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# An Exploratory Trial of Cyclooxygenase Type 2 Inhibitor in HIV-1 Infection: Downregulated Immune Activation and Improved T Cell-Dependent Vaccine Responses▽

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**Chronic HIV infection is characterized by chronic immune activation and dysfunctional T cells with elevated intracellular cyclic AMP (cAMP), which inhibits the T cell activation capability. cAMP may be induced by prostaglandin $E_2$ following lipopolysaccharide (LPS)-induced upregulation of cyclooxygenase type 2 (COX-2) in monocytes due to the elevated LPS levels in patients with chronic HIV infection. This hypothesis was tested using celecoxib, a COX-2 inhibitor, for 12 weeks in HIV-infected patients without antiretroviral treatment in a prospective, open, randomized exploratory trial. Thirty-one patients were randomized in the trial; 27 completed the study, including 13 patients on celecoxib. Celecoxib reduced chronic immune activation in terms of CD38 density on $CD8^+$ T cells (−24%; $P = 0.04$), IgA levels ($P = 0.04$), and a combined score for inflammatory markers ($P < 0.05$). Celecoxib further reduced the inhibitory surface receptor programmed death 1 (PD-1) on $CD8^+$ T cells ($P = 0.01$), including PD-1 on the HIV Gag-specific subset ($P = 0.02$), enhanced the number of $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{lo/-}$ Treg or activated cells ($P = 0.02$), and improved humoral memory recall responses to a T cell-dependent vaccine ($P = 0.04$). HIV RNA ($P = 0.06$) and D dimers ($P = 0.07$) tended to increase in the controls, whereas interleukin-6 (IL-6) possibly decreased in the treatment arm ($P = 0.10$). In conclusion, celecoxib downmodulated the immune activation related to clinical progression of chronic HIV infection and improved T cell-dependent functions *in vivo*.**

Chronic immune activation accompanies chronic HIV infection and accelerates the development of immune deficiency. Whereas the hallmark of primary HIV infection is a substantial $CD4^+$ T cell loss at mucosal sites and elsewhere (20), chronic HIV infection is characterized by the inability of the immune system to control viral replication (22) as well as by a state of chronic immune activation. Mucosal translocation of microbial bioactive substances such as lipopolysaccharides (LPS) may be an important stimulus of this chronic immune activation (4, 5, 9, 11, 14, 20, 29, 40, 42, 49). In fact, markers of chronic activation, such as CD38 on $CD8^+$ T cells, appear to be better suited to predicting clinical progression in HIV infection than HIV RNA levels and $CD4^+$ T cell counts (18, 24, 26, 28, 46). Targeting the HIV-related chronic immune activation may therefore be a therapeutic strategy *per se*.

The current trial was based on our observations that augmented levels of cyclic AMP (cAMP) contribute to T cell dysfunction in HIV-infected patients (2, 3, 30). In T cells, cAMP triggers a protein kinase A-Csk-Lck inhibitory pathway that inhibits the proximal T cell receptor (TCR) signaling events (2, 3, 48, 54). This mechanism may also be involved in the inhibitory function of regulatory T cells (Tregs) (39). Elevated levels of cAMP in T cells from HIV-infected individuals (2) may result from increased production of prostaglandin $E_2$ ($PGE_2$) in lymphoid tissues following activation-induced synthesis of cyclooxygenase type 2 (COX-2). Although we have identified COX-2-positive T cells in HIV-infected individuals (36), activated monocytes may be the major source of $PGE_2$ (25). High levels of COX-2 are produced *de novo* in monocytes, particularly upon exposure to LPS (10, 27). Circulating LPS is indeed increased in untreated chronic HIV infection due to enhanced translocation of microbial material from the gut and correlates with chronic immune activation and disease progression (20).

We tested this hypothesis by abrogating COX-2 function with a COX-2 inhibitor (COX-2i) in HIV-infected patients off antiretroviral treatment (ART) and studied whether this drug could downregulate chronic immune activation and improve T cell functions in a proof-of-concept exploratory clinical trial, with the possibility that COX-2 inhibition could provide clini-

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cal benefit. In two previous clinical exploratory trials, we demonstrated that COX-2 inhibition improved immune functions in HIV-positive patients on ART (30, 36). In this trial, downregulation of chronic immune activation was assessed primarily by measuring CD38 on $CD8^+$ T cells. T cell responsiveness was tested *in vitro* as well as *in vivo* in a vaccine substudy where humoral vaccine responses to a T cell-dependent vaccine were tested, with coadministration of a T cell-independent vaccine as a control.

Chronic immune activation *per se* may contribute to enhanced cardiovascular risk in HIV-infected individuals (33, 45, 49). If COX-2i reduces chronic immune activation, this excess cardiovascular risk could be lowered by COX-2i. Although COX2i has been associated with cardiovascular events in predisposed patients (47, 51), rofecoxib actually reduced the risk markers interleukin-6 (IL-6) and C-reactive protein (CRP) in patients with ischemic heart disease (8). Patients having cardiovascular risk factors were therefore excluded, and parameters reflecting activated coagulation and endothelial damage were monitored. This *a priori* risk-versus-benefit analysis justified a limited exploratory proof-of-concept trial.

## MATERIALS AND METHODS

**Patients, study regulations, and end points.** Adult (18 to 65 years), asymptomatic, HIV-1-positive patients off ART were recruited into this open, randomized, explorative trial. All patients gave their informed consent. The study was approved by the Norwegian Medicines Agency (European Union Drug Regulating Authorities; clinical trial no. 2006-001882-41) and the Regional Committee for Medical Research Ethics, with the following inclusion criteria: confirmed diagnosis of HIV infection less than 8 years prestudy, no HIV-related clinical manifestations, no current indication for or use of ART according to European guidelines, HIV RNA level of >6,000 copies/ml, and $CD4^+$ T cell count of $>300 \times 10^6$/liter. If patients were ART experienced, treatment should have been terminated more than 1 year prestudy. Exclusion criteria were concomitant use of nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, or alpha interferon (IFN-α), total cholesterol of >7 μM, elevated serum creatinine, creatinine clearance of <30 ml/min, diabetes, hypertension, heart failure, ischemic heart disease, peripheral arteriosclerosis and/or cerebrovascular disease, cardiovascular events or stroke in parents, siblings, or offspring of <55 years of age, pregnancy, deranged liver function, or inflammatory bowel disease. Additionally, exclusion criteria from the celecoxib summary of product characteristics were applied.

Patients who met the inclusion criteria were randomized into an open control arm receiving no drug or a treatment arm receiving high-dose celecoxib (400 mg twice a day [BID] [800 mg/day]) for 12 weeks. One patient received 200 mg BID (400 mg/day) due to low body weight. The study participants were examined at the times of screening (week −4) and inclusion (week 0) and at weeks 6, 12, and 18, with sampling for analyses at 0 and 12 weeks.

The density of CD38 on $CD3^+$ $CD8^+$ T cells served as the primary end point of the study. Secondary end points included $CD4^+$ T cell counts, HIV RNA levels, immunoglobulin and $\beta_2$-microglobulin levels, HIV-related clinical events, and indication for ART. Safety was evaluated by the need for dose reduction or cessation due to adverse effects.

**Sample processing and routine laboratory parameters.** Plasma was snap-frozen at −70°C from three different tubes (Becton Dickinson [BD], San Diego, CA), containing either EDTA or buffered citrate or presupplemented with 30 IU of LPS-free heparin at 100 IU/ml (Leo Pharma A/S, Ballerup, Denmark). Serum was prepared at the same time in a serum separator tube and immediately stored in aliquots at −70°C. Peripheral blood mononuclear cells (PBMC) were isolated at baseline and at study week 12 in cell preparation tubes (CPTs; BD) with sodium heparin and both analyzed immediately and stored in 40% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) at −145°C until analysis. Other markers, including CD4+ T cell counts and HIV RNA levels, were quantified by use of a TriTEST reagent kit (BD) and the Cobas Amplicor HIV-1 Monitor test (Roche, Branchburg, NJ), respectively.

**Reagents and antibodies.** The following antibodies and reagents were obtained from BD: anti-CD3–allophycocyanin (APC) and Pacific Blue-anti-CD4–peridinin chlorophyll protein (PerCP) and –phycoerythrin (PE)-Cy7, anti-CD5–APC, anti-CD8–PerCP and –AmCyan, anti-CD19–PerCP, anti-CD28 (unconjugated [1 mg/ml]), anti-CD28–fluorescein isothiocyanate (FITC), anti-CD38–PE and –Quantibrite PE, –Quantibrite PE beads, anti-CD45RA–APC, anti-CD45RO–FITC, anti-CD107a–FITC, anti-CD127–PE, anti-CCR7–PE and –PE-Cy7, anti-HLA-DR–APC and –FITC, anti-IFN-γ–APC, anti-PD-1–FITC and –PE, anti-perforin–FITC, anti-tumor necrosis factor alpha–Alexa 700 (anti-TNF-α–Alexa 700), and IgG (IgG1 and IgG2a/b) isotype control antibodies. The following were purchased from eBioscience (San Diego, CA): anti-CD3–FITC, anti-CD25–APC, anti-CD27–APC and –Alexa 700, anti-CD154–PE, costimulatory anti-CD28, and monensin. In addition, anti-CD3–PE-Texas Red and anti-CD25–PE were delivered by Beckman Coulter and Miltenyi Biotec, respectively.

**Flow cytometry and activation assay.** The CD38 density on $CD8^+$ T cells and the presence of PD-1 were determined as described previously (28) and simultaneously poststudy for all samples to avoid day-to-day variation in the assay, despite an expected loss of CD38 expression in thawed samples (28). Cutoffs were determined by the use of a parallel fluorescence-minus-one (FMO) control and phenotype profiles of naïve/central memory (CM) ($CCR7^+$ $CD27^+$) or effector memory (EM) ($CCR7^+$ $CD27^-$, $CCR7^-$ $CD27^+$, or $CCR7^-$ $CD27^-$) $CD4^+$ and $CD8^+$ T cells on a FACSCanto II flow cytometer (BD). Fresh EDTA-blood was analyzed on a FACSCalibur flow cytometer (BD) as previously described (32), identifying CM ($CD45RO^+$ $CCR7^+$ $CD27^+$), intermediate memory (IM) ($CD45RO^-$ $CCR7^-$ $CD27^+$), and EM ($CD45RO^-$ $CCR7^-$ $CD27^-$) subsets of $CD4^{hi}$ lymphocytes. Tregs were identified by a $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{lo/-}$ phenotype (37).

$CD8^+$ and $CD4^+$ T cell-specific responses were determined in fresh PBMC cultured for 6 h in RPMI containing anti-CD107a and anti-CD154, respectively, in addition to 10% autologous serum, antigen, costimulatory anti-CD28 (1 μg/ml), and monensin (2 μM) (6, 23, 43). HIV-1 group M panels of overlapping 15-mer peptides from Gag, Env, or Nef at 2 mg/liter (NIH AIDS Research and Reference Reagent Program, Bethesda, MD) (38) or cytomegalovirus (CMV) lysate proteins (35) were used as antigens. Cells were finally surface stained with monoclonal antibodies (MAbs) to CD3, CD4 or CD8, and PD-1 before data acquisition and subsequent analyses with WinList 6.0 software (Verity SH, Topsham, ME). Antigen-specific responses were obtained by subtracting responses in control cultures without antigen.

**Soluble factors and parameters of activated coagulation or endothelial damage.** Tumor necrosis factor receptors (TNFRs) I and II, IL-6, and soluble CD14 (sCD14) were analyzed in plasma and serum by Quantikine enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN). Endotoxin from Gram-negative bacteria was assessed with a chromogenic *Limulus* amebocyte lysate test (LAL QCL-1000; Lonza Group Ltd., Basel, Switzerland) on thawed heparin-plasma.

von Willebrand factor (vWF) antigen, tissue factor pathway inhibitor (TFPI)-free antigen, and D dimers were analyzed with Asserachrom kits (Diagnostica Stago, Asnière, France). E-selectin, P-selectin, sICAM-1, and sVCAM-1 were assayed with respective Quantikine kits (R&D Systems Europe, Abingdon, United Kingdom). F1+2 and TAT were assayed with Enzygnost F1+2 and Enzygnost TAT kits, respectively (Dade-Behring, Marburg, Germany). Tissue factor (TF) antigen was assayed with an Imubind tissue factor ELISA kit (American Diagnostica, Stamford, CT). Plasminogen activator inhibitor type 1 (PAI-1) activity was analyzed using Spectrolyse/pL PAI (Biopool Trinity Biotech, Wicklow, Ireland).

***In vitro* stimulation setup.** Fresh PBMC were cultured in RPMI GlutaMAX (Gibco) containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 1× nonessential amino acids and used directly or depleted of $CD25^+$ cells prior to stimulation using human CD25 microbeads II (Miltenyi Biotec) at a high concentration (20 μl/100 μl cell suspension). The magnetically labeled cells were run through an LD depletion column (Miltenyi Biotec), providing highly purified $CD25^-$ cells (>99%). PBMC or $CD25^-$ cells ($10^6$ cells/ml) were stimulated specifically with a 15-mer Gag peptide pool (2 μg of each peptide/ml) in the presence of anti-CD28 (1 μg/ml) or nonspecifically with anti-CD2/3/28-coated microbeads (Miltenyi Biotec; bead-to-cell ratio of 1:5). Samples were pretreated for 12 h with the nonselective COX inhibitor indomethacin (25 μM; Sigma-Aldrich) or the cAMP antagonist Rp-8-Br-cAMPS (1 mM; BioLog) and run in parallel at 37°C and 5% $CO_2$ in a humidified atmosphere. PBMC and $CD25^-$ cells treated with anti-CD28 alone were used as background controls. Following preincubation, the cells were stimulated with Gag peptides or microbeads, as indicated, and incubated for 5 h, with the last 4 h conducted in the presence of brefeldin A (10 μg/ml).

Flow cytometric analyses of intracellular cytokine levels were performed on a FACS Aria flow cytometer (BD) after fixation, permeabilization, and staining of

TABLE 1. Description of study cohort at week 0 ($n = 27$)[c]

| Parameter[a] | Value for group[b] | |
|---|---|---|
| | Control | COX-2i treatment |
| No. of males:no. of females | 13:1 | 11:2 |
| No. of patients in risk group (MSM:HS:IDU:UK) | 10:3:0:1 | 6:4:0:3 |
| No. of Caucasians:no. of Asians:no. of black patients | 12:0:2 | 10:1:2 |
| Age (yr) | 40 (34–42) | 39 (31–46) |
| Time of HIV infection (mo) | 35.7 (14.5–69.4) | 27.7 (14.7–40.8) |
| CD4 cell count ($\times 10^6$/liter) | 405 (340–490) | 450 (300–550) |
| CD8 cell count ($\times 10^6$/liter) | 1,110 (800–1,550) | 1,320 (920–1,930) |
| HIV RNA ($\times 10^3$ copies/ml) | 46 (22–190) | 36 (17–91) |
| $\beta_2$-Microglobulin (mg/liter) | 3.2 (2.4–4.1) | 2.8 (2.6–3.1) |

[a] MSM, men who have sex with men; HS, heterosexual; IDU, intravenous drug use; UK, unknown.
[b] Data are means and IQR unless shown otherwise.
[c] Differences in age, time of HIV infection, CD4 and CD8 cell counts, HIV RNA levels, and $\beta_2$-microglobulin levels were not significant by MWU.

the cells as described previously (52). Fixed and permeabilized cells were stained with anti-CD3–PE-Texas Red, anti-CD4–PE-Cy7, anti-CD25–PE, anti-IFN-γ–APC, and anti-TNF-α–Alexa 700 in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA and were analyzed with FlowJo v8.5.2 (Tree Star).

**Vaccination substudy with T cell-dependent and -independent antigens.** In a substudy, we tested the response to one injection of each of two standard vaccines at study week 6; 23 volunteers among the study participants gave their separate informed consent and received the T cell-dependent tetanus toxoid (TT) vaccine (Tetavax; Sanofi Pasteur MSD), and 21 also received the T cell-independent pneumococcal polysaccharide vaccine (PP) (Pneumovax 23; Sanofi Pasteur MSD). IgG antibodies to the tetanus toxoid (50) and 23-valent pneumococcal polysaccharide vaccines were both measured with ELISA in the same assay poststudy, using thawed sera, the latter after C polysaccharide adsorption of sera (1). Vaccine responses to TT and PP vaccines were assessed as differences in specific IgG levels between study week 12 and the time of vaccination (week 6).

**Statistical analyses.** Statistical analyses were performed with Statistica v7 (StatSoft Inc., Tulsa, OK) and SPSS Statistics v16 (SPSS Inc., Chicago, IL). Data are presented as median values (25th to 75th interquartile range [IQR]) unless stated otherwise. Nonparametric two-tailed statistical methods were used throughout, including Spearman rank correlation analysis, the Mann-Whitney U test (MWU) for groupwise comparisons, and the two-tailed Wilcoxon matched-pair test (WMP), to determine time-dependent changes in dependent variables within each study arm. WMP was preferred for the "as treated" analyses presented here because of a potential selection bias caused by the exclusion of patients with rash in the treatment group which prohibited groupwise comparisons (see below). The two-tailed Fisher exact test (FiT) was used to test proportional changes. *P* values are presented throughout, without comments on significance levels.

## RESULTS

**Patient inclusion and adverse drug reactions.** In the present study, 31 HIV-infected volunteers who met the study inclusion and exclusion criteria were enrolled consecutively; 14 individuals were randomized to the control arm, and 17 individuals were randomized to receive treatment with high-dose celecoxib for 12 weeks. Four patients receiving celecoxib were excluded after developing generalized urticaria-like exanthema by day 12 or 13 without concurrent systemic manifestations or signs of internal organ dysfunction. After immediate discontinuation of COX-2i, the skin reactions resolved quickly and spontaneously ($n = 1$) or by administration of antihistamines ($n = 2$) or corticosteroids ($n = 1$).

The remaining 13 patients who were randomized to receive celecoxib completed the study. One of them experienced an elevation of liver enzymes (≤5× upper limit of normal [ULN]) at week 12, which normalized rapidly upon treatment discontinuation. Dose reduction to 200 mg BID was undertaken in one patient due to transient celecoxib-related tinnitus. Another patient on celecoxib experienced substantial improvement of generalized muscle and joint pain which he had suffered since before he was diagnosed with HIV. The baseline data for the 27 patients who completed the study are presented in Table 1.

**Decrease in CD38 density (primary end point).** The change in the density of CD38 on $CD8^+$ T cells was the formal primary end point of the study, and the densities in both study groups were similar at inclusion ($P = 0.28$) (see Fig. 1A for gating strategy). However, when only the 27 patients who completed the study were evaluated in "as treated" analyses, the baseline CD38 levels tended to be lower for the remaining 13 patients on celecoxib than for the controls ($P = 0.09$; MWU) (Fig. 2, upper left panels). This suggested that the four patients who experienced rash may have had higher levels of immune activation at baseline, with higher median CD38 densities, than those in the whole treatment arm (7,125 [4,011 to 9,260] molecules/cell versus 3,587 [2,741 to 4,494] molecules/cell). Moreover, three (75%) of them had elevated $\beta_2$-microglobulin levels, above 3.0 mg/liter, versus four patients (31%) among the controls. Because this potential bias weakened the conditions

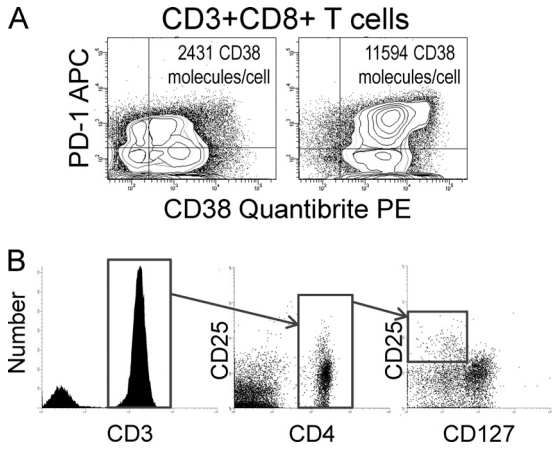


FIG. 1. Gating strategies for CD38 density and the $CD4^+$ $CD25^+$ $CD127^{lo/-}$ T cell subset. (A) Scatter plots on CD38 and PD-1 after primary gating on the lymphocyte live gate in forward scatter (FSC)-side scatter (SSC) plots and secondary gating on $CD3^+$ $CD8^+$ T cells in CD3-CD8 scatter plots for two different patients with relatively high and low CD38 densities. FMO cutoffs are indicated by horizontal and vertical lines. CD38 densities within the $CD3^+$ $CD8^+$ $PD\text{-}1^+$ $CD38^+$ T cells are indicated in quadrant 2 (2,095 and 10,054 molecules/cell). PBMC were stained with Quantibrite PE–CD38 MAb having one PE molecule per MAb. A PE standard curve was obtained with a mixture of Quantibrite PE bead batches, each with a defined number of PE molecules per bead. The CD38 density in a cell population was determined from extrapolation of the median fluorescence intensity of the anti-CD38–Quantibrite PE signal to the standard curve according to the manufacturer's instructions. (B) One- and two-parameter histograms after primary gating on the lymphocyte live gate in FSC-SSC plots, demonstrating the identification of $CD3^+$ $CD4^+$ $CD25^+$ $CD127^{lo/-}$ T cells.

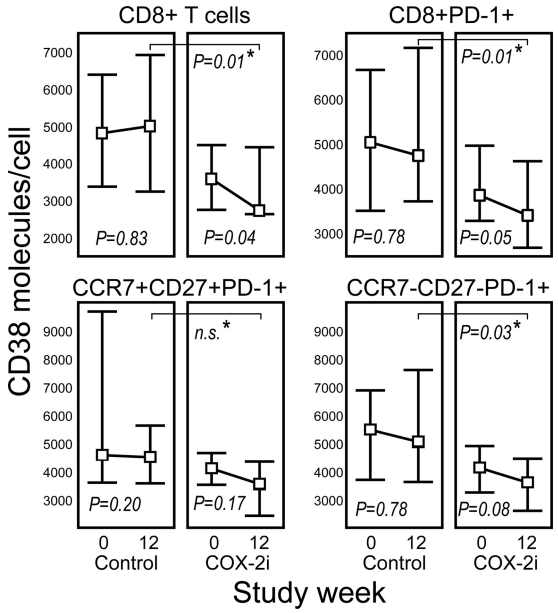


FIG. 2. Reduced expression of CD38 following celecoxib treatment. Changes in CD38 density (molecules/cell) in celecoxib (COX-2i)-treated and control groups from weeks 0 to 12 are shown for different T cell subsets, including total $CD8^+$ and $CD8^+$ $PD\text{-}1^+$ T cells (top panels) and the PD-1-positive subsets of $CCR7^+$ $CD27^+$ and $CCR7^-$ $CD27^-$ memory $CD8^+$ T cells (bottom panels). Median values and interquartile ranges (25th to 75th percentiles) are indicated. *P* values of <0.10 are shown in the relevant panels (Wilcoxon matched-pair test). Groupwise differences between the COX-2i-treated and control groups at week 12 were assessed by MWU and are indicated by asterisks. n.s., not significant.

required for comparisons between randomized groups, pairwise statistics (WMP) were applied within each group. By this analytical approach, the median CD38 density on $CD8^+$ T cells was reduced in the COX-2i-treated group (−24% [3,587 molecules/cell at week 0 to 2,722 molecules/cell at week 12]; $P = 0.04$) but was stable in the control arm (+4%; $P = 0.83$) (Fig. 2, upper left panels).

CD38 densities on PD-1-positive $CD8^+$ T cells and on certain differentiated subsets may predict $CD4^+$ T cell loss rates better than the median CD38 density for all $CD8^+$ cells (28). In this case, CD38 density on PD-1-positive $CD8^+$ T cells again decreased in the treatment arm ($P = 0.05$) but not in controls ($P = 0.78$) (Fig. 2, upper right panels), with statistically similar levels at baseline ($P = 0.17$; MWU).

Among other $CD8^+$ T cell subsets, CD38 probably decreased in celecoxib-treated patients in both $CD8^+$ effector memory ($CCR7^-$ $CD27^-$) T cells ($P = 0.09$) (data not shown) and their PD-1-positive subset ($P = 0.08$) (Fig. 2, lower right panels). For the latter subset, groupwise comparison between the two study arms suggested that there was a difference between the two ($P = 0.09$; MWU) at baseline which was not present when all patients were taken into account, i.e., including those who developed a celecoxib-related rash ($P = 0.36$; MWU).

No changes in the control and treatment arms were found for CD38 densities on $CD4^+$ $CD38^+$ T cells ($P = 0.83$ and 0.55, respectively) and $CD4^+$ $CD38^+$ $PD\text{-}1^+$ T cells ($P = 0.88$ and 0.70, respectively).

**Effects of celecoxib on other markers of chronic immune activation.** HIV-related hyperimmunoglobulinemia was reduced by celecoxib, as IgA decreased among the celecoxib-treated patients (−19% [$P = 0.04$]; difference for controls, +3% [$P = 0.19$]) (Table 2). Among the IgG subclasses, IgG2, which is produced by plasma cells in response to T cell-independent antigens such as polysaccharides, possibly changed in opposite directions in the celecoxib arm (decreasing; $P = 0.06$) and the control arm (increasing; $P = 0.07$) (Table 2).

Notably, median values for micro-CRP, sICAM-1, TNFR-II, IgM, IgG1, and sCD14 levels appeared numerically lower for celecoxib-treated patients at week 12 and higher or stable for controls, but not significantly so for each individual parameter. However, combined binary trend scores for all immune function parameters for each patient differed between the study arms ($P < 0.05$; data not shown).

**HIV-related parameters.** $CD4^+$ and $CD8^+$ T cell counts were stable and similar in both groups, whereas HIV RNA levels in the control group probably increased (+10,000 copies/ml; $P = 0.06$), in contrast to those in the treatment group ($P = 0.25$) (Table 2).

**Celecoxib-related reduction of PD-1.** HIV-related T cell dysfunction has been associated with high expression of PD-1 (28, 53). Interestingly, PD-1 density appeared to decrease after celecoxib treatment in both $CD4^+$ and $CD8^+$ T cells ($P = 0.06$ and $P = 0.01$, respectively) and among the activated $CD38^+$ and $CD38^+$ $HLA\text{-}DR^+$ $CD8^+$ T cell subsets ($P = 0.02$ and $P = 0.04$, respectively) (Fig. 3A). Further analyses showed that the celecoxib-related reduction in PD-1 levels between week 0 and week 12 was most prominent within the $CCR7^-$ $CD27^-$ effector $CD8^+$ T cell subset ($P = 0.03$) (Fig. 3A).

**Celecoxib-related changes in HIV-specific T cells.** The frequencies and total numbers of Gag, Env, and Nef peptide panel-specific $CD8^+$ T cell subsets were evaluated in fresh PBMC at weeks 0 and 12. In agreement with the COX-2i-related general reduction of PD-1 density on T cells discussed above, celecoxib-treated patients had a 14% reduction in the frequency of Gag-specific $CD8^+$ T cells expressing PD-1 ($P = 0.02$) (Fig. 3B). Other changes in antigen-specific cells were observed only among control patients, whose total numbers of Env- and Nef-specific cells became smaller ($P = 0.04$ and 0.05, respectively), whereas the corresponding frequencies of their CMV-specific T cells increased ($P = 0.02$) (data not shown).

**COX-2i-related changes in other T cell subsets.** The number of circulating Tregs may decrease over time in chronic HIV infection (31). Tregs, defined here as $CD4^+$ $CD25^+$ $CD127^{lo/-}$ cells (see Fig. 1B for gating strategy), increased by 84% ($P = 0.02$) in the celecoxib-treated group, in contrast to the case for controls (−13%; $P = 0.83$) (Fig. 4). Similarly, the absolute number of circulating Tregs displayed an upward trend in COX-2i-treated individuals ($P = 0.06$) (Table 2; Fig. 4). Furthermore, central memory $CD4^+$ T cells decreased in the control arm over the 12-week study period ($P = 0.02$; data not shown).

**T cell responsiveness *in vitro*.** To explore the effects of celecoxib on T cell responsiveness, fresh PBMC were isolated and stimulated *ex vivo* at baseline and at the end of the study without any further supplementation of COX inhibitors *in*

TABLE 2. Immunological and hemostatic parameters

| Parameter | Value for control group[a] | | *P* value[b] | Direction of change[c] | Value for Cox-2i-treated group[a] | | *P* value[b] | Direction of change[c] |
|---|---|---|---|---|---|---|---|---|
| | Week 0 | Week 12 | | | Week 0 | Week 12 | | |
| Markers of chronic immune activation | | | | | | | | |
| CD4 cell count ($\times 10^6$/liter) | 405 (340–490) | 380 (290–470) | 0.37 | | 400 (300–550) | 390 (340–540) | 0.66 | |
| CD8 cell count ($\times 10^6$/liter) | 1,110 (800–1,550) | 1,170 (770–1,740) | 0.59 | | 1,320 (920–1,710) | 1,340 (1,150–1,740) | 0.75 | |
| HIV-RNA level ($\times 10^3$ copies/ml) | 46 (22–190) | 56 (26–120) | **0.06** | ↑ | 36 (17–91) | 41 (29–110) | 0.25 | |
| $\beta_2$-Microglobulin level (mg/liter) | 3.2 (2.4–4.1) | 3.0 (2.4–3.1) | 0.97 | | 2.8 (2.6–3.1) | 2.8 (2.2–3.2) | 0.21 | |
| IgA level (g/liter) | 3.1 (2.8–3.8) | 3.2 (2.6–4.2) | 0.19 | | 3.6 (2.7–4.3) | 2.9 (2.6–3.7) | **0.04** | ↓ |
| Total IgG level (g/liter) | 21.2 (16.3–22.6) | 19.3 (16.7–26.6) | 0.48 | | 18.8 (17.0–20.4) | 19.1 (15.8–20.2) | 0.30 | |
| IgG2 level (g/liter) | 2.0 (1.5–3.0) | 2.0 (1.6–3.4) | **0.07** | ↑ | 2.8 (2.0–3.4) | 2.6 (1.7–3.0) | **0.06** | ↓ |
| CRP level (mg/liter) | 1.3 (0.8–2.7) | 2.6 (1.3–3.9) | 0.26 | | 1.5 (0.7–3.3) | 1.3 (1.2–4.4) | 0.97 | |
| IL-6 level (pg/ml) | 2.4 (1.8–3.0) | 1.7 (1.4–2.4) | 0.78 | | 1.8 (1.5–2.8) | 1.8 (1.2–2.2) | **0.10** | ↓ |
| sCD14 level (ng/ml) | 1,660 (1,414–1,870) | 1,769 (1,582–1,881) | 0.55 | | 1,786 (1,560–1,901) | 1,682 (1,610–1,989) | 0.86 | |
| LPS level (EU/liter) | 544 (476–616) | 514 (472–528) | 0.55 | | 488 (448–584) | 536 (432–600) | 0.63 | |
| sICAM-1 level (ng/ml) | 290 (238–311) | 288 (263–386) | **0.07** | ↑ | 329 (264–347) | 290 (259–346) | 0.70 | |
| sVCAM-1 level (ng/ml) | 1,171 (1,030–1,452) | 1,311 (1,024–1,430) | 0.51 | | 1,242 (923–1,330) | 1,247 (1,143–1,367) | 0.31 | |
| Lymphocyte subsets | | | | | | | | |
| % B cells | 4.5 (3.5–7.0) | 5.4 (3.6–6.5) | 0.51 | | 4.6 (3.5–6.5) | 5.0 (3.5–6.7) | 0.48 | |
| % $CD4^+$ T regulatory cells | 4.6 (2.8–5.8) | 4.0 (2.4–5.7) | 0.83 | | 3.2 (2.7–3.8) | 5.9 (5.0–7.0) | **0.02** | ↑ |
| Absolute no. of Treg cells ($\times 10^6$/liter) | 19.3 (15.2–22.3) | 20.1 (10.6–23.9) | 0.13 | | 11.0 (10.0–22.5) | 22.9 (18.7–27.2) | **0.06** | ↑ |
| HIV specific responses on $CD8^+$ $CD107a^+$ T cells | | | | | | | | |
| % Gag responses | 0.91 (0.35–2.70) | 1.06 (0.69–2.67) | 0.94 | | 2.06 (1.26–3.36) | 0.65 (0.48–1.50) | 0.27 | |
| % PD-1 on Gag-responsive cells | 87.6 (77.3–90.3) | 87.5 (79.3–94.4) | 0.64 | | 86.5 (82.2–94.3) | 75.0 (65.9–90.9) | **0.02** | ↓ |
| % Env responses | 0.12 (0.09–0.61) | 0.12 (0.03–0.48) | 0.31 | | 0.26 (0.11–0.97) | 0.12 (0.04–0.34) | 0.21 | |
| % PD-1 on Env-responsive cells | 56.7 (40.8–70.8) | 45.3 (25.0–68.8) | 0.52 | | 77.5 (62.5–85.7) | 71.7 (48.3–77.8) | **0.09** | ↓ |
| % Nef responses | 0.86 (0.55–1.98) | 0.85 (0.38–1.30) | 0.31 | | 0.87 (0.38–1.70) | 1.21 (0.69–2.25) | 0.53 | |
| % PD-1 on Nef-responsive cells | 87.1 (64.8–88.7) | 68.4 (65.1–86.7) | 0.12 | | 82.0 (76.4–87.5) | 77.9 (56.6–86.1) | 0.14 | |
| Parameters of coagulation and endothelial damage | | | | | | | | |
| D dimer level (ng/ml) | 266 (178–398) | 332 (222–455) | **0.10** | ↑ | 303 (211–339) | 315 (251–505) | 0.70 | |
| % von Willebrand factor antigen | 123 (96–147) | 129 (103–182) | **0.07** | ↑ | 144 (114–191) | 137 (95–164) | 0.53 | |

[a] Data are medians (IQR).
[b] By Wilcoxon matched-pair test.
[c] The direction of change (up or down) in medians between baseline and the end of the study is listed when the *P* value is <0.10.

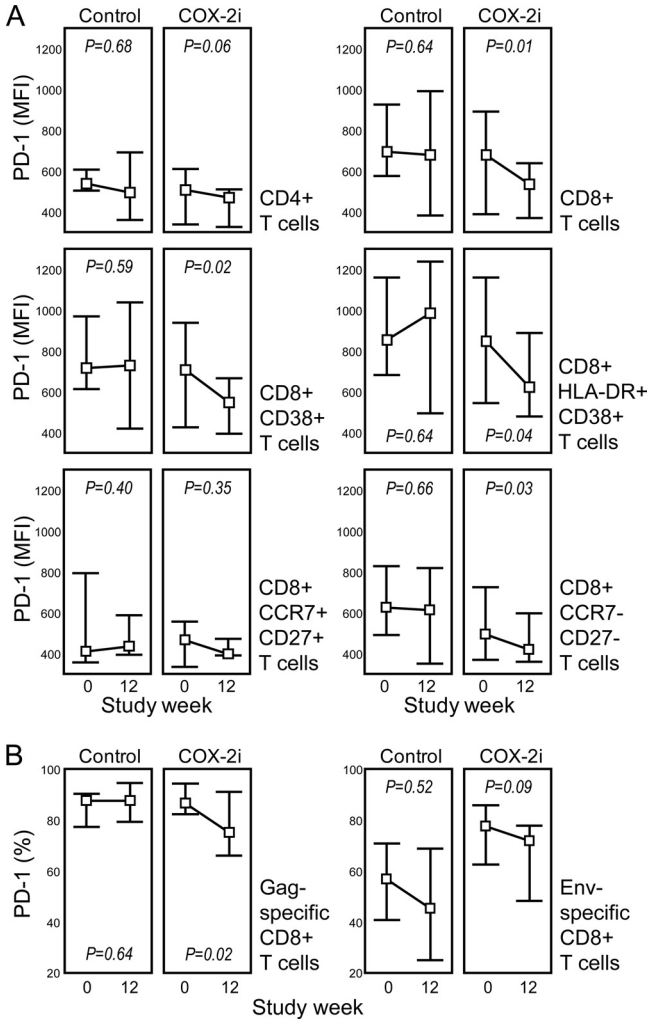


FIG. 3. Celecoxib causes downregulation of surface-expressed PD-1 on T cells. (A) MFI of PD-1 on $CD4^+$ and $CD8^+$ T cells (top row), activated $CD8^+$ T cells (HLA-$DR^{+/-}$ $CD38^+$; second row), and $CCR7^-$ $CD27^-$ and $CCR7^+$ $CD27^+$ $CD8^+$ memory T cell subsets (third row). (B) Percentages of PD-1-positive cells among Gag- and Env-specific $CD8^+$ T cells at 0 and 12 weeks in both study arms, as indicated. Median values and interquartile ranges (25th to 75th percentiles) are indicated.

*vitro*, except for samples that were treated with indomethacin. Ten and nine complete pairs of fresh samples from weeks 0 and 12 were obtained from the treatment and control groups, respectively, due to low yields of PBMC from the remaining individuals. The responses to *ex vivo* treatment with indomethacin of PBMC from the celecoxib-treated group improved significantly from week 0 to week 12. Thus, $CD8^+$ and $CD4^+$ T cells from celecoxib-treated but not control individuals displayed only moderate increases in both IFN-γ ($P = 0.02$ and 0.06, respectively) and TNF-α ($P = 0.02$ and 0.04, respectively) production in the presence of indomethacin (Fig. 5).

In order to address possible effects of celecoxib treatment on Treg cells, the pan-T bead-mediated stimulation of PBMC was compared to that of PBMC depleted of $CD25^+$ cells ($CD25^-$ cells). Notably, the effect of removing $CD25^+$ cells may be positive or negative, depending on the relative contributions of

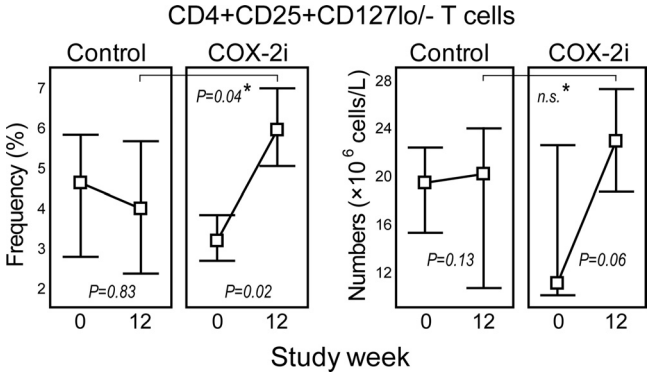


FIG. 4. Increases in absolute numbers and frequencies of regulatory T cells upon celecoxib treatment. The relative proportions (percentages of T cells) (left) and absolute numbers ($\times 10^6$ cells/liter) (right) of Tregs increased in the celecoxib treatment study arm. Median values and interquartile ranges (25th to 75th percentiles) are indicated. *P* values of <0.10 are shown in the relevant panels (Wilcoxon matched-pair test). Groupwise differences (MWU) between the COX-2i-treated and control groups at week 12 are indicated by asterisks.

Treg and activated effector cells to the $CD25^+$ population. Indeed, removal of $CD25^+$ cells resulted in diminished TNF-α responses in the $CD25^-$ PBMC at baseline in both groups ($P < 0.02$), suggesting that the depleted $CD25^+$ cell fractions con-

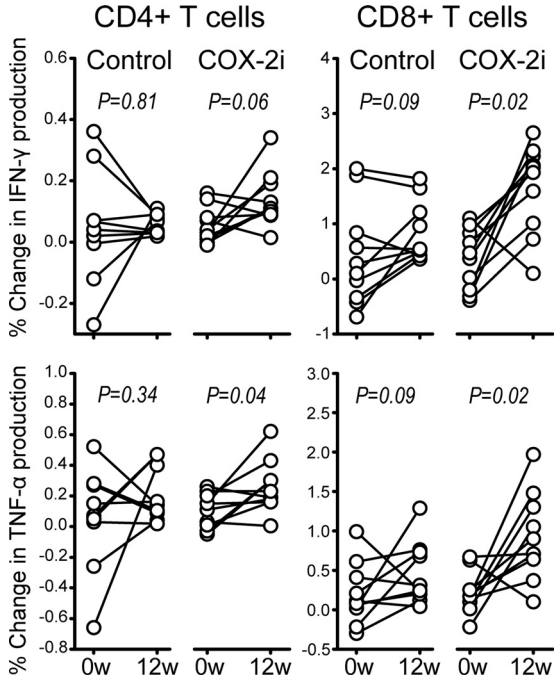


FIG. 5. Celecoxib treatment potentiates the effects of indomethacin on T cell responsiveness *in vitro*. We measured the effects of indomethacin on the ability of $CD4^+$ and $CD8^+$ T cells to produce the proinflammatory cytokines IFN-γ and TNF-α in response to stimulation with CD2/3/28-coated microbeads (compared to beads alone). PBMC were pretreated with indomethacin overnight prior to stimulation with beads. Celecoxib (COX-2i) treatment for 12 weeks potentiated the effects of indomethacin with respect to production of both IFN-γ and TNF-α in $CD4^+$ as well as $CD8^+$ T cells. Individual effects of indomethacin pretreatment are indicated for baseline and the study end at week 12.

tained mainly activated T cells (data not shown). The patients in the celecoxib arm demonstrated consistent reductions in both IFN-γ and TNF-α responses among $CD8^+$ $CD25^-$ T cells from week 0 to week 12, resulting in lower production of TNF-α and, possibly, IFN-γ at week 12 than the CD25-depleted $CD8^+$ T cell responses in controls ($P = 0.02$ and $P = 0.07$, respectively; MWU), as well as to reduced production of IFN-γ by CD25-depleted $CD4^+$ T cells ($P = 0.03$; MWU) (data not shown).

**Soluble factors of activation, coagulation, and endothelial damage.** No differences were found in the plasma levels of either LPS or sCD14 between the study arms during the study period (data not shown). IL-6, which is linked to both activation of HIV and development and destabilization of atherosclerotic plaques (8), developed differently in the two study groups. In the celecoxib group, IL-6 probably decreased ($P = 0.10$; WMP), in contrast to the case with controls ($P = 0.78$). No other changes in biological markers typically associated with increased cardiovascular risks (8, 15) were detected in the COX-2i-treated study arm. In contrast, in 11 of 14 control patients, D-dimer levels as well as vWF antigen percentages seemed to increase over the 12-week study period ($P = 0.07$ and $P = 0.10$, respectively) (Table 2).

**Vaccination substudy.** Twenty-three patients (9 on celecoxib) were voluntarily vaccinated with the TT vaccine at week 6; 21 of them (8 on celecoxib) were also vaccinated with the PP vaccine at the same time. The vaccination history was incomplete for most patients, and particularly so for the TT vaccine. The prevaccination antibody levels to the TT and PP vaccines were nevertheless similar at baseline and before the vaccinations at study week 6 ($P > 0.55$) (data not shown). Overall, the antibody levels increased from prevaccination at week 6 to the end of study at week 12 for both vaccines ($P < 0.05$).

Antibody levels to the T cell-dependent TT vaccine increased after vaccination, up to week 12, in both control ($P < 0.01$) and COX-2i-treated ($P = 0.01$) individuals. However, for adequate protection against TT in individuals with very low or undetectable antibody levels to this rarely encountered antigen, three vaccine doses are generally recommended in order to mount adequate responses, in contrast to the single TT dose given in this study. We therefore examined primarily the COX-2i-related immune memory recall responses and checked for a boosting effect by including patients with clearly detectable antibody levels at baseline only (>25th percentile at 0.29 IU/ml). Among these individuals, TT responses between weeks 6 and 12 were nearly three times higher in COX-2i-treated patients (0.70 IU/ml) than in controls (0.25 IU/ml) ($P = 0.04$) (Fig. 6).

For the T cell-independent PP vaccine, inverse response patterns were seen compared to those for the TT vaccine. A modest median increase in antibody levels among the COX-2i-treated individuals (50%; $P = 0.01$) was seen from week 6 to week 12, with corresponding responses in the control group reaching 342% ($P < 0.01$), with the latter showing an almost three times larger increase in anti-PP (median, 11.3 versus 4.0 U/ml; $P = 0.03$) (Fig. 6). These opposing response patterns to the two vaccines, with superior TT recall and inferior PP responses for the COX-2i group, were linked, as demonstrated by a higher TT/PP response ratio (0.88 [median]) for the COX-2i group than for control individuals (0.29 [median]) ($P < 0.01$) (Fig. 6).

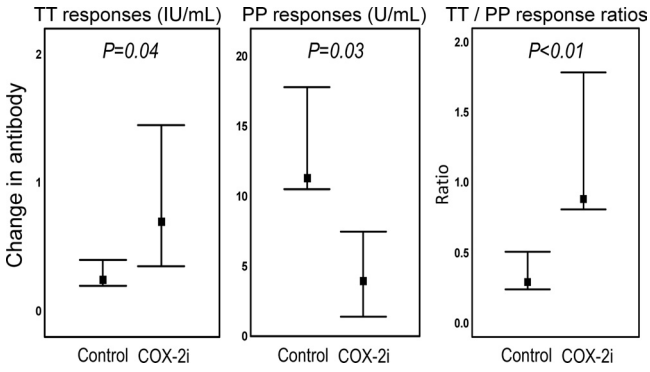


FIG. 6. Results of vaccine substudy. The two left panels show changes in antibody levels from weeks 6 to 12 after vaccination with TT and PP vaccines. For TT responses, only recall responses are shown, i.e., responses for those who had measurable anti-TT at baseline and for whom a single vaccine would be expected to be sufficient to induce a recall response. The right panel shows the groupwise variations in TT/PP response ratios, with a relative dominance of TT responses in the celecoxib group. Medians and interquartile ranges are indicated.

## DISCUSSION

This exploratory study is the first to test the hypothesis that COX-2i might abrogate an LPS-$PGE_2$-driven mechanism of chronic immune activation, which also explains the increase of cAMP in T cells of HIV-infected individuals, leading to reduced T cell responsiveness (48, 54). This drug or drug class may offer a new and affordable approach to partly decrease the detrimental effects of HIV-associated chronic immune activation, improve T cell functions, and ultimately modulate clinical progression.

Although HIV RNA tended to increase in controls, sustained changes in $CD4^+$ T cell counts, the need for initiation of ART, or the appearance of HIV-related opportunistic infections could not be expected within the limited time frame of this study. Therefore, quantitative changes in the density of CD38 on $CD8^+$ T cells, currently the best surrogate marker of clinical progression (24, 28), were chosen as the primary end point. This is in keeping with recent trials attempting to downregulate immune activation by other means (41). Indeed, a reduction of CD38 was shown here for the celecoxib group only, although any clinical benefit of such a reduction remains to be shown. However, based on the correlation between CD38 and the actual CD4 loss prestudy (28), this decrease in CD38 could be extrapolated to a reduction in CD4 loss of approximately $30 \times 10^6$ cells/liter/year (data not shown). This positive yet exploratory observation both encourages and requires larger and longer trials with clinical end points for further support. Moreover, the possibility exists that COX-2i may have a beneficial potential for patient groups other than those included here, perhaps particularly ART-naïve patients with low $CD4^+$ T cell counts and restricted access to ART, and possibly patients with limited ART-related increases in their CD4 counts despite adequate control of HIV replication.

Whereas CD38 levels were similar in both study arms at inclusion, a postrandomization bias was likely introduced due

to the exclusion of patients with celecoxib-related rash who, in addition, showed signs of higher immune activation. Thus, for those patients who completed the study, a group difference in CD38 at baseline was plausible. This unfortunate bias made group comparison statistics less reliable. Paired statistics were therefore used to test changes within each study arm for most variables in the "as treated" analysis.

COX-2i treatment further resulted in decreased serum IgA, in accordance with our previous observations of patients on ART (36). Since IgA is produced mainly at mucosal sites, COX-2 inhibition might reduce chronic immune activation in mucosal lymphoid compartments and counteract the translocation of microbial antigens typically observed in these patients (4, 5, 9, 11, 14, 29, 40, 42, 49). We therefore measured circulating LPS, which did not change. Still, longer periods on COX-2i treatment may be required to reduce leakage across the mucous membranes, as the gastrointestinal lining is not immunologically normalized even after long-term ART (13). Alternatively, COX-2i treatment may dampen only the consequences of microbial translocation by blocking the effects following LPS-mediated activation of monocytes and macrophages, which include upregulation of COX-2. These notions should be addressed in future studies.

We also monitored whether COX-2i treatment was associated with longitudinal changes in phenotypic profiles in fresh blood, as well as performing a simultaneous analysis of thawed cells from different time points, with the latter allowing comparison of expression densities (mean fluorescence intensities [MFI]). Three celecoxib-related effects were observed. First, an overall reduction in PD-1 density (MFI) on $CD8^+$ T cells was found, which may imply improved T cell responsiveness (17, 21, 53). Second, the fraction of PD-1 on Gag-specific $CD8^+$ T cells was reduced, which might indicate improved functionality of HIV Gag-specific T cells. This may be particularly relevant because $CD8^+$ T cells responsive to Gag epitopes have been related to slow disease progression (7, 43). Finally, circulating cells with the Treg phenotype increased, although the definition used in this study ($CD4^+$ $CD25^+$ $CD127^{lo/-}$) was later questioned for this patient group (19). An increase of this phenotype may therefore represent Tregs, in keeping with the stable levels of circulating Tregs observed in HIV elite controllers (12), or comprise even activated $CD25^+$ effector T cells. The latter possibility was supported by the fact that depletion of $CD25^+$ cells *ex vivo* in the celecoxib arm resulted in reduced polyclonal T cell activation.

We also tested the effects of COX-2i on T cell-related functions *in vivo* by immunization with TT. To test our hypothesis, a vaccine with a T cell-independent antigen (PP) was also included for comparison. Indeed, the data showed that the humoral memory recall responses to the T cell-dependent TT vaccine were improved selectively in the celecoxib-treated group. Whether COX-2i in this patient group also facilitates naïve immune responses should be addressed in future studies. This first observation of COX-2i-related improvement of vaccine responses may be particularly relevant for HIV-infected patients, because most of the recommended vaccines for these patients are T cell dependent. Whether COX-2i might have potential as an inexpensive and easily administered adjuvant to vaccination will likewise be tested in future studies. In contrast, the PP responses were clearly reduced but not abolished by COX-2i. In our experience, responses to PP are unpredictable in HIV-infected individuals and are barely related to CD4 T cell counts (34). One may therefore speculate that HIV-related immune activation actually facilitates this IgG2-dominated rudimentary pathway for B cell activation.

The restricted treatment period, the high drug dosage used to overcome any dose-response limitation effects on efficacy, and a number of study-specific exclusion criteria with emphasis on cardiovascular risk factors made us conduct the trial with fairly small groups, and the study is therefore exploratory in nature. Celecoxib was chosen because it had already been tested in patients on ART (36). The high dose of celecoxib may have caused the unexpected and relatively large number of generalized exanthemas (4 of 17 patients) compared to that in our previous study involving similar doses of celecoxib administered to patients on ART (0 of 19 patients; $P < 0.05$ by FiT) (36). These exanthemas occurred at almost the same time point for all four subjects. Because these patients probably had higher levels of immune activation, one might speculate that these adverse events resulted from processes of immune modulation rather than classical drug allergy, similar to the transient rash occasionally seen after initiating ART. Whether these skin reactions represent a limitation for celecoxib or the overall drug class and whether lower drug dosages or dose escalation schedules can reduce such adverse events need to be explored further. Additionally, before data from such new studies testing conventional doses of celecoxib or other COX inhibitors become available, one may question how generalizable the current data are.

The use of COX inhibitors has been associated with cardiovascular events, particularly in individuals at risk of cardiovascular disease (47, 51). A number of exclusion criteria were therefore enforced to avoid any cardiovascular events. However, assessment of cardiovascular risk in chronic HIV infection is considerably more complex than that for the general population. In fact, untreated HIV infection and immune activation *per se*, as well as certain combinations of ART, appear to increase the risk for cardiovascular events (16, 33). It is noteworthy that no evidence in this study suggested a celecoxib-related enhancement of cardiovascular risk. On the contrary, IL-6 tended to decrease in the celecoxib arm only, in keeping with the controlled study by Bogaty et al. (8) of another COX-2 inhibitor in patients with ischemic heart disease. Furthermore, D dimers, a possible marker of cardiovascular risk (33), tended to increase in the control arm only.

We earlier demonstrated high levels of cAMP in T cells from HIV-infected individuals (2), and studies with a retrovirus-induced murine AIDS model showed that COX-2 inhibitor could reduce intracellular cAMP in T cells (44), both of which helped us in forming our hypothesis. It would therefore have been relevant to sort out T cells and to measure cAMP in this study. However, in our experience, such assays would require a substantial fraction of the PBMC available, and these were instead prioritized for functional T cell assays. Future studies are in progress to study the relationship between this signal system and LPS, COX-2 activation, and T cell activation, including regulation of CD38 and other activation markers.

In conclusion, this first exploratory clinical trial of COX-2i in patients with untreated chronic HIV infection supported our hypothesis that COX-2 activity is enhanced in patients with

chronic HIV infection and may be modulated by celecoxib. Signs of reduced chronic immune activation were observed, as indicated by reduced CD38 densities, reduced IgA levels, reduced general expression of PD-1 on $CD8^+$ cells as well as on HIV Gag-specific $CD8^+$ T cells, and selectively improved T cell responsiveness to a T cell-dependent vaccine. Data from this exploratory trial justify larger studies with a focus on potential clinical benefit, mechanisms of action, and optimal drug dosage.

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E. M. Aandahl and K. Taskén are inventors of a patent on the use of COX-2 inhibitors for the treatment of immunodeficiency diseases and own shares in Lauras AS, a spin-off company from research at the University of Oslo through Oslo Research Park, University of Oslo. Aandahl and Taskén had no clinical role in the study.

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