Immune modulatory effects of cyclooxygenase type 2 inhibitors in HIV patients on combination antiretroviral treatment

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\textbf{Objectives:} To examine the immune modulating effects of cyclooxygenase type 2 (COX-2) inhibitors (COX-2i) in HIV-infected patients on combination antiretroviral treatment (CART).

\textbf{Design:} In-depth substudy from an approved, open, controlled, randomized study comparing the immune modulating effects of CART in combination with COX-2i after 12 weeks.

\textbf{Methods:} Patients ($n = 38$) on long-term CART with stable viral load (VL) $< 50,000$ copies/ml and CD4$^+$ T-cell counts $> 100/\mu$l were randomized to CART and rofecoxib 25 mg bid ($n = 12$) or celecoxib 400 mg bid ($n = 12$), or CART only without placebo ($n = 14$). Routine clinical chemistry, CD4$^+$ and CD8$^+$ counts and VL were safety parameters. Immunological parameters included C-reactive protein, $\beta_2$-microglobulin, Ig isotypes and IgG subclasses as well as several T-lymphocyte subsets. Non-parametric analyses were used throughout.

\textbf{Results:} Prestudy experiments showed higher median intracellular expression of COX-2 in CD4$^+$ ($P = 0.048$) and possibly CD8$^+$ ($P = 0.09$) T cells from patients on CART compared with uninfected controls. In the clinical study, increased CD4$^+$ T-cell counts were observed only in patients on COX-2i with VL $< 50$ copies/ml ($P = 0.02$). Decreased expression of CD38$^+$ on CD8$^+$ T cells and subsets as well as reductions in IgA and IgM ($P < 0.03$) were most pronounced in patients on COX-2i who had detectable VL ($n = 6$). COX-2i treatment enhanced the perforin content particularly in the differentiated CD27$^-$/CD8$^+$ T-cell subsets compared with controls ($P = 0.05$).

\textbf{Conclusions:} COX-2i together with CART improved markers for persistent immune activation, particularly in patients with viraemia, as well as enhanced perforin expression, and thereby strengthened COX-2 as a potential therapeutic target in HIV infection.

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\textbf{Keywords:} HIV, cyclooxygenase type 2, cyclooxygenase type 2 inhibitors, combination antiretroviral treatment, therapy, clinical trial, perforin
Introduction

Combination antiretroviral treatment (CART) shown to be clinically efficient over the last decade. However, the inability of CART to eradicate HIV-1, completely suppress HIV replication and completely restore T-cell functions [1] as well as the substantial cost, long-term side effects and the increasing incidence of drug-resistant virus call for additional treatment modalities.

Two important non-viral factors contribute significantly and perhaps synergistically to the immunopathology and progression of chronic HIV infection, namely T-cell dysfunction and persistent immune activation [1,2]. Persistent immune activation per se enhances HIV replication, reduces the naive T-cell pool, accelerates depletion of CD4+ T cells, and impedes both cellular and humoral immunity [1,2]. Dysfunctional T cells, which are present in all patients with chronic HIV including those taking CART, may play a part in maintaining the virus reservoir and in predisposing to opportunistic infections [1]. Our hypothesis is that reversal of this T-cell dysfunction in HIV may be obtained by antagonizing hyperactivated protein kinase A (PKA), a signalling enzyme which becomes activated in the presence of cyclic AMP (cAMP). We have previously characterized a PKA-COOH-terminal Src kinase (Csk)-lymphocyte-specific protein tyrosine kinase (Lck) inhibitory pathway in cell membrane-associated lipid rafts whereby elevated levels of intracellular cAMP abolishes T-cell receptor-mediated activation [3–8]. We later found that HIV-infected patients have higher levels of PKA-mediated inhibition of both TCR signalling and T-cell activation than uninfected, healthy controls [9,10]. Inhibition of PKA via reduced CAMP levels might therefore reverse this process and restore T-cell responsiveness.

Prostaglandin E₂ (PGE₂) is one of the most effective ligands that upregulate intracellular cAMP levels and might thereby inhibit T-cell receptor-mediated immune activation [11,12]. PGE₂ is produced by cyclooxygenase type 2 (COX-2), perhaps mainly in monocytes [13], and its synthesis can be blocked by COX-2 inhibitors (COX-2i). Three recent observations strengthen COX-2 as a candidate target in HIV infection. First, we showed that COX-2-mediated PGE₂ synthesis was involved in retrovirus-induced T-cell dysfunction in mice [14]. Second, we characterized the existence of regulatory T cells (Treg) in HIV [15] infection and new data show that these inhibitory T cells do upregulate COX-2 upon stimulation (M. Mahic, C.C. Johansson, S. Yaqub, K. Taskén, E.M., unpublished data). Thus, COX-2i may help to downregulate HIV-specific Treg and thereby improve cellular immune responses to HIV. Third, we found that COXi given for 14 days improved T-cell proliferative responses in an open, exploratory phase II clinical trial [16]. Moreover, COX-2i and reduced PGE₂ levels may theoretically also bring down the persistent immune activation in HIV infection through other pathways, in keeping with some of the beneficial clinical observations with glucocorticoids that unfortunately cause unacceptable side effects [17]. We here report immune modulatory effects of COX-2i in 40 HIV-infected patients on stable CART who were randomized to in addition openly receive celecoxib, rofecoxib or only CART (no COX-2i or placebo) for 12 weeks.

Materials and methods

Inclusion criteria

The present substudy is part of a longer COX-2i study including 60 HIV-infected patients on long-term CART with stable viral load (VL) < 50 000 copies/ml and CD4+ T-cell counts > 100/µl for at least 6 months. The patients were recruited from two clinics in Oslo and were randomized after informed consent to either of the three following groups: (i) open control who did not receive any drug or placebo but were followed clinically for 24 weeks and subjected to the same tests as the treated groups; (ii) rofecoxib 25 mg bid for 24 weeks; or (iii) celecoxib 400 mg bid for 24 weeks. All patients continued their regular CART throughout the study. The study was approved by the Regional Ethics Committee. The current substudy ended at week 12 and incorporated all of the patients from one of the clinics (Ullevål University Hospital, n = 40).

Safety parameters

Routine clinical chemistry profiles were monitored and included haematology, electrolytes, kidney and liver function tests. CD4+ and CD8+ T lymphocytes in blood were quantified using the TriTEST reagent kit (Becton Dickinson, Biosciences; San Jose, California, USA). HIV RNA was monitored by the COBAS Amplicor HIV-1 monitor test (Roche, Branchburg, New Jersey, USA) with a detection limit of 50 copies/ml.

Immunological parameters

C-reactive protein, β₂-microglobulin and Ig isotypes and IgG subclasses were quantified by standard methods. T-lymphocyte subsets were analysed in fresh EDTA blood which was rapidly subjected to direct staining for 20 min with combinations of fluorescence-labelled monoclonal antibodies to CD3+, CD4+, CD8+, CD38+, HLA-DR, CD28+, CD45RA (all from Becton Dickinson) and CD27+ (eBioscience, San Diego, California, USA) or labelled IgG isotype control antibodies (IgG1, IgG2a, and IgG2b; Becton Dickinson). Four-colour flow cytometry (FACS Calibur flow cytometer; Becton Dickinson) was performed after fixation and lysis using lysis buffer (Becton Dickinson) and analysed with Winlist analysis software (Verity SH, Topsham, Maine, USA) as described [18]. The isotype control monoclonal antibodies were used to individually calibrate the flow cytometer for each patient sample in all four colour channels. T cells were
considered positive for CD38+, HLA-DR, CD27+, CD28+ and CD45+RA if they had a fluorescence intensity more than twice the cut-off limits determined by isotype control mAb.

Cells were also permeabilized after staining for surface markers and subsequent lysis, fixation, permeabilization and co-stained with anti-CD8+, anti-CD3+ and anti-CD14+ as described elsewhere [19]. In addition, intracellular expression of COX-2 enzyme was quantified after staining with a COX-2 mAb (Becton Dickinson) in pre-study experiments. Because the manufacturer did not provide a truly matched isotype control mAb in the COX-2 kit (i.e., identical mAb concentration and fluorescence intensity), cut-off limits were determined according to the dominating COX-2-negative cell population. However, before each batch staining of patient samples, a positive control was prepared where both COX-2 and isotype control mAb were used: Control peripheral blood mononuclear cells (PBMC) were stimulated with lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich, St. Louis, Missouri, USA) for 4 h, which particularly induces COX-2 in monocytes, and these control cells were subjected to both specific and isotype control mAb staining (Fig. 1a). Intracellular perforin was detected in CD8+ T cells with a phycoerythrin (PE)-labelled monoclonal antibody to perforin (BD Pharmingen, clone G9); a matched control mAb in the kit with identical PE-intensity and fluorescence intensity, cut-off limits were determined by the COX-2-negative cell populations, as outlined in Fig. 1b, because the supplier did not provide exactly matched isotype mAb controls.

Patient cohort and major outcomes
Forty patients (four women, 36 men) were initially included from our clinic in the current substudy. One 41-year-old male on celecoxib suffered a sudden death only 12 days after initiating treatment; this incident was classified as possibly related to study drug because a clear cause of death could not be established at post-mortem. A moderate stenosis in the left anterior descendent coronary artery was noted without signs of infarction. In addition, chronic bronchitis and traces of cannabis in the blood were noted. Another patient was excluded from the study after hospitalization for an intoxication which was not drug-related. Thus, 38 patients (14 controls, 12 on rofecoxib, and 12 on celecoxib) were re-examined after 12 weeks. The baseline characteristics of the patients are given in Table 1; these are patients on long-term CART with adequate CD4 T lymphocyte counts who were mainly on typical protease inhibitor- or non-nucleoside reverse transcriptase-based regimens with only two exceptions (both of whom had HIV RNA < 50 copies/ml). No differences in baseline parameters were observed between the three study arms (data not shown). Five patients were seropositive for hepatitis C virus but had normal liver function test results. Only one patient had additional medication (anticonvulsant) that did not interfere with COX-2i. Eight patients had HIV RNA > 400 copies/ml, six of whom were randomized to the COX-2i arms. No differences in effects or side effects were found between the two COX-2i-treated groups (data not shown); both COX-2i arms were therefore combined in the following data analyses. It was noted that the tolerance to maximal COX-2i dosages was good among most of these patients who already had a drug burden; only one patient (celecoxib) needed a dose reduction.

No groupwise differences were found between the COX-2i and control arms in HIV-related variables such as nadir, pre-CART and baseline CD4+ lymphocyte counts or pre-CART HIV RNA and no differences developed in the safety parameters. Increase in CD4+ T-cell counts was observed only among COX-2-treated patients who, in addition, had effective CART with either VL < 50 (n = 15; P = 0.02) or VL < 400 copies/ml (n = 18; P = 0.07) at baseline. In contrast, COX-2i had no effect on VL in either of the study subgroups.

Elevated T-cell expression of COX-2 in patients on CART
In pre-study experiments we measured intracellular expression of COX-2 enzyme in CD4 and CD8 T-cells in eight HIV-infected patients on CART and compared these results with those found in five healthy, HIV seronegative controls. Despite the small number of patients, we found that COX-2 enzyme was expressed more frequently in CD4+ T cells from patients than from controls (P = 0.048) and possibly also in CD8+ T cells (P = 0.09) (Fig. 1b, c). Staining with isotype control mAb showed insignificant non-specific staining at the concentraions tested in parallel positive control experiments (Fig. 1a). Cut-off limits were determined by the COX-2-negative cell populations, as outlined in Fig. 1b, because the supplier did not provide exactly matched isotype mAb controls.

COX-2i-associated CD38+ decrease in CD8+ T-cell subsets
Decreased expression of CD38+ in CD8+ subsets was most frequently observed in COX-2i patients having VL > 400 copies/ml (VLhi), although the statistical power...
Fig. 1. COX-2 expression in T cells in HIV-positive patients on CART (HIV+) and healthy seronegative controls (control). (a) Example of positive control experiment in parallel with each intracellular COX-2 batch staining of patients. PBMC from controls were stimulated (lower four panels) or not (upper four panels) with LPS for 4 h prior to staining. This control was stained intracellularly with PE-labelled COX-2 mAb in parallel with patient samples and in addition to PE-labelled IgG isotype control.
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was hampered by the low number of patients in this group (n = 6) (Fig. 2). Furthermore, we observed a reduction in the CD38+ fraction of both the CD28+ and HLA-DR-negative CD8+ T-cell subsets, but only among these few VLna COX-2i-treated patients (P = 0.03) (Fig. 2). However, other signs of elevated immune activation at baseline in the VLna COX-2i group, such as the higher CD8+ T lymphocyte counts and DR–positive fraction of CD8+ T cells still persisted after 12 weeks of treatment (data not shown).

**COX-2i treatment in relation to serum markers indicating persistent immune activation**

Changes in Ig isotypes, IgG subclasses, and β2-microglobulin were subsequently examined. The median and interquartile Ig isotype and IgG subclass levels were all within normal reference values, but with skewed levels for some individual patients having higher than normal levels of total IgG, IgG1 and IgG3 or lower IgG2 levels. Only COX-2i treated patients developed small reductions in IgA (P = 0.01) and IgM (P < 0.03) levels that were quantitatively most pronounced in patients having HIV RNA > 50 copies/ml. In contrast, no significant reductions in β2-microglobulin levels were observed during COX-2i treatment, but it should be pointed out that all patients had values of β2-microglobulin well within the normal reference limits.

**COXi enhances perforin expression in CD8+ T-cell subsets**

We quantified the frequencies of perforin–containing CD8+ T lymphocytes within the CD27+CD28+ (38.2%, 3.1% perforin+), CD27+CD28− (13.9%, 38.0% perforin+), CD27−CD28+ (41.9%, 87.0% perforin+), CD27−CD45RA− (23.9%, 71.6% perforin+), and CD27−CD45RA+ (20.1%, 80.9% perforin+) subsets (medians at baseline). For COX-2 patients the expression of perforin increased significantly from baseline to week 12 in all of the five CD8+ perforin+ subsets studied according to paired tests (P ≤ 0.05), whereas this was not the case for control patients (Fig. 3). Thus, groupwise differences in perforin expression became evident between COX-2i patients and controls only at week 12 within the terminally differentiated CD27+CD28−CD8+ and CD27−CD28−CD8+ T cell subsets (P = 0.07 and P = 0.05, respectively, Mann–Whitney U test) (Fig. 3).

**Discussion**

In a preceding study we found that proliferative T-cell responses in HIV-infected patients on CART were improved after 2 weeks with the non-selective COXi indomethacin and more potently with the selective COX-2i rofecoxib [16]. Here we first examined the expression of COX-2 enzyme in circulating T cells from CART-treated HIV patients and found increased levels compared with healthy controls, particularly in the CD4+ subset. These differences might be mirrored in other compartments than blood as circulating T cells originate from large reservoirs in lymphatic tissue. Thus, COX-2i may target even tissue T cells as well as other cell types in patients on CART [20]. To our knowledge, COX-2 enzyme levels have not been recorded in T cells during other virus infections in man; we have recently observed this phenomenon in mice infected with Rad-LS virus causing murine AIDS [14]. However, we are in the process of mapping COX-2 expression in lymphocyte subsets from more HIV-infected patients as well as from other infections with persistent antigenaemia such as hepatitis C virus, in order to understand our preliminary observations in a broader context.

In this exploratory and controlled study we examined immune modulating effects in patients taking two different COX-2i for a longer period of time, with focus on markers indicating persistent immune activation and on changes in T-cell subsets, including those representing differentiated CD8+ cytotoxic T cells. Because no differences in efficacy or side effects were found between the two COX-2i study drugs, they were combined in the analysis.

Persistent immune activation can be reflected by several parameters such as increased serum levels of β2-microglobulin and polyclonal hypergammaglobulinaemia [20]. The relatively low baseline concentrations of these markers in this study suggested that the activity of such processes were modest and probably a result of long-term CART. However, a more significant parameter for persistent immune activation is probably CD38+ CD8+ T cell lymphocytes, because this subset is an equally good progression predictor of HIV as HIV RNA in early HIV stages, and a better indicator of progression than CD4 T-cell counts in the later stages of the infection [21,22].

**Fig. 1.** (continued)

mAb (IgG). Plots shown were gated on live gate for lymphocytes and monocytes and co-stained for CD3+ T cells (four left panels) and CD14+ monocytes (four right panels). The positive control showns intense COX-2 staining of LPS-stimulated CD14+ monocytes (91.0%) with 0.22% weakly positive in isotype control (lower right panels). In unstimulated cells very little non-specific binding of IgG isotype controls compared to COX-2 was observed, particularly in CD3+ T cells. The numbers denote percentage COX-2 positives among CD3+ and CD14+ cells, respectively. (b) Examples of flow cytometric analysis of PBMC gated on viable CD3+ lymphocytes showing fractions of COX-2-positive T cells, both in CD8+ (upper right quadrants) and in CD8− (mainly CD4+) T cells (lower right quadrants). The total fractions of COX-2-positive T cells are 1.29% and 4.59% in the seronegative controls and HIV-positive individuals, respectively. (c) Fractions of COX-2 positive CD4+ and CD8+ T cells as well as total CD3+ T cells. P values for groupwise differences between HIV seronegative controls and HIV-positive patients are indicated. Box plots represent medians, 25–75 percentiles and minimum and maximum.
Recent data even suggest that CD38+CD8 T cells have prognostic significance in CART-patients with drug-resistant virus [23]. It should be noted that the mechanisms which induce CD38+ on CD8 T cells in chronic HIV and which could explain its clinical predictive role remain elusive, possibly because CD38+ may have a number of different functions [21].

Our inclusion criteria opened for recruitment of patients with persistent viraemia and clinically resistant HIV. In this study we included eight such patients with HIV RNA > 400 copies/ml (six COX-2i and two control patients), which is a small number for statistical analysis. Our data nevertheless suggested that this COX-2i patient subgroup possibly reduced the CD38+ expression on CD8 T cells and significantly so in the CD28+CD8+ and HLA-DR+CD8 subset, although the impact of decreased CD38+ on these subsets is unclear. We also found small but significant reductions in serum IgA and IgM mainly in the viremic patients which altogether indicated that persistent immune stimulation might be reduced by COX-2i, particularly in patients with incomplete suppression of HIV despite long-term CART.

Here, CD38+ surprisingly decreased in the HLA-DR-negative CD8+ T cells among the few COX-2i patients with viraemia. However, stimulatory mechanisms and regulation of CD38+ and HLA-DR may be just as different as their immunological functions and thus explain why increased expression of CD38+ and not HLA-DR on CD8 T cells relates to unfavourable prognosis in HIV-infected patients. Moreover, co-expression of these two molecules on T cells is not consistent: First, the frequency of DR-positive cells was much lower than CD38+ in CD8+ T cells, even in the viremic patients (20% DR versus 48% CD38+). Second, HLA-DR cells are abundant during lymphocyte development, particularly in B cells, and in the absence of CD38+, and vice versa in other tissues including epithelial cells, which may be CD38+ in the absence of HLA-DR [21]. Together, our data should stimulate further research on the clinical significance of CD38+ expression in CD8+ T cell subsets, preferably through use of calibrators that make it possible to calculate the number of CD38+ molecules per cell. Such techniques would also allow estimates of changes within the CD38+ subsets over time, including more subtle transitions between CD38dim and CD38bright cells [21].

We also explored the overall terminal differentiation of CD8+ cytotoxic T cells including their intracellular...
content of perforin, because expression of this important effector substance is generally reduced in CD8\(^+\) T cells from HIV-infected patients [24]. Perforin was measured in increasingly differentiated CD8\(^+\) T-cell subsets having subsequent loss of CD28\(^+\) and CD27\(^+\), respectively, and re-expression of CD45-RA in the terminally differentiated CD27\(^-\)/CD28\(^-\) subset [25]. Perforin-positive fractions increased in all these CD8\(^+\) T-cell subsets in the COX-2i group, which thereby developed more perforin-positive cells than control patients in the end-differentiated CD8\(^+\) T-cell subset. We also observed increased fluorescence intensities related to perforin content in positive cells over time among COX-2i patients (data not shown). How COX-2i exerts such effects and whether increased perforin concentrations improve functional cytotoxicity require further studies.

If COX–2i does reduce persistent immune stimulation as our data suggest, delayed disease progression must be proven in new, clinical trials. One might speculate whether more clear-cut COX–2i-related effects on the parameters studied would have been observed in other HIV cohorts with stronger persistent immune activation, such as in treatment-naive patients or in patients on CART with drug-resistant HIV. On the other hand, we also observed viraemia-independent COX–2i-related effects such as the early increase in CD4 T-cell counts among COX–2i patients on effective CART and the enhancement of perforin in CD8\(^+\) T cells.

Clinical efficacy studies of COX–2i in HIV must take into consideration a small but increased risk for cardiovascular side effects in predisposed individuals [26]. Such risk data were not available at the time of inclusion, and it cannot be completely excluded that one incident was recorded in this study. Nevertheless, the impact of such risk factors has to be weighed against potentially beneficial effects on disease progression and the unfavourable effects on lipid metabolism by CART and even by HIV infections per se. The possibility also exists that COX–2i behave differently when combined with CART. Anyway, cardiovascular risk factors should be carefully monitored in future trials and possibly be used as exclusion criteria. Moreover, although COX–2 expression is more inducible than COX–1, it may be that even non-selective COX-inhibitors should be compared with COX–2-specific ones in future studies, in keeping with our previous study [16], with emphasis on comparing both effects and side effects.

In conclusion, preliminary experiments found elevated frequencies of COX–2 enzyme-positive peripheral T cells from HIV-infected patients on CART. This exploratory trial showed that the COX–2i rofecoxib and celecoxib together with CART improved markers for persistent immune activation, particularly in patients with viraemia. Moreover, a COX–2i-related increase in perforin-containing CD8\(^+\) T-cells was observed. Functional and clinical consequences must be determined in future clinical trials which might focus on patients with chronic viraemia, such as treatment-naive patients and those having multi-resistant HIV.

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