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Prostaglandin E_2 Increases Bovine Leukemia Virus *tax* and *pol* mRNA Levels via Cyclooxygenase 2: Regulation by Interleukin-2, Interleukin-10, and Bovine Leukemia Virus

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Prostaglandin E_2 (PGE_2), produced by macrophages, has important immune regulatory functions, suppressing a type 1 immune response and stimulating a type 2 immune response. Type 1 cytokines (interleukin-2 [IL-2], IL-12, and gamma interferon) increase in freshly isolated peripheral blood mononuclear cells (PBMCs) of animals with an early disease stage of bovine leukemia virus (BLV) infection, while IL-10 increases in animals with a late disease stage. Although IL-10 has an immunosuppressive role in the host immune system, IL-10 also inhibits BLV *tax* and *pol* mRNA levels in vitro. In contrast, IL-2 stimulates BLV *tax* and *pol* mRNA and p24 protein expression in cultured PBMCs. The inhibitory effect of IL-10 on BLV expression depends on soluble factors secreted by macrophages. Thus, we hypothesized that PGE_2, a cyclooxygenase 2 (COX-2) product of macrophages, may regulate BLV expression. Here, we show that the level of COX-2 mRNA was decreased in PBMCs treated with IL-10, while IL-2 enhanced the level of COX-2 mRNA. Addition of PGE_2 stimulated BLV *tax* and *pol* mRNA levels and reversed the IL-10 inhibition of BLV mRNA. In addition, the specific COX-2 inhibitor, NS-398, inhibited the amount of BLV mRNA detected. Addition of PGE_2 increased BLV *tax* mRNA regardless of NS-398 addition. PGE_2 inhibited antigen-specific PBMC stimulation, suggesting that stimulation of BLV *tax* and *pol* mRNA levels by PGE_2 is independent of cell proliferation. These findings suggest that macrophage-derived COX-2 products, such as PGE_2, regulate virus expression and disease progression in BLV infection.

Bovine leukemia virus (BLV), closely related to human T-cell leukemia virus type 1 (HTLV-1), is a type C retrovirus that infects bovine B cells and leads to enzootic bovine leukosis (16). The genomes of BLV and HTLV-1 are similarly arranged. In particular, the 3′ region that contains the *tax*, *rex*, R3, and G4 genes is unique to BLV and HTLV-1 (2). In addition, there are several features of pathogenesis that are shared by BLV and HTLV-1. For both viruses, many infected individuals develop antibodies, but clinical symptoms are relatively rare. Disease progression in BLV-infected animals is divided into three stages: serologically positive, persistent lymphocytosis negative (alymphocytotic [AL]); serologically positive, persistent lymphocytosis positive (persistently lymphocytotic [PL]); and tumor-bearing stages (usually lymphosarcoma). Most infected animals never display outward signs of disease and are referred to as asymptomatic or aleukemic. Fewer than 5% of infected animals develop malignant lymphosarcoma (11), while 30% of infected animals progress to persistent lymphocytosis, in which nonneoplastic B cells proliferate and leukocyte counts may exceed 10,000 cells/mm^3 (17). Usually there is a long duration between these disease stages. The mechanism of disease progression from AL to PL or tumor-bearing stage is not clear.

Recent investigation has revealed that cytokine production plays a critical role in the progression of many different diseases (9, 32). In previous studies, we found cytokine polarization in BLV-infected animals at different disease stages (27, 29). Type 1 cytokines, such as interleukin-2 (IL-2), IL-12, and gamma interferon (IFN-γ), were expressed in high amounts in AL animals, while IL-10 was increased in PL and tumor-bearing animals. Other studies showed that antigen-specific lymphocyte proliferation (25) and cytotoxic γδ T-cell activity (P. S. Lundberg and G. A. Splitter, submitted for publication) were reduced in PL and tumor-bearing animals. Because IL-10 had an inhibitory effect on type 1 immune responses, increased IL-10 expression in late disease stages may result in suppressed cell-mediated immune responses. Nevertheless, we found that IL-10 inhibited BLV *tax* and *pol* mRNA levels, while IL-2 stimulated detection of BLV in peripheral blood mononuclear cells (PBMCs) in vitro (28). These data suggest that increasing levels of IL-10 during BLV infection may have a beneficial effect to suppress BLV expression in infected B cells. In addition, we found that macrophages have a critical role in BLV expression and secrete certain factor(s) to activate BLV *tax* and *pol* mRNA.

We hypothesized that one of the soluble factor(s) may be prostaglandin E_2 (PGE_2) that is produced by cyclooxygenase 2 (COX-2). PGE_2 is the only prostaglandin produced by macrophages in response to inflammatory cytokines, such as IL-1 and tumor necrosis factor alpha (TNF-α), that is known to inhibit cell-mediated immune response and stimulate type 2 cytokine production (12, 14, 18, 26, 36). There are two COX enzymes, referred to as COX-1 and COX-2, that produce prostaglandins from arachidonic acid (10). While COX-1 is a constitutive enzyme that is expressed at its highest concentrations in the kidney, stomach, platelets, and vascular endothelium, COX-2 is an inducible enzyme whose expression is regulated by growth factors, tumor promoters, or cytokines. COX-2 is expressed in a few specialized tissues and cells, including macrophages and

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follicular dendritic cells. The functions of COX-2 and prostaglandins are very important in regulating normal physiological processes (8, 19), as well as the immune response. Here, we demonstrate that IL-10 decreased detection of COX-2 mRNA by PBMCs, while conversely, IL-2 increased COX-2 mRNA. Although PGE_2 reduced antigen-specific PBMC proliferation, PGE_2 increased detection of BLV and reversed the IL-10 inhibition of BLV *tax* and *pol* mRNA levels. In addition, BLV acts as an autocrine stimulator to increase the levels of BLV *tax* and *pol* mRNA and COX-2 mRNA.

COX-2 mRNA from PBMCs was inhibited by IL-10 and enhanced by IL-2. In a previous study, we found that human recombinant IL-10 (hrIL-10) inhibited BLV *tax* and *pol* mRNA, while IL-2 enhanced the detection of BLV *tax* and *pol* mRNA and BLV p24 protein in PBMCs in vitro (27). Also, IL-10-mediated regulation of BLV expression was macrophage dependent. Because COX-2, an important enzyme for prostaglandin synthesis, is mainly expressed by macrophages (18, 26, 36), we hypothesized that COX-2 and its products may affect BLV expression regulated by IL-2 and IL-10. Quantitative competitive PCR (QC-PCR) was performed to determine COX-2 mRNA levels in PBMCs cultured with or without hrIL-10. Transcripts of COX-2 mRNA were quantified by a competitive reverse transcriptase PCR (RT-PCR) assay using standard curve methodology. Validation of this assay and synthesis of native and competitor standards has been published previously (37, 38). A standard curve was created by RT-PCR using a constant amount of competitor RNA (2 aM) together with twofold serial dilutions of native RNA (15 to 0.11 aM). Unknown mRNA samples were diluted as needed, reverse transcribed and amplified with the same amount of competitor RNA, and compared to the standard curve. Reverse transcription was carried out in 1× RT buffer (Promega), 0.2 mM deoxynucleoside triphosphates, 100 μM random hexamer, and 40 U of Moloney murine leukemia virus RT for 1.5 h at 37°C, followed by 95°C for 10 min in a final volume of 20 μl. Four microliters of RT reaction mixture were then PCR amplified (30 s, 95°C; 30 s, 57°C; 30 s, 72°C) for 30 cycles, followed by 72°C for 5 min in 20 μl of 1× PCR buffer (Promega, Madison, Wis.), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.4 μM upstream and downstream primers, and 0.5 U of *Taq* DNA polymerase. PCR products were separated on a 5% polyacrylamide gel electrophoresis (PAGE) gel and stained with ethidium bromide. Bands observed in gels were quantified using Collage[a] software (Fotodyne, Heartland, Wis.). Densitometric values for the standard curve were plotted as follows: log([native RNA]/[competitor RNA]) versus log [native RNA (aM)]. Addition of hrIL-10 reduced the detection of COX-2 mRNA in PBMCs from both AL and PL animals (Fig. 1a). These results paralleled the effects of IL-10 inhibition of BLV *tax* and *pol* mRNA levels (27). In contrast, IL-2 dramatically increased detection of COX-2 mRNA (Fig. 1b). These results suggest that cytokines, such as IL-2 and IL-10, can have opposing effects on the regulation of COX-2 mRNA.

PGE_2, a COX-2 product, enhances detection of BLV *tax* and *pol* mRNA and reverses IL-10 inhibition of BLV *tax* and *pol* mRNA. PGE_2 is the only COX-2 product expressed by macrophages (14). To determine whether PGE_2 affects BLV expression, BLV-infected PBMCs were cultured with or without PGE_2 in the presence of hrIL-10. Subsequently, the *tax* and *pol* mRNA produced by PBMCs from BLV-infected animals was measured by QC-PCR (27). For standardization, PCR was performed using this serially twofold diluted standard plasmid, with concentrations ranging from 8,192 to 16 fg/μl for *tax* and from 2,048 to 4 fg/μl for *pol* and with a fixed amount of mimic (10 fg/μl). Synthesized cDNA from each sample and fixed

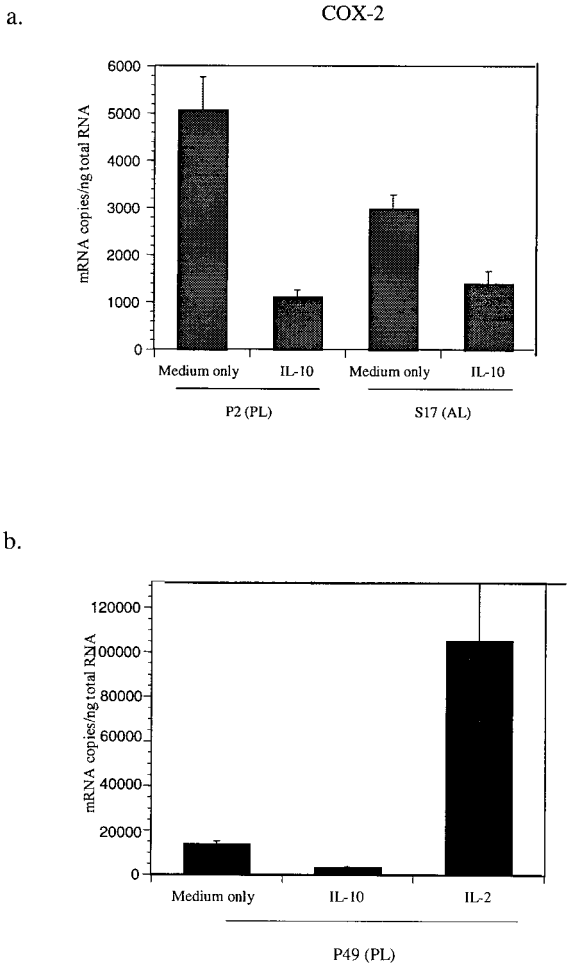

FIG. 1. IL-10 inhibits detection of COX-2 mRNA (a and b), while IL-2 stimulates detection of COX-2 mRNA (b). PBMCs from PL animals and AL animals were cultured for 3 days with or without IL-10 and IL-2, and QC-PCR was performed. PCR products were separated on a 5% PAGE gel and stained with ethidium bromide. Quantification of gels was accomplished using Collage[a] software. Densitometric values for the standard curve were plotted as follows: $\log_{10}$([native RNA]/[competitor RNA]) versus $\log_{10}$ [native RNA (aM)]. The amounts of COX-2 mRNA were calculated based on the standard curve. The data is representative of three different PL animals tested (P2 [a] and P49 [b]) and three AL animals tested (S17 [a]). Standard errors of means are shown from at least three experiments on cells from the same animal.

amounts of mimic were added to the same tube and amplified simultaneously with tubes for a standard reaction. Gel photographs were scanned, and the amplified DNA bands were analyzed by densitometry using the NIH Image program, version 1.61, with standard curves constructed with Cricket graph. The amount of cytokine produced was determined by comparing the density ratios of sample and standard reaction mixtures. As expected, we observed that different animals have different viral loads. Usually, PL animals have more viral load than AL animals. While IL-10 reduced detection of BLV *tax* and *pol* mRNA, PGE_2 reversed this IL-10 inhibitory effect (Fig. 2a). Addition of PGE_2 alone also enhanced BLV *tax* and *pol* mRNA levels (Fig. 2b). These results suggest that macrophage-derived PGE_2 may stimulate BLV expression from infected B cells and that IL-10 may inhibit BLV expression by reducing PGE_2 production. At the higher concentration of PGE_2, BLV *tax* and *pol* mRNA levels were slightly diminished, indicating that the stimulatory effect of PGE_2 was maximal at

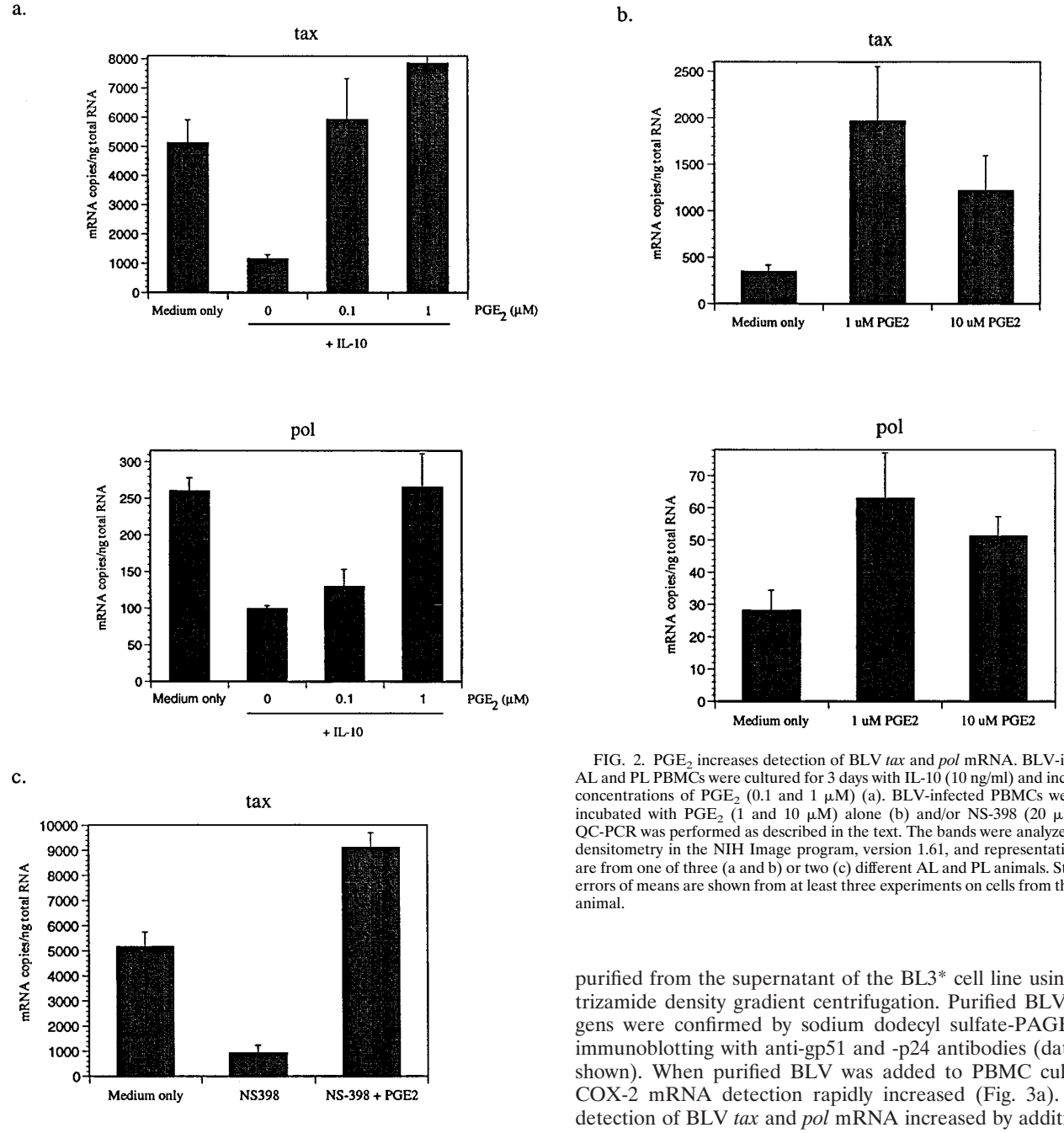

FIG. 2. PGE_2 increases detection of BLV *tax* and *pol* mRNA. BLV-infected AL and PL PBMCs were cultured for 3 days with IL-10 (10 ng/ml) and increasing concentrations of PGE_2 (0.1 and 1 μM) (a). BLV-infected PBMCs were also incubated with PGE_2 (1 and 10 μM) alone (b) and/or NS-398 (20 μM) (c). QC-PCR was performed as described in the text. The bands were analyzed using densitometry in the NIH Image program, version 1.61, and representative data are from one of three (a and b) or two (c) different AL and PL animals. Standard errors of means are shown from at least three experiments on cells from the same animal.

1 μM. To confirm the effects of PGE_2 to increase BLV mRNA levels, a selective COX-2 inhibitor, NS-398 (21), was added to PBMC cultures. As expected, the level of BLV *tax* mRNA was dramatically suppressed by the COX-2 inhibitor (Fig. 2c). In contrast, addition of PGE_2 increased BLV *tax* mRNA detection regardless of NS-398 addition (Fig. 2c). These data suggest that a selective COX-2 inhibitor may reduce the level of BLV mRNA in infected B cells and that PGE_2 addition may bypass the NS-398 inhibition of COX-2 activity.

Purified BLV increases the level of COX-2 mRNA and has an autocrine effect that increases the levels of BLV *tax* and *pol* mRNA. Numerous factors can stimulate COX-2 and PGE_2 expression by macrophages. To investigate how BLV infection might affect COX-2 and BLV expression, BLV proteins were purified from the supernatant of the BL3* cell line using metrizamide density gradient centrifugation. Purified BLV antigens were confirmed by sodium dodecyl sulfate-PAGE and immunoblotting with anti-gp51 and -p24 antibodies (data not shown). When purified BLV was added to PBMC cultures, COX-2 mRNA detection rapidly increased (Fig. 3a). Also, detection of BLV *tax* and *pol* mRNA increased by addition of purified BLV (Fig. 3b), suggesting that BLV antigens regulate BLV expression by an autocrine mechanism. To remove the possibility that other factors isolated during BLV antigen purification could affect COX-2 and BLV expression, a BLV-negative reagent was prepared from the BL3 cell line, which does not produce any BLV antigens. BLV gp51 and p24 were not detected by immunoblotting in the purified BLV-negative material. The BL3 purified material could not enhance COX-2 mRNA or BLV *tax* and *pol* mRNA (Fig. 3).

PGE_2 suppresses antigen-specific PBMC proliferation. To determine if PBMC proliferation and BLV expression were correlated, different concentrations of PGE_2 were added to the PBMC cultures with or without BL3* supernatant as a viral antigen source (23). Following incubation of PBMCs for 3 to 5 days, cells were harvested for further experiments. In cell pro-

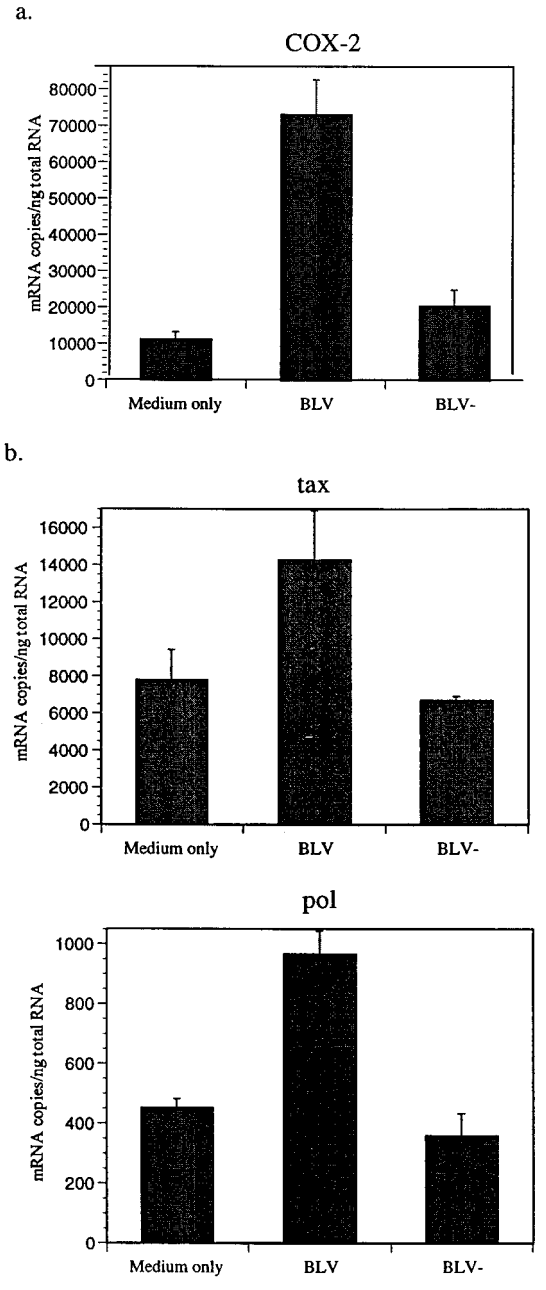

FIG. 3. BLV proteins stimulate COX-2 mRNA (a) and BLV *tax* and *pol* mRNA (b). BLV proteins were purified from BL3* supernatant, and BLV-negative reagent was prepared from BL3 supernatant using metrizamide density gradient centrifugation. BLV-infected PBMCs were cultured for 3 days with similar volumes of purified BLV (10 μg/ml) and BLV-negative (BLV−) materials. COX-2 and BLV *tax* and *pol* mRNA were quantified as described above. Shown are representative data (PL animals) of experiments with three different AL and PL animals. Standard errors of means are shown from at least three experiments on cells from the same animal.

liferation assays, [^{3}H]thymidine was added 8 to 12 h before harvest and the radioactivity of the harvested cells was measured by a β-counter (MATRIX 9600; Packard, Meriden, Conn.). Medium alone was used as a control in proliferation assay. Addition of BL3* supernatant increased PBMC proliferation more than 10-fold. However, antigen-specific PBMC proliferation was dramatically suppressed with increasing concentrations of PGE_2 (Fig. 4). Spontaneous cell proliferation in the absence of antigens was also slightly reduced by addition of PGE_2 (Fig. 4). Thus, PGE_2 enhanced BLV detection but suppressed antigen-specific PBMC proliferation. The concentration of cells differed between transcription and proliferation assays, as fewer cells were optimal in the U-bottom wells for the proliferation assay.

The results presented here demonstrate that IL-10 inhibited detection of BLV *tax* and *pol* mRNA and reduced COX-2 transcription from macrophages, while PGE_2 activated BLV *tax* and *pol* mRNA. These data indicate that regulation of BLV is closely related to signals induced by PGE_2. Both AL and PL animals, but not noninfected animals, produced similar findings when tested with IL-2, IL-10, and PGE_2, supporting a common mechanism of pathogenesis in infected animals. Also, in spite of the relatively low IL-10 levels, AL animals produced more IL-10 than uninfected animals, and AL and PL animals responded similarly to IL-10 and PGE_2. These findings suggest that macrophages produce PGE_2 and have a central role in regulating BLV expression in infected B cells (Fig. 5).

The E series of prostaglandins are widely known as immunosuppressive products produced by macrophages, follicular dendritic cells, and fibroblasts (10, 17). These prostaglandins, especially PGE_2, can downregulate many aspects of B- and T-cell functions. PGE_2 production is triggered by inflammatory cytokines, such as IL-1 and TNF-α, that are produced in viral and bacterial infections (4, 20). Increasing PGE_2 negatively regulates type 1 cytokines, such as IL-2, IFN-γ, and TNF-α, by increasing production of type 2 cytokines, such as IL-10 (13). Thus, PGE_2 may have a central role in regulating production of type 1 and type 2 cytokines. PGE_2 activates a humoral immune response, stimulating B-cell differentiation and immunoglobulin class switching (26). In this paper, we show that PGE_2 stimulated detection of BLV *tax* and *pol* mRNA and inhibited PBMC proliferation. These findings suggest that enhancement of BLV expression by PGE_2 may not depend on cell proliferation. Ironically, B cells are the only PBMCs that are significantly infected with BLV (22), while macrophages are the only source of PGE_2 in PBMCs (14). However, B cells

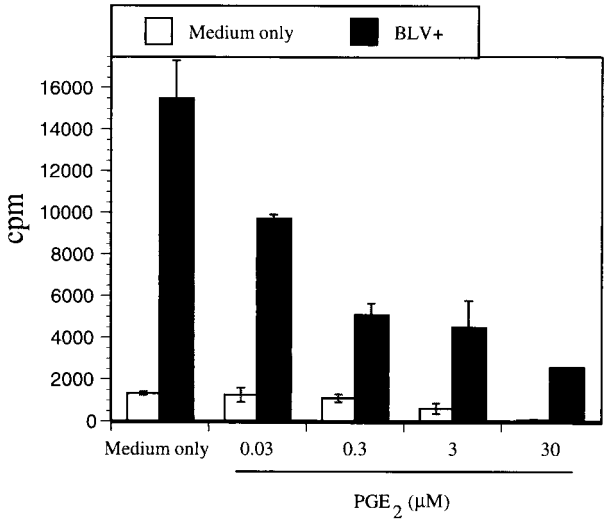

FIG. 4. PGE_2 inhibits BLV-specific PBMC proliferation. BLV-infected PBMCs from AL and PL animals were cultured with BL3* supernatant (BLV+) or medium alone (BLV−) for 5 days with different concentrations of PGE_2 (0.03 to 30 μM). Proliferation was assessed by [^{3}H]thymidine incorporation using a β-scintillation counter. The data are representative of experiments with four different AL and PL animals. Standard errors of means are shown.

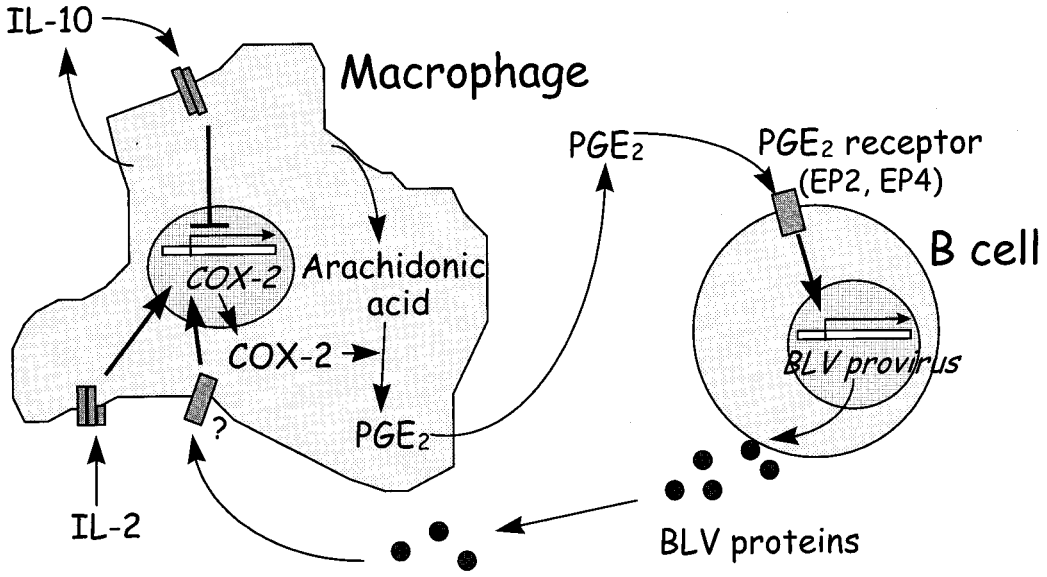

FIG. 5. Possible linkage between BLV expression by B cells and COX-2 and PGE_2 produced by macrophages. Arrows represent positive regulation, and a blunt line represents negative regulation.

have a number of PGE_2 receptors that regulate B-cell activation (7). Signal transduction by PGE_2 receptors mediates increased cyclic AMP (cAMP) production (3). BLV long terminal repeats (LTRs) contain a cAMP-response element (CRE) that facilitates BLV gene transcription (1, 39). Tax stabilizes CRE-binding protein (CREB) to bind CRE in LTRs (5). Thus, CREB and Tax may activate BLV expression of infected B cells. In addition, protein kinase C (PKC) increases BLV expression with increased Ca^{2+} influx (15). Therefore, increased PGE_2 production by macrophages may stimulate BLV *tax* and *pol* mRNA expression through cAMP-dependent PKA and/or PKC signal transduction pathway. BLV LTRs also contain NF-κB binding sites that facilitate BLV transcription (6). Recently, antiinflammatory agents, such as aspirin and salicylate, reportedly inhibit the activity of IκB kinase-β, which facilitates the degradation of IκB and activates NF-κB (40). Antiinflammatory agents that inhibit prostaglandin synthesis may suppress BLV expression via NF-κB inhibition.

We demonstrate that BLV functions as a stimulant of COX-2 expression. Although PGE_2 enhances IL-10 expression (13) to inhibit COX-2 and BLV expression, a synergistic effect of BLV expression, opportunistic infections (35), pregnancy (19), and/or stress (8) could induce disease progression in BLV infection. COX-2 and PGE_2 also inhibit programmed cell death and facilitate tumor formation (33, 34), and thus these activities might promote lymphosarcoma and leukemia with other carcinogenic factors, such as Bcl-2 and BLV Tax protein, in BLV infection. Thus, in other retrovirus infections, the inhibitory function of IL-10 on human immunodeficiency virus expression has been reported (24, 30, 31). Most studies have utilized macrophage cell lines and primary macrophages, while the studies with T-cell lines or primary T cells failed to demonstrate the IL-10 inhibition of human immunodeficiency virus expression. Therefore, macrophages may have a direct role in regulating retrovirus expression responding to IL-10 (32). Our preliminary data showed that bovine herpes virus type 1 and *Brucella abortus*, two common opportunistic infections in cattle, activated COX-2 mRNA expression (D. Pyeon and G. A. Splitter, unpublished data). We anticipate that further research regarding PGE_2 and opportunistic infections will reveal additional clues to solve the complicated mechanisms of disease progression in retrovirus infections.

To examine whether inhibitors of PGE_2 or COX-2 would be efficacious for treatment of BLV infection, in vivo studies are necessary. Infected animals could be treated with a PGE_2 inhibitor, such as indomethacin, and viral load and BLV expression could be measured. Treatment with a PGE_2 inhibitor may reduce BLV load in BLV-infected animals and would support a role for PGE_2 to stimulate BLV replication and disease progression in vivo. This study provides an additional strategy to treat retrovirus infection combined with currently available antiretroviral treatment.

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