in citing the page number of the original article titled "*Helicobacter pylori* disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release" by Lee-Ann H. Allen, Benjamin R. Beecher, Jeffrey T. Lynch, Olga V. Rohner, and Lara M. Wittine. The correct page number associated with the article is 3658. The error has been corrected in the online version, which now differs from the print version as originally published.

Soren Schenk, Danielle D. Kish, Chunshui He, Tarek El-Sawy, Eise Chiffoleau, Chuangqui Chen, Zihao Wu, Sigrid Sandner, Anton V. Gorbachev, Kiyotaka Fukamachi, Peter S. Heeger, Mohamed H. Sayegh, Laurence A. Turka, and Robert L. Fairchild. Alloreactive T Cell Responses and Acute Rejection of Single Class II MHC-Disparate Heart Allografts Are under Strict Regulation by $CD4^+CD25^+$ T Cells. *The Journal of Immunology*, 2005, 174: 3741–3748.

The sixth author's first name is misspelled. The correct name is Chuangqi Chen.