Precursor Forms of Vitamin D Reduce HIV-1 Infection In Vitro

Wbeimar Aguilar-Jimenez, Biol, MSc, Simon Villegas-Ospina, Biol,* Sandra Gonzalez, Bact, MSc,* Wildeman Zapata, Bact, MSc, PhD,*† Irma Saulle, PhD,‡ Micaela Garziano, PhD,‡ Mara Biasin, PhD,‡ Mario Clerici, PhD,§‖ and Maria T. Rugeles, MSc, PhD**

Background: Although the anti-HIV-1 effects of vitamin D (VitD) have been reported, mechanisms behind such protection remain largely unexplored.

Methods: The effects of two precursor forms (cholecalciferol/calciol at 0.01, 1 and 100 nM and calcidiol at 100 and 250 nM) on HIV-1 infection, immune activation, and gene expression were analyzed in vitro in cells of Colombian and Italian healthy donors. We quantified levels of released p24 by enzyme-linked immunosorbent assay, of intracellular p24 and cell-surface expression of CD38 and HLA-DR by flow cytometry, and mRNA expression of antiviral and immunoregulatory genes by real-time reverse transcription-polymerase chain reaction.

Results: Cholecalciferol decreased the frequency of HIV-1-infected $p24^+CD4^+$ T cells and levels of p24 in supernatants in a dose-dependent manner. Moreover, the $CD4^+CD38^+HLA\text{-}DR^+$ and $CD4^+CD38^-HLA\text{-}DR^+$ subpopulations were more susceptible to infection but displayed the greatest cholecalciferol-induced decreases in infection rate by an X4-tropic strain. Likewise, cholecalciferol at its highest concentration decreased the frequency of $CD38^-HLA\text{-}DR^+$ but not of $CD38^+HLA\text{-}DR^+$ T-cell subsets. Analyzing the effects of calcidiol, the main VitD source for immune cells and an R5-tropic strain as the most frequently transmitted virus, a reduction in HIV-1 productive infection was also observed. In addition, an increase in mRNA expression of *APOBEC3G* and *PI3* and a reduction of *TRIM22* and *CCR5* expression, this latter positively correlated with p24 levels, was noted.

Conclusions: VitD reduces HIV-1 infection in T cells possibly by inducing antiviral gene expression, reducing the viral co-receptor CCR5 and, at least at the highest cholecalciferol concentration, by promoting an HIV-1-restrictive $CD38^+HLA\text{-}DR^-$ immunophenotype.

Key Words: HIV-1, vitamin D, immune activation, T lymphocytes, CD38, HLA-DR, infection susceptibility

(*J Acquir Immune Defic Syndr* 2016;73:497–506)

INTRODUCTION

Chronic immune activation and massive depletion of $CD4^+$ T cells are the hallmarks of HIV-1 infection, leading to AIDS.[1] Consequently, low basal immune activation[2] and high expression of anti-HIV-1 molecules[3,4] seem to act as key HIV-1 resistance mechanisms by reducing the susceptibility of target cells while restricting viral infectivity. Interestingly, vitamin D (VitD) is an immunoregulatory element[5,6] that could induce these HIV-1 protective effects as elicited in other viral infections.[7,8] Indeed, immune cells possess the metabolic machinery including the cytochrome (CY) P450 hydroxylases and the VitD receptor (VDR), allowing VitD conversion from cholecalciferol (VitD3) into calcidiol (25[OH]D3) and then into calcitriol ($1,25\text{-}[OH]_2D3$), the metabolically active form.[5] Once calcitriol interacts to VDR, the complex binds to VitD response elements (VDREs) located in a large number of genes, either inducing or repressing their expression. Indeed, the calcitriol/VDR complex can block NF-κB signaling and promote regulatory T cells and interleukin-10 (IL-10) secretion[8–11] while inducing the production of antimicrobial peptides.[12,13]

Received for publication March 31, 2016; accepted July 29, 2016.

From the *Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia; †Grupo Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia; ‡Dipartimento di Scienze Biomediche e Cliniche- L. Sacco, Università Degli Studi di Milano, Milan, Italy; §Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, Milan, Italy; and ‖Fondazione Don C. Gnocchi IRCCS, Milan, Italy.

Supported by Colciencias (code 111565740508), by the Universidad de Antioquia (Sostenibilidad and CODI, act 2014-938), and by the Universidad Cooperativa de Colombia (Sostenibilidad 2015–2016, Grupo Infettare). W.A.-J. is recipient of a doctoral scholarship from Colciencias. The funders had no role in study design, data collection and analyses, decision to publish, or preparation of the manuscript.

Preliminary results were presented in an international congress: Villegas-Ospina S, Aguilar-Jiménez W, Gonzalez SM, et al. Vitamin D reduces HIV-1 infection of $CD4^+$ T cells in an in vitro model. *Front Immunol*. 2015. Conference Abstract: IMMUNOCOLOMBIA2015—11th Congress of the Latin American Association of Immunology—10o. Congreso de la Asociación Colombiana de Alergia, Asma e Inmunología. doi: 10.3389/conf.fimmu.2015.05.00311.

The authors have no conflicts of interest to disclose.

W.A.-J. and S.V.-O. contributed equally to this study. W.A.-J., M.T.R., and M.B. conceived and designed the study. W.A.-J. and S.V.-O. performed the experiments, analyzed the data, and drafted the manuscript. I.S., M.G., and M.B. assisted with the experiments. S.G., W.Z., and M.T.R. assisted with interpretation of the results and manuscript writing. M.C. and M.B. contributed with reagent/material/analysis tools. M.B., M.C., and M.T.R. guided and reviewed the research and writing of the manuscript. All authors approved the final version of the manuscript.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

Correspondence to: Maria T. Rugeles, MSc, PhD, Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín 050010, Colombia (e-mail: maria.rugeles@udea.edu.co).

Supporting its protective role during HIV-1 exposure, we found higher plasma calcidiol levels and higher *VDR* mRNA expression in the blood and mucosa of HIV-1-exposed uninfected individuals than of healthy controls[14]; in addition, *VDR* mRNA expression was positively correlated with expression of the anti-inflammatory cytokine IL-10 and the antimicrobial defensins.[14] Moreover, in vivo supplementation with cholecalciferol increases the frequency of antigen-specific T cells expressing HIV-1-blocking chemokines, raises peripheral lymphocyte counts, and reduces productive infection.[15–17]

However, studies evaluating the effect of VitD on HIV-1 infection in vitro have yielded contrasting results,[17–21] most likely due to variations in the experimental design. Furthermore, the potential effects of VitD on the T-cell activation status or its ability to induce well-recognized anti-HIV-1 molecules have so far not been explored. Therefore, we examined the anti-HIV-1 effects of cholecalciferol and calcidiol in an in vitro model of acute infection of peripheral blood mononuclear cells (PBMCs). We also determined the effects of cholecalciferol on the $CD4^+$ T-cell infection rate as well as on the expression of the CD38 and Human Leukocyte Antigen–antigen D Related (HLA-DR) activation markers and of calcidiol on the mRNA expression of immunoregulatory and antiviral molecules.

METHODS

Study Population

PBMCs from 12 healthy donors from Medellin, Colombia, were separated through a blood density gradient using the Histopaque reagent (Sigma-Aldrich, St Louis, MO) and used to analyze the effects of cholecalciferol on HIV-1 infection and immune activation. Likewise, PBMCs from 27 healthy donors recruited in Milan, Italy, were used to study the effects of calcidiol on HIV-1 infection and gene expression. Since not all methods were applied to all samples, the number of samples included in each analysis is provided in the corresponding figure legends.

The study was performed according to the Helsinki declaration (1975, revised in 2000) and was approved by the bioethics boards of both research facilities. All subjects provided written informed consent to participate in this study.

VitD Treatments

Cholecalciferol (Enzo Life Sciences, Farmingdale, NY) and calcidiol (Sigma-Aldrich) were dissolved in absolute EtOH. Three concentrations of cholecalciferol were evaluated [10^{-11} M (0.01 nM), 10^{-9} M (1 nM), and 10^{-7} M (100 nM)], including 100 nM that has been previously reported in cell cultures.

Calcidiol was evaluated at 250 M (100 ng/mL) and in some assays at 100 M (40 ng/mL), both of them within the physiological range (32–100 ng/mL).[22] EtOH 0.1% or 0.01% v/v was used as vehicle control for the cholecalciferol and calcidiol treatments, respectively.

HIV-1 Infection Assays

The PBMCs from the Colombian subjects were cultured for 24 hours in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and cholecalciferol at the concentrations mentioned above, or 0.1% vol/vol EtOH as vehicle control. Proliferation of PBMCs was induced with 8 μg/mL phytohemagglutinin (PHA) and 100 IU/μL IL-2 (Sigma-Aldrich) for 72 hours. Per treatment, 1.6×10^6 PBMCs were infected with 6.5 ng of X4-tropic HIV-1 p24 [obtained from supernatants of the cell line H9-HTLV-IIIB (ATCC-CRL-8543)], in medium supplemented with the respective cholecalciferol treatments or EtOH along with 10 μg/mL polybrene (Sigma-Aldrich) for 3 hours, following the protocol reported by O'Doherty et al.[23] After washing, the cells were taken back to their respective cholecalciferol- or EtOH-supplemented medium, with cholecalciferol replenishment after 48 hours. The cells were harvested 72 hours postinfection for flow cytometry analyses and the supernatants collected for p24 quantification by enzyme-linked immunosorbent assay (ELISA) using the "Lentivirus-associated p24" ELISA kit (Cell Biolabs, San Diego, CA).

Similarly, PBMCs from the Italian subjects were cultured for 24 hours with calcidiol at the concentrations mentioned above or EtOH 0.01%. After 48-hour activation with 7.5 μg/mL PHA and 15 ng/mL IL-2, 2×10^6 activated PBMCs per well were infected with 1 ng of R5-tropic HIV-1 p24 ($HIV\text{-}1_{Ba\text{-}L}$ courtesy of the National Institutes of Health AIDS Reagent Program[24]) in medium supplemented with calcidiol or EtOH for 3 hours, as previously described.[25] Subsequently, the cells were washed and cultured in calcidiol- or EtOH-supplemented medium, with calcidiol replenishment every 48 hours. Some cells were harvested immediately before and others 20 hours postinfection and kept at −80°C in Isol-RNA lysis reagent (5 PRIME, Hilden, Germany) until RNA extraction; after 5 days, supernatants were collected for p24 quantification using the ELISA Kit XB-1000 (XpressBio, Frederick, MD).

Cultures, one per donor, for both calcidiol and cholecalciferol treatments were incubated at 37°C and 5% CO_2.

Flow Cytometry to Test Cholecalciferol Effects on Cell Activation and Infection Rate in Colombian Individuals

Expression of activation markers and frequency of infected cells were determined by flow cytometry after the HIV-1 infection assays performed in the Colombian samples. For each treatment, cell viability was tested by 7-AAD/DIOC6 (eBioscience, Santa Clara, CA) staining. After washing, the cells were stained with anti-CD3 APC, anti-CD4 eFluor-405, anti-CD8 PE-Cy7, anti-HLA-DR FITC, and anti-CD38 AlexaFluor-700 (eBioscience). The cells were washed and fixed using the "Foxp3 staining kit" (eBioscience), and intracellular staining with anti-p24 PE (Beckman-Coulter, Brea, CA) was performed. Lectures of 1.5×10^5 events per tube were performed on a FACSCanto II flow cytometer, and analysis was performed using the FACSDiva v.8.0.1 software. Gating

strategy is shown in Figure S1, Supplemental Digital Content, http://links.lww.com/QAI/A877.

Real-Time Reverse Transcription-Polymerase Chain Reaction to Test Calcidiol Effects on Gene Expression in Italian Subjects

A total of 21 genes, belonging to the VitD pathway (CYP27B1, CYP24A1, and VDR), antiviral response (APOBEC3G, SLPI, PI3, TRIM22, RNASE4, ANG, CAMP, CH25H, and MX2), and immunoregulators (IL10, HAVCR2, FOXP3, NFKBIA, ERAP2, TNFA, IFNG, and TLR2) and the viral co-receptor CCR5 were selected for gene expression assays for their relevance in the context of HIV-1 infection (reviewed in Zapata et al,[4] Aguilar-Jiménez et al,[14] Merindol and Berthoux,[26] Biasin et al,[27] Kajaste-Rudnitski et al,[28] Bedoya et al,[29] and Aguilar-Jimenez et al[30]). RNA was extracted with the Isol-RNA lysis reagent (5 PRIME) from calcidiol- or EtOH-treated PBMCs of the Italian individuals pre- (n = 14 in each group) or postinfection (n = 27 in each group). RNA from calcidiol- or EtOH-treated PBMCs, stimulated with lipopolysaccharide (LPS) for 12 hours (1 μg/mL, n = 6 in each group) were also analyzed as positive controls of mRNA expression of calcidiol-to-calcitriol converting 1a-hydroxylase (*CYP27B1*).[31–33] Following DNAse I treatment (Promega, Madison, WI), RNAs were retrotranscribed using the Moloney murine leukemia virus retrotranscriptase (Promega, Fitchburg, WI), and reverse transcriptase negative controls were performed to rule out contamination with genomic DNA in PCR amplifications. Real-time reverse transcription-polymerase chain reactions were performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) (genes and primers are detailed in Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A876), running melting curves to ensure specific amplification. The results are presented as the median of the relative expression units to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) reference genes calculated by the ΔCt method using CFX manager 3.1 (Bio-Rad). Samples that did not amplify in the real-time reverse transcription-polymerase chain reaction were excluded from the analysis of the respective gene; thus, the number of samples is detailed in the corresponding figure legends.

Statistical Analysis

Data were analyzed on the GraphPad Prism v.6.05 software. Normality was tested by the Shapiro–Wilk test, and pairwise differences between treatments were tested by the paired *t* test or Wilcoxon test. One-way analysis of variance (with Geisser-Greenhouse sphericity correction and post-hoc Bonferroni's multiple comparisons) or Friedman (with Dunn's multiple comparisons) tests were used to compare differences between the VitD treatments and the vehicle control. Correlations between VDR mRNA and transcript levels of the molecules analyzed were evaluated using the Spearman coefficient rank (r). Two-tailed hypotheses were considered, and a corrected *P*-value <0.05 was considered statistically significant. The results are presented as mean with 95% confidence interval or median with interquartile range. The statistical power in each analysis was calculated using PASS 14 Power Analysis and Sample Size Software, 2015 (NCSS, LLC, Kaysville, UT, www.ncss.com/software/pass). The open-access database of transcription factor binding profiles (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) was used to find putative VDRE sequences in the genes potentially modulated by VitD in gene expression analysis.

RESULTS

Cholecalciferol Decreases the Percentage of Infected Cells and p24 Levels

We first tested if cholecalciferol (the most consumed form of VitD, and endogenously synthesized) treatment modifies HIV-1 infection by an X4 strain in cells from healthy Colombian individuals. For all cholecalciferol doses tested, cell viability was >75% and the loss of mitochondrial potential was <1.5% (Fig. S2A, Supplemental Digital Content, http://links.lww.com/QAI/A878). Cholecalciferol decreased by 9.8% at 10^{-9} M and by 23.7% at 10^{-7} M the percentage of $CD4^+p24^+$ infected cells compared with the EtOH controls ($P = 0.0681$ and $P = 0.0233$, respectively; Figs. 1A, B). There was also a 10.5% reduction in the p24-PE mean fluorescence intensity at 10^{-7} M treatment compared with EtOH ($P = 0.0216$; Fig. S2B, Supplemental Digital Content, http://links.lww.com/QAI/A878). Furthermore, cholecalciferol significantly decreased the concentration of p24 released into the supernatants in a dose-dependent manner compared with the EtOH controls (47.38% at 10^{-11} M, $P = 0.0006$; 56.8% at 10^{-9} M, $P = 0.0008$; and 63.0% at 10^{-7} M, $P = 0.0005$; Fig. 1C).

Cholecalciferol modulates HLA-DR and CD38 expression in $CD4^+$, $CD8^+$ and in $CD4^+p24^+$ T cells

The differential expression of the activation markers CD38 and HLA-DR leads to specific phenotypes in $CD4^+$ and $CD8^+$ T cells, modulating their function and susceptibility to infection.[34,35] Thus, we examined whether the cholecalciferol-induced decrease in viral production and infection was associated with changes in the expression of CD38 and HLA-DR molecules.

In $CD4^+$ T cells, a significant reduction in the frequency of the $CD38^-HLA\text{-}DR^-$ (30.4%, $P = 0.0001$) and $CD38^-HLA\text{-}DR^+$ (52.6%, $P < 0.0001$) subpopulations was observed at the 10^{-7} M treatment when compared with EtOH control. In addition, there was an increase in the frequency of the $CD38^+HLA\text{-}DR^-$ subpopulation at 10^{-7} M treatment compared with EtOH control (18.1%, $P = 0.0002$), whereas the $CD38^+HLA\text{-}DR^+$ subset showed no significant changes after cholecalciferol treatment (Fig. 2A).

In $CD8^+$ T cells, cholecalciferol treatment did not modify the frequencies of the $CD38^+HLA\text{-}DR^+$ or the $CD38^-HLA\text{-}DR^-$ subpopulations, whereas it reduced the $CD38^-HLA\text{-}DR^+$ subset at the 10^{-7} M treatment compared with the EtOH control (50.5%, $P = 0.0531$). In addition, there was an increase in the frequency of the $CD38^+HLA\text{-}DR^-$ subpopulation at the 10^{-7} M compared with the EtOH control (27.4%, $P = 0.0285$) (Fig. 2B).

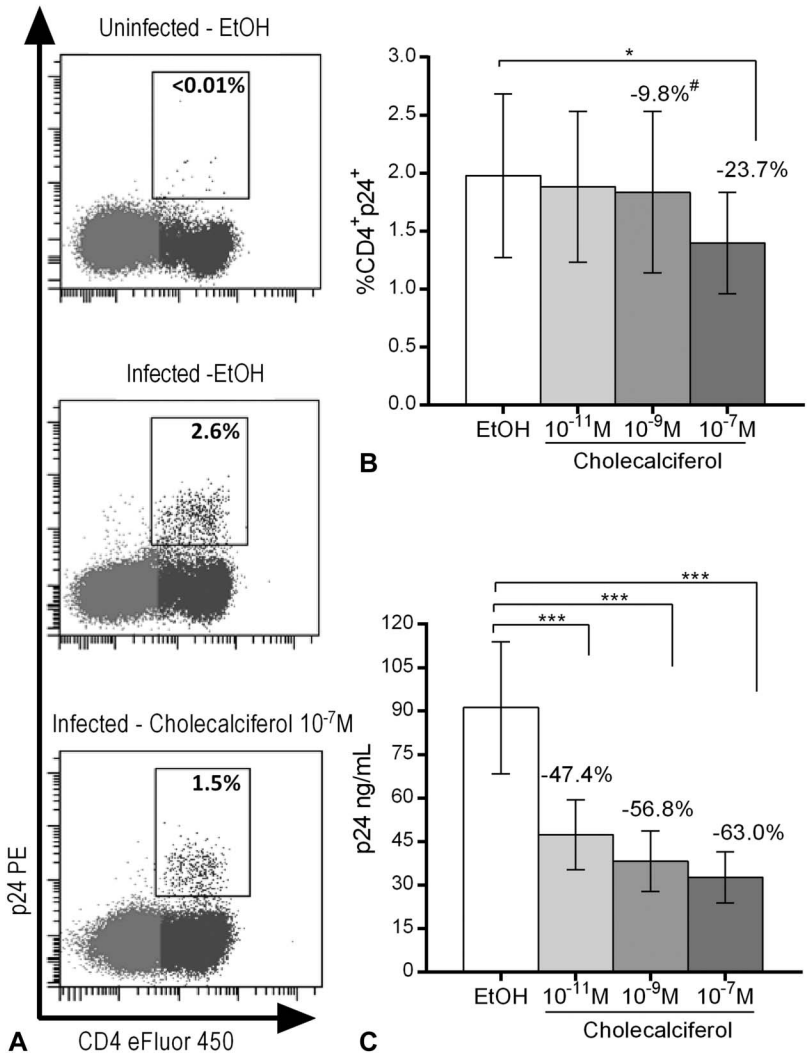

FIGURE 1. Cholecalciferol reduces the percentage of infected cells and p24 levels in supernatants. PBMCs from 12 healthy Colombian subjects were treated with 3 concentrations of cholecalciferol [10^{-11} M (light grey bars), 10^{-9} M (medium grey bars), and 10^{-7} M (dark grey bars)] or with 0.1% v/v EtOH control (white bars); activated with PHA/IL-2 and infected with an X4 strain (H9-HTLV-IIIB) for 3 days. A, Representative flow cytometry dot plots of infected $CD4^+p24^+$ cells: uninfected control (top), EtOH vehicle (middle), and cholecalciferol 10^{-7} M treatment (bottom). B, Statistical analysis to determine the percentage of infected $CD4^+p24^+$ cells. The viral protein p24 was quantified in supernatants by ELISA (C). After a Shapiro–Wilk normality test, one-way analysis of variance with post-hoc Bonferroni's correction was performed for statistical analyses; bars represent the mean with 95% confidence intervals. #$P = 0.0681$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, and percent (%) decrease are depicted.

We further explored the frequency of these subsets within the infected $CD4^+p24^+$ T cells. Similar to the total $CD4^+$ population, none of the cholecalciferol concentrations modified the $CD38^+HLA\text{-}DR^+$ subpopulation. Furthermore, there was a reduction in the $CD38^-HLA\text{-}DR^-$ (53.2%, $P = 0.0015$) and $CD38^-HLA\text{-}DR^+$ (73.2%, $P = 0.0008$) subpopulations, and an increase in the $CD38^+HLA\text{-}DR^-$ subpopulation (39.6%, $P = 0.0047$) (Fig. 2C), at the 10^{-7} M treatment compared with the EtOH controls.

Cholecalciferol Decreases Infection in More- and in Less-Susceptible $CD4^+$ T-Cell Subpopulations

We then examined the susceptibility to infection in the $CD4^+$ subpopulations analyzing the percentage of infected cells under EtOH treatment and found that $CD4^+CD38^+HLA\text{-}DR^+$ and $CD4^+CD38^-HLA\text{-}DR^+$ had higher infection rates than $CD4^+CD38^-HLA\text{-}DR^-$ (4.5-fold, $P = 0.0002$ and 2.9-fold, $P = 0.0160$, respectively; Fig. 3A). Furthermore, the $CD4^+CD38^+HLA\text{-}DR^+$ subpopulation had higher levels of intracellular p24 that were 26.5% and 25.0% brighter than $CD4^+CD38^-HLA\text{-}DR^-$ and $CD4^+CD38^-HLA\text{-}DR^+$ ($P = 0.0009$ and $P = 0.0054$, respectively; Fig. S1C, Supplemental Digital Content, http://links.lww.com/QAI/A877). Moreover, when compared with the EtOH controls, cholecalciferol decreased the percentage of infected $CD4^+CD38^+HLA\text{-}DR^+$ cells in a dose-dependent manner (10.3% at 10^{-9}M, $P = 0.0342$ and 38.9% at 10^{-7} M, $P = 0.0047$) and decreased by 70.8% the percentage of infection in the $CD4^+CD38^-HLA\text{-}DR^+$ T-cell subpopulation at the 10^{-7} M compared with the EtOH control ($P = 0.0272$) (Fig. 3B). Finally, cholecalciferol at 10^{-7} M also decreased the mean fluorescence intensity of intracellular p24 in the $CD4^+CD38^+HLA\text{-}DR^+$ (8.6%, $P = 0.0531$) and $CD4^+CD38^-HLA\text{-}DR^+$ (43.4%, $P = 0.0109$) subpopulations (Fig. S1D, Supplemental Digital Content, http://links.lww.com/QAI/A877).

Calcidiol Reduces Productive HIV-1 Infection

Once we had found that cholecalciferol had anti-HIV-1 activity, we evaluated the anti-HIV-1 activity of calcidiol, the

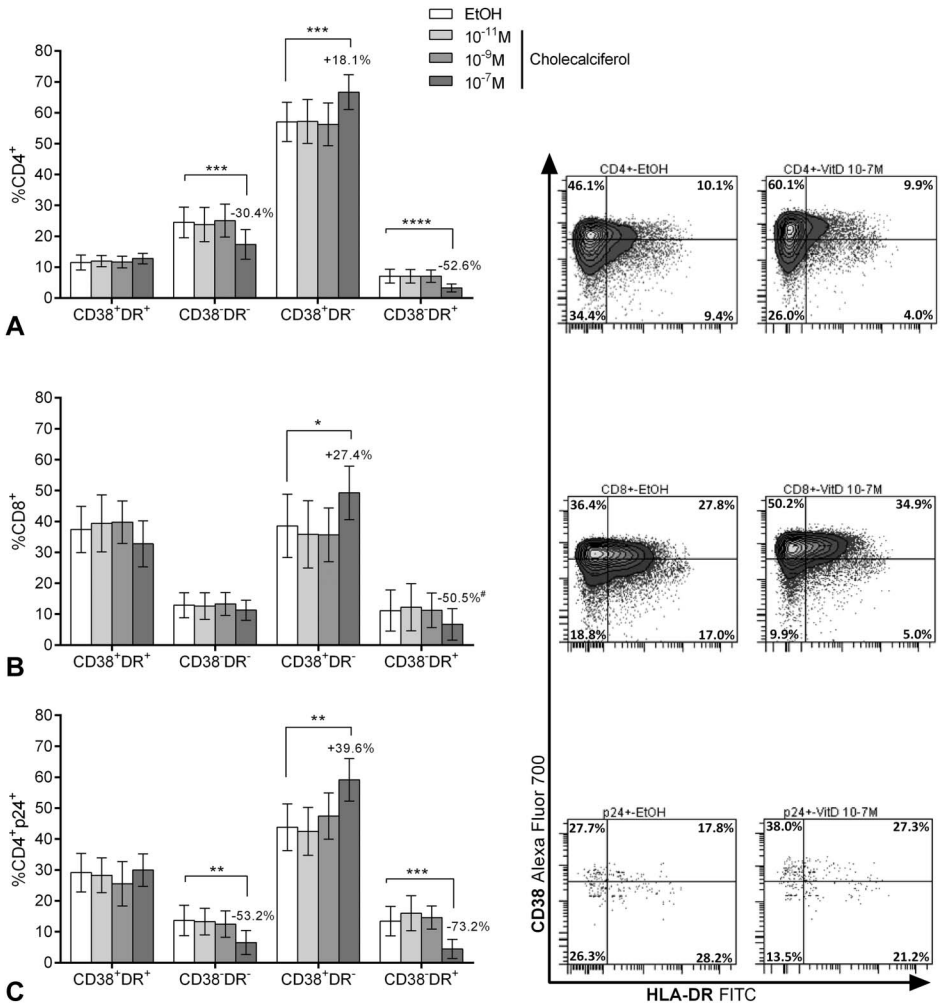

FIGURE 2. High concentrations of cholecalciferol modulate the frequency of HLA-DR- and CD38-expressing T cells. We explored the coexpression of CD38 and HLA-DR in $CD4^+$ (A) and $CD8^+$ (B) cells, as well as in infected $CD4^+p24^+$ (C) cells after HIV-1 infection (H9-HTLV-IIIB) at 3 cholecalciferol concentrations [10^{-11} M (light grey bars), 10^{-9} M (medium grey bars), and 10^{-7} M (dark grey bars)] or 0.1% EtOH control (white bars). Representative flow cytometry density plots on the right for $CD4^+$ (A) and $CD8^+$ (B), and dot plot for $CD4^+p24^+$ (C) are presented for the EtOH control (left) and VitD 10^{-7} M treatment (right). After the Shapiro–Wilk normality test, a one-way analysis of variance with post-hoc Bonferroni's correction was performed on $CD4^+$ cells, and bars represent the mean with 95% confidence interval; whereas Friedman's test with post-hoc Dunn's correction was used on $CD8^+$ and $CD4^+p24^+$ cells, and bars represent the median with interquartile range. #$P = 0.0531$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, and percent (%) increase/decrease are depicted.

main source of VitD for immune cells,[5,22] and using an R5-tropic strain, as the most frequently transmitted virus.[36] These experiments were performed in PBMCs from an independent population from Italy trying to replicate our results in a demographically different population.

We found that calcidiol at physiological concentrations of 100 ng/mL (250 nM) and 40 ng/mL (100 nM) decreased by 27.4% ($P = 0.0025$) and 47.0% ($P = 0.0189$), respectively, the levels of the viral p24 protein in supernatants when compared with the EtOH controls (Figs. 4A, B).

Calcidiol Modulates mRNA Expression of Genes Involved in the VitD and Antiviral Pathways

The mRNA expression level of 3 genes (*VDR*, *CYP27B1*, and *CYP24A1*) involved in the VitD pathway was investigated. No differences in *CYP27B1* mRNA expression were observed between EtOH and calcidiol treatments either pre- or postinfection, or in the LPS control; however, the *CYP27B1* mRNA levels preinfection (mean ± SD relative expression units: 0.142 ± 0.069) were similar to those produced by LPS control stimulus (0.166 ± 0.053) (Fig. 4C). In addition, calcidiol increased by 17.2% the *VDR* mRNA expression level postinfection compared with the EtOH treatment ($P = 0.0135$; Fig. 4D). Although no significant differences in the *CYP24A1* mRNA expression levels were detected between calcidiol- and EtOH-treated PBMCs (data not shown), the mRNA levels of *VDR* were positively correlated with those of *CYP24A1* in the calcidiol-treated cells postinfection (r = 0.42, $P = 0.0347$; Fig. 4E).

We subsequently quantified the mRNA expression level of the viral co-receptor *CCR5* and of several genes involved in immunoregulation and in the antiviral response (see Materials and Methods and Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A876), aiming to identify the association with the anti-HIV-1 effect of calcidiol.

The reduction in HIV-1 infection by calcidiol was associated with 9.3% reduction of *CCR5* mRNA expression postinfection ($P = 0.0079$; Fig. 5A) that in turn positively correlated with p24 levels 5 days postinfection (r = 0.54, $P = 0.0219$; Fig. 5B). Furthermore, reduction in infection was also associated with 29.5% increase of *PI3* mRNA (which encodes elafin) postinfection ($P = 0.0199$; Fig. 5C) and with 24.1% increase of *APOBEC3G* mRNA level preinfection followed by 12.0% reduction postinfection ($P = 0.0309$ and $P = 0.04351$,

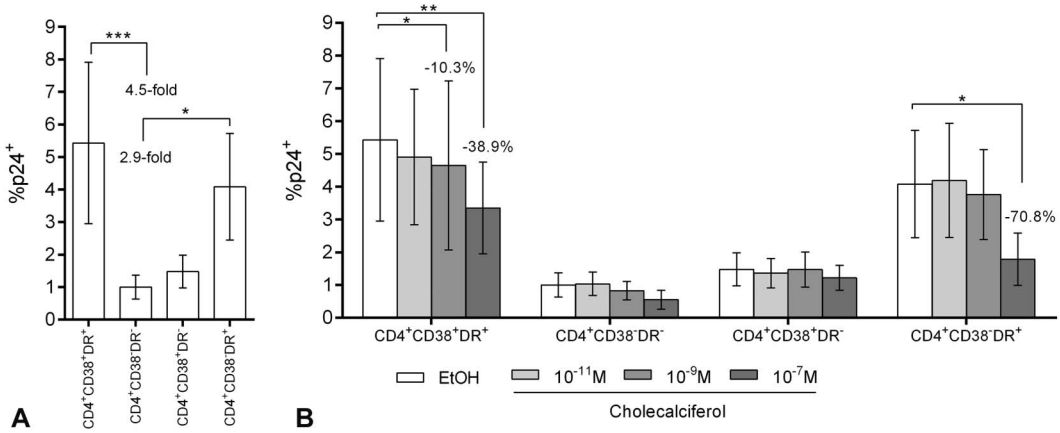

FIGURE 3. Cholecalciferol decreases the proportion of infected cells in CD4+ subpopulations. We explored the degree of HIV-1 (H9-HTLV-IIIB) susceptibility within 0.1% EtOH control treatments (white bars) (A) and the decrease of infection levels (B) in each of the CD4+ T-cell subpopulations after cholecalciferol treatment [10^{-11} M (light grey bars), 10^{-9} M (medium grey bars), and 10^{-7} M (dark grey bars)]. Friedman's test with post-hoc Dunn's multiple comparisons was performed for statistical analysis, and bars represent the median with interquartile range. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and percent (%) decrease are depicted.

respectively; Fig. 5D). A 9.2% reduction of *TRIM22* mRNA expression level postinfection was also observed ($P = 0.0061$; Fig. 5E). In addition, in calcidiol-treated PBMCs 20 hours postinfection, the mRNA of *VDR* was positively correlated with the mRNA of *PI3* (r = 0.71, $P = 0.0004$; Fig. 5F), of *APOBEC3G* (r = 0.47, $P = 0.0142$; Fig. 5G), and of *ANG* (that encodes angiogenin) (r = 0.68, $P < 0.0001$; Fig. 5H). Additionally, almost all genes modulated by calcidiol, excluding only *PI3*, display putative VDREs in their promoter sequences, irrespective of whether their gene expression was promoted or inhibited.

DISCUSSION

VitD is a key immunoregulatory element[5,6] that could play an important role during exposure to HIV-1 and/or infection as proposed in other viral infections.[7,8]

Remarkably, we observed that two precursor forms of VitD, even at physiological concentrations, act as protecting factors on exposure to the R5 and X4 HIV-1 strains by reducing the infection of CD4+ T cells and viral replication in PBMCs of individuals with different genetic backgrounds. Our findings are supported by previous evidence showing that oral supplementation with VitD in different cohorts worldwide reduces the risk of infection and possibly disease progression, becoming a simple, cost-effective intervention, especially in resource-poor settings.[15–17]

When we analyzed the activation phenotype according to HLA-DR and CD38 expression, the CD4+CD38+HLA-DR+ and CD4+CD38−HLA-DR+ T cells were preferentially infected compared with CD4+CD38+HLA-DR− and CD4+CD38−HLA-DR− T cells (Fig. 3A), confirming a higher susceptibility of these subsets to HIV-1 infection as previously reported.[35,37] HLA-DR expression, associated with increased proliferative rates[34,38] and the sizable CCR5 and CXCR4 expression on the first 2 subpopulations might account for their higher viral permissiveness.[35] Strikingly, the more susceptible CD4+CD38+HLA-DR+ and CD4+CD38−HLA-DR+ T-cell subsets displayed a greater decrease in the infection rate in response to cholecalciferol (Fig. 3B).

Furthermore, cholecalciferol at the highest, but most commonly used concentration decreased the frequency of the CD38−HLA-DR+, while increasing the CD38+HLA-DR− subpopulations in both CD4+ and CD8+ T cells (Fig. 2). However, the proportion of highly activated CD38+HLA-DR+ CD4+ and CD8+ T cells[35,37,39] was not affected by cholecalciferol. Therefore, this VitD precursor seems to downregulate HLA-DR expression on T cells, thus probably decreasing infection through a reduction of HLA-DR+ proliferative cells.[40,41] Besides, cholecalciferol upregulates CD38 expression on T cells, a marker associated with better functional capabilities,[42] IL-2 and IFN-γ production,[43] degranulation of cytotoxic cells,[44] and suppressive capacities in T- and B-regulatory cells,[45–47] which is supported by previously described findings[41,48–50] and own observations (Villegas-Ospina et al, under review for publication). Interestingly, CD38 can impair CD4-gp120 interaction, probably by decreasing HIV-1 binding and entry into target cells,[51,52] whereas soluble CD38 peptides have been studied as potential anti-HIV-1 strategies.[53]

On the other hand, we further hypothesized that a potential conversion of cholecalciferol and calcidiol into calcitriol might have occurred, possibly explaining the anti-HIV-1 effects observed. Although, we did not measure calcitriol levels directly, both 25-hydroxilase CYP27A1 and 1α-hydroxylase CYP27B1 are expressed in PBMCs.[30] Indeed, we observed high mRNA expression levels of the VitD activating *1α-hydroxylase CYP27B1*[5] before infection (after PHA stimulation), comparable to its levels in LPS-stimulated PBMCs, and reported as sufficiently high to allow calcidiol-to-calcitriol conversion.[31–33] Likewise, the *VDR* mRNA levels were also increased postinfection after calcidiol treatment and were positively correlated with those of *CYP24A1* (Figs. 4C, D), both being target genes of the VitD/VDR complex.[5,9,13] Interestingly, other authors have reported a significant

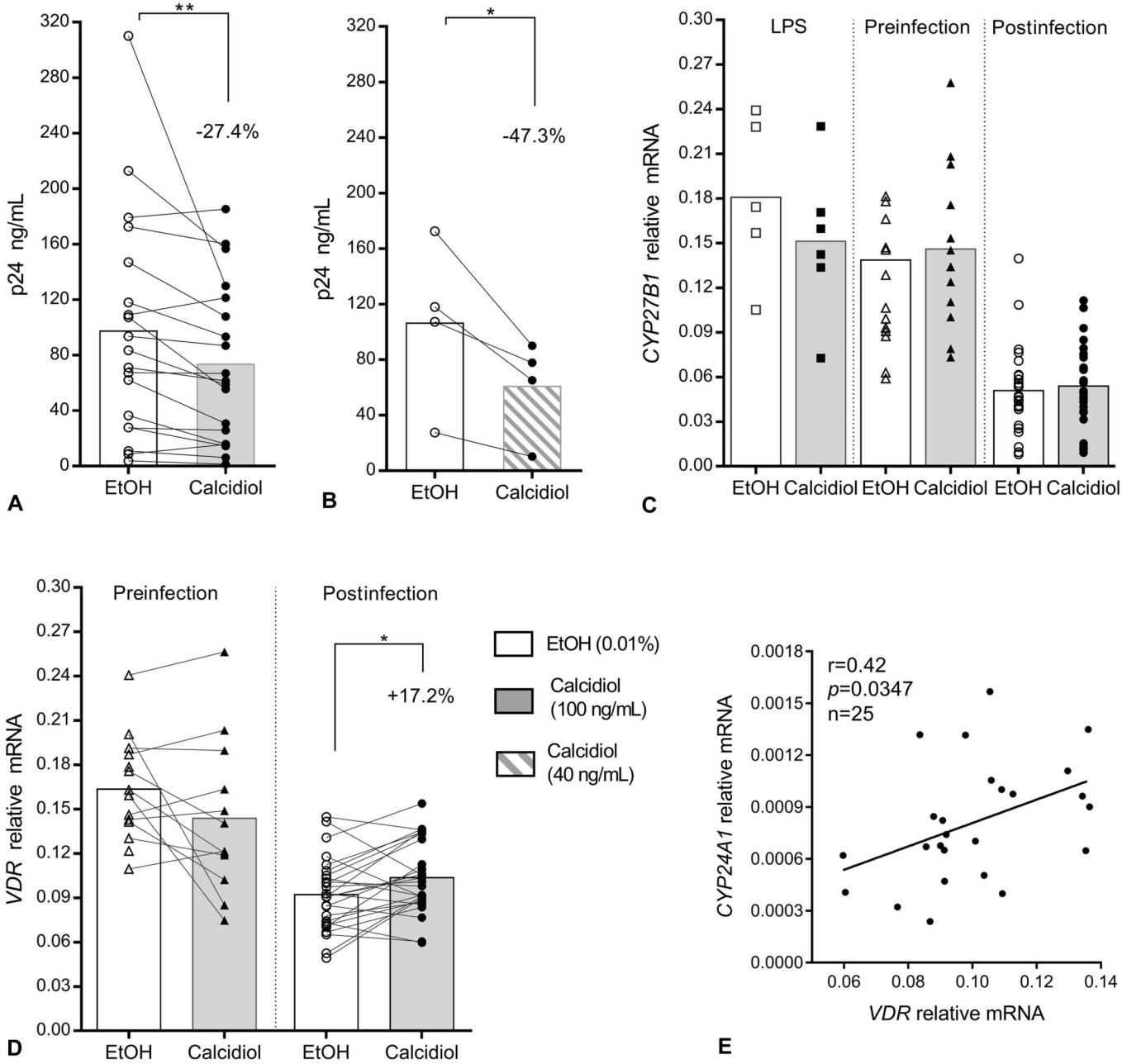

FIGURE 4. Calcidiol reduces HIV-1 infection after activating the VitD pathway. PBMCs from healthy Italian individuals were PHA/IL-2-activated and treated with physiological concentrations of calcidiol [250 nM (100 ng/mL), grey bars and filled symbols], [100 nM (40 ng/mL), slashed bars], or EtOH 0.01% control (white bars and empty symbols), and infected with an R5-tropic strain (HIV-1_{Ba-L}). The concentration of the viral protein p24 in supernatants 5 days postinfection under calcidiol at 100 ng/mL (n = 19) (A) or 40 ng/mL (n = 4) (B) was quantified by ELISA; and the mRNA expression of genes of the VitD pathway and immunoregulatory and antiviral response (for details see Materials and Methods and Table S1, Supplemental Digital Content, http://links.lww.com/QAI/A876) was calculated as the ratio of median mRNA relative units to GAPDH and HPRT reference genes after LPS control treatment (for *CYP27B1* mRNA) (in squares), preinfection (in triangles) and 20 hours postinfection (in circles). The mRNA expression levels of *CYP27B1* in preinfection conditions were similar to the levels observed after LPS control stimulus, but no calcidiol effects were observed (C). The mRNA expression of the VitD receptor (*VDR*) was higher after calcidiol treatment 20 hours postinfection (n = 26) but not preinfection (n = 12) (D). Furthermore, the mRNA levels of *VDR* and of *CYP24A1* were positively correlated in the calcidiol-treated cells postinfection (n = 25) (E). Paired *t* test and Spearman correlation tests were performed for statistical analyses and bars represent the mean. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and percent (%) increase/decrease.

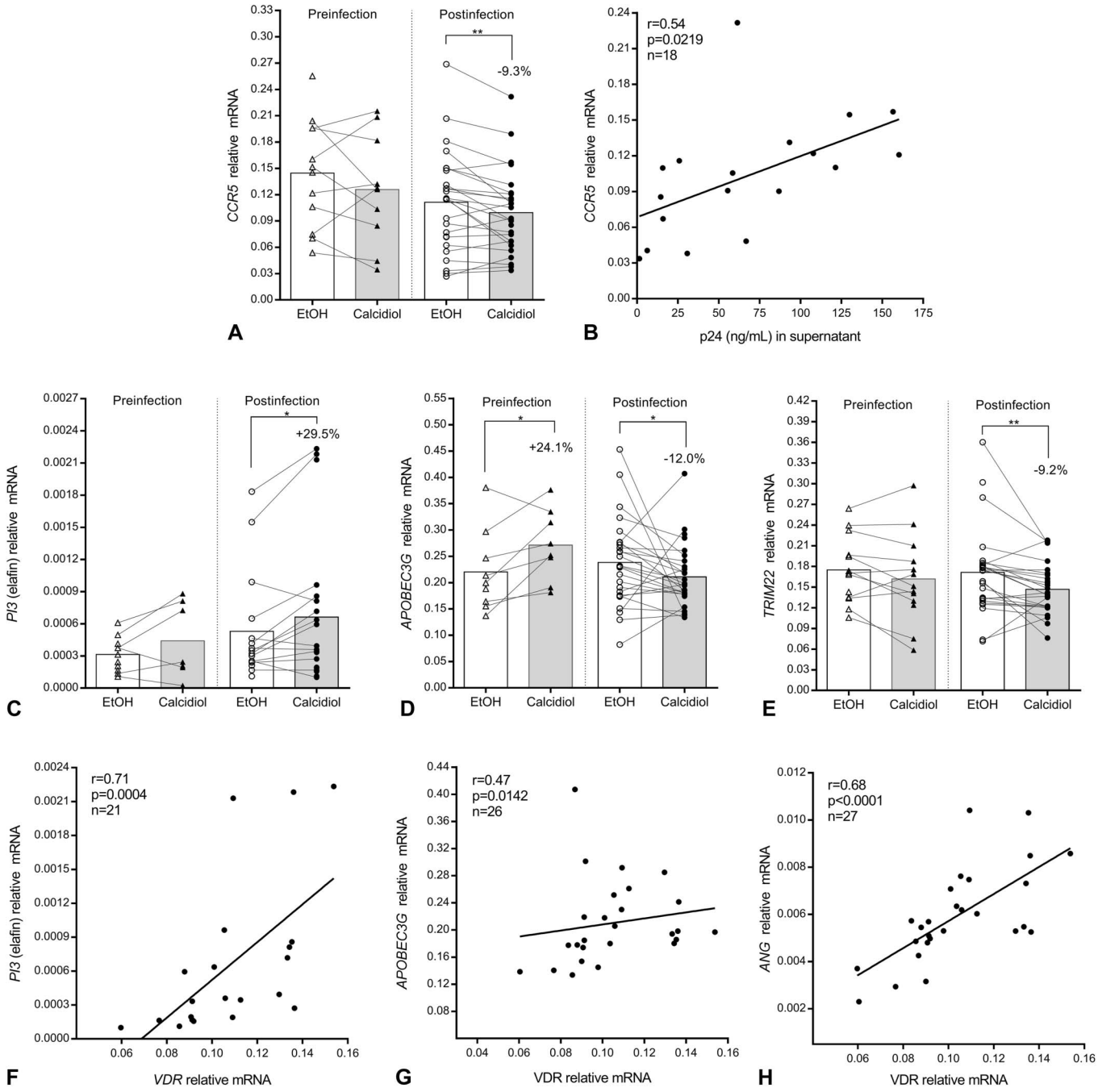

FIGURE 5. Calcidiol modulates the mRNA expression level of viral co-receptor and antiviral molecules. The mRNA levels of the viral co-receptor CCR5 and antiviral molecules relative to the *GAPDH* and *HPRT* reference genes were measured preinfection (in triangles) and 20 hours postinfection (in circles). We observed significant calcidiol-induced changes in *CCR5* (A) that positively correlated with released levels of p24 (B). Significant calcidiol-induced changes were also observed in antiviral genes *PI3* (C), *APOBEC3G* (D), and *TRIM22* (E). Furthermore, the mRNA levels of *VDR* correlated with those of *PI3* (F), *APOBEC3G* (G), and *ANG* (H). Bars represent the mean. All comparisons preinfection were performed by using paired *t* test (n = 7–13), whereas differences postinfection were analyzed by paired *t* test for CCR5 (n = 26) and *PI3* (n = 19) and Wilcoxon test for *APOBEC3G* (n = 26) and *TRIM22* (n = 26). Correlations were evaluated using the Spearman coefficient rank (r). $*P < 0.05$, $**P < 0.01$, and percent (%) increase/decrease are presented.

quantifiable calcidiol-to-calcitriol activation supporting the anti-hepatitis C virus activity exhibited by calcidiol.[7] Alternatively, a direct anti-HIV-1 effect particularly by calcidiol cannot be ruled out, since it is an agonistic VDR ligand with direct gene regulatory and anti-proliferative properties, although it is at least 100-fold less potent than calcitriol.[54,55]

Next, we evaluated the expression of several antiviral molecules downstream of the VitD/VDR pathway. Surprisingly, calcidiol increased the antiprotease elafin (*PI3*) mRNA expression level postinfection, and the mRNA levels of *APOBEC3G*, *PI3*, and *ANG* were positively correlated with those of *VDR* (Fig. 5). It is known that APOBEC3G restricts HIV-1 replication by hypermutation of the viral genome[56]; elafin has anti-HIV-1 and tissue repair properties,[57] angiogenin inhibits HIV-1 replication in vitro, and is over-expressed in endocervical mucosa of HIV-1-exposed uninfected individuals,[4,29] suggesting an interesting VitD-modulated anti-HIV-1 axis. These results support our previous findings that individuals who naturally resist HIV-1 infection exhibit higher plasma levels of calcidiol than healthy controls and have a higher mRNA expression level of *VDR* in PBMCs and genital mucosa that correlates with the production of antiviral peptides.[14,30] Likewise, calcidiol may promote other anti-HIV-1 responses not observed in this study, such as autophagy induced by the antimicrobial peptide cathelicidin.[58] Intriguingly, the increase of *PI3* mRNA levels seems to require both the calcidiol and the viral stimuli, whereas *APOBEC3G* and *TRIM22* mRNAs (TRIM22 is another HIV-1 restricting factor[28]) were downregulated after infection, suggesting that they were either inhibited by the virus or consumed for protein production to block infection; however, a contrasting unknown calcidiol effect cannot be ruled out. Interestingly, except for *PI3*, these genes modulated by calcidiol were predicted to have VDREs in their sequence analysis, partially supporting our gene expression results.

Furthermore, calcidiol downregulates the *CCR5* mRNA level after infection, and its levels were positively correlated with those of p24, suggesting that VitD could also decrease infection by modulating the expression of this viral co-receptor. A VitD-mediated decrease in CXCR4 expression cannot be ruled out either, since the expression pathways of both viral co-receptors seem to be linked,[59] and both genes have putative VDREs in their promoters, somehow explaining why cholecalciferol reduced the infection rates of $CD4^+CD38^+HLA\text{-}DR^+$ and $CD4^+CD38^-HLA\text{-}DR^+$ cells that substantially express the CCR5 and CXCR4 co-receptors.[35,59] However, we did not measure the mRNA expression of this co-receptor to confirm this possibility.

Unexpectedly, the mRNAs of the VitD-target genes *CYP24A1* and cathelicidin (*CAMP*)[5,12] were not upregulated after calcidiol treatment, possibly due to the kinetics of gene expression required to detect quantifiable levels[5] or to the strong PHA stimulus that might have modified the expression of several genes,[60] possibly confounding the interpretation of these results. In addition, the evident differences between the 2 experimental designs used here prevent us from unequivocally concluding that the mechanisms behind anti-HIV-1 activity of cholecalciferol and calcidiol are equivalent. Further evaluations with a higher sample size, protein quantification, and using gain- and loss-of-function approaches directed toward *CYP27A1*, *CYP27B1*, *PI3*, *APOBEC3G*, *HLA-DR*, *CD38*, *CCR5*, and *CXCR4* are required to validate our results and to thoroughly elucidate the role of VitD during HIV exposure.

Our results clearly show that precursors of VitD inhibit HIV-1 infection and, although requiring confirmation, the more likely mechanisms were the increase of antiviral factors expression, the decrease of viral co-receptors expression, and at least at higher cholecalciferol concentrations, the promotion of a less proliferative HIV-1-restrictive $CD38^+HLA\text{-}DR^-$ immunophenotype. Finally, our findings provide evidence that VitD presents a potential use for the development of preventive strategies during HIV-1 exposure.

ACKNOWLEDGMENTS

The authors thank the volunteers who kindly participated in this study, Ana L. Rodriguez and Paula Velilla from the Universidad de Antioquia for their support in flow cytometry analyses and for their constructive comments, and Anne-Lise Haenni from the Université Paris-Diderot for her positive revision and English proofreading. They also thank Dr Suzanne Gartner, Dr Mikulas Popovic, and Dr Robert Gallo, and the AIDS Reagent Program, Division of AIDS, NIAID, NIH, for kindly providing the HIV-$1_{Ba\text{-}L}$ strain.

REFERENCES

1. Grossman Z, Meier-Schellersheim M, Paul WE, et al. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med.* 2006;12:289–295.
2. Songok EM, Luo M, Liang B, et al. Microarray analysis of HIV resistant female sex workers reveal a gene expression signature pattern reminiscent of a lowered immune activation state. *PLoS One*. 2012;7:e30048.
3. Iqbal SM, Ball TB, Levinson P, et al. Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition. *AIDS*. 2009;23:1669–1677.
4. Zapata W, Aguilar-Jiménez W, Feng Z, et al. Identification of innate immune antiretroviral factors during in vivo and in vitro exposure to HIV-1. *Microbes Infect.* 2016;18:211–219.
5. Baeke F, Korf H, Overbergh L, et al. Human T lymphocytes are direct targets of 1,25-dihydroxyvitamin D3 in the immune system. *J Steroid Biochem Mol Biol.* 2010;121:221–227.
6. Liu PT, Krutzik SR, Modlin RL. Therapeutic implications of the TLR and VDR partnership. *Trends Mol Med.* 2007;13:117–124.
7. Gal-Tanamy M, Bachmetov L, Ravid A, et al. Vitamin D: an innate antiviral agent suppressing hepatitis C virus in human hepatocytes. *Hepatology*. 2011;54:1570–1579.
8. Hansdottir S, Monick MM, Lovan N, et al. Vitamin D decreases respiratory syncytial virus induction of NF-kappaB-linked chemokines and cytokines in airway epithelium while maintaining the antiviral state. *J Immunol.* 2010;184:965–974.
9. Korf H, Wenes M, Stijlemans B, et al. 1,25-Dihydroxyvitamin D3 curtails the inflammatory and T cell stimulatory capacity of macrophages through an IL-10-dependent mechanism. *Immunobiology*. 2012;217:1292–1300.
10. Cohen-Lahav M, Shany S, Tobvin D, et al. Vitamin D decreases NFkappaB activity by increasing IkappaBalpha levels. *Nephrol Dial Transpl.* 2006;21:889–897.
11. Khoo A, Joosten I, Michels M, et al. 1,25-Dihydroxyvitamin D3 inhibits proliferation but not the suppressive function of regulatory T cells in the absence of antigen-presenting cells. *Immunology*. 2011;134:459–468.
12. Wang TT, Nestel FP, Bourdeau V, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J Immunol.* 2004;173:2909–2912.
13. McMahon L, Schwartz K, Yilmaz O, et al. Vitamin D-mediated induction of innate immunity in gingival epithelial cells. *Infect Immun.* 2011;79:2250–2256.
14. Aguilar-Jiménez W, Zapata W, Caruz A, et al. High transcript levels of vitamin D receptor are correlated with higher mRNA expression of human beta defensins and IL-10 in mucosa of HIV-1-exposed seronegative individuals. *PLoS One*. 2013;8:e82717.
15. Lachmann R, Bevan MA, Kim S, et al. A comparative phase 1 clinical trial to identify anti-infective mechanisms of vitamin D in people with HIV infection. *AIDS*. 2015;29:1127–1135.

16. Stallings VA, Schall JI, Hediger ML, et al. High-dose vitamin D3 supplementation in children and young adults with HIV: a randomized, placebo-controlled trial. *Pediatr Infect Dis J.* 2015;34:e32–e40.
17. Coussens AK, Naude CE, Goliath R, et al. High-dose vitamin D3 reduces deficiency caused by low UVB exposure and limits HIV-1 replication in urban Southern Africans. *Proc Natl Acad Sci U S A.* 2015;112:8052–8057.
18. Skolnik PR, Jahn B, Wang MZ, et al. Enhancement of human immunodeficiency virus 1 replication in monocytes by 1,25-dihydroxycholecalciferol. *Proc Natl Acad Sci U S A.* 1991;88:6632–6636.
19. Pauza CD, Kornbluth R, Emau P, et al. Vitamin D3 compounds regulate human immunodeficiency virus type 1 replication in U937 monoblastoid cells and in monocyte-derived macrophages. *J Leukoc Biol.* 1993;53: 157–164.
20. Connor RI, Rigby WF. 1 alpha,25-dihydroxyvitamin D3 inhibits productive infection of human monocytes by HIV-1. *Biochem Biophys Res Commun.* 1991;176:852–859.
21. Campbell GR, Spector SA. Hormonally active vitamin D3 (1alpha,25-dihydroxycholecalciferol) triggers autophagy in human macrophages that inhibits HIV-1 infection. *J Biol Chem.* 2011;286:18890–18902.
22. Holick MF. Vitamin D status: measurement, interpretation, and clinical application. *Ann Epidemiol.* 2009;19:73–78.
23. O'Doherty U, Swiggard WJ, Malim MH. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J Virol.* 2000;74:10074–10080.
24. Gartner S, Markovits P, Markovitz DM, et al. The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science*. 1986;233:215–219.
25. Sironi M, Biasin M, Cagliani R, et al. A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. *J Immunol.* 2012;188:818–823.
26. Merindol N, Berthoux L. Restriction factors in HIV-1 disease progression. *Curr HIV Res.* 2015;13:448–461.
27. Biasin M, De Luca M, Gnudi F, et al. The genetic basis of resistance to HIV infection and disease progression. *Expert Rev Clin Immunol.* 2013; 9:319–334.
28. Kajaste-Rudnitski A, Marelli SS, Pultrone C, et al. TRIM22 inhibits HIV-1 transcription independently of its E3 ubiquitin ligase activity, Tat, and NF-kappaB-responsive long terminal repeat elements. *J Virol.* 2011; 85:5183–5196.
29. Bedoya VI, Boasso A, Hardy AW, et al. Ribonucleases in HIV type 1 inhibition: effect of recombinant RNases on infection of primary T cells and immune activation-induced RNase gene and protein expression. *AIDS Res Hum Retroviruses.* 2006;22:897–907.
30. Aguilar-Jimenez W, Zapata W, Rugeles MT. Antiviral molecules correlate with vitamin D pathway genes and are associated with natural resistance to HIV-1 infection. *Microbes Infect.* 2016;18:510–516.
31. Fritsche J, Mondal K, Ehrnsperger A, et al. Regulation of 25-hydroxyvitamin D3-1 alpha-hydroxylase and production of 1 alpha, 25-dihydroxyvitamin D3 by human dendritic cells. *Blood.* 2003;102:3314–3316.
32. Pinzone MR, Di Rosa M, Celesia BM, et al. LPS and HIV gp120 modulate monocyte/macrophage CYP27B1 and CYP24A1 expression leading to vitamin D consumption and hypovitaminosis D in HIV-infected individuals. *Eur Rev Med Pharmacol Sci.* 2013;17:1938–1950.
33. Stoffels K, Overbergh L, Giulietti A, et al. Immune regulation of 25-Hydroxyvitamin-D3-1α-Hydroxylase in human monocytes. *J Bone Miner Res.* 2005;21:37–47.
34. Hua S, Lécuroux C, Sáez-Cirión A, et al. Potential role for HIV-specific CD38−/HLA-DR+ CD8+ T cells in viral suppression and cytotoxicity in HIV Controllers. *PLoS One.* 2014;9:e101920.
35. Meditz AL, Haas MK, Folkvord JM, et al. HLA-DR+ CD38+ CD4+ T lymphocytes have elevated CCR5 expression and produce the majority of R5-tropic HIV-1 RNA in vivo. *J Virol.* 2011;85:10189–10200.
36. Shaw GM, Hunter E. HIV transmission. *Cold Spring Harb Perspect Med.* 2012;2.
37. Horikoshi H, Kinomoto M, Sasao F, et al. Differential susceptibility of resting CD4(+) T lymphocytes to a T-tropic and a macrophage (M)-tropic human immunodeficiency virus type 1 is associated with their surface expression of CD38 molecules. *Virus Res.* 2001;73:1–16.
38. Speiser DE, Migliaccio M, Pittet MJ, et al. Human CD8(+) T cells expressing HLA-DR and CD28 show telomerase activity and are distinct from cytolytic effector T cells. *Eur J Immunol.* 2001;31:459–466.
39. Sachdeva M, Fischl MA, Pahwa R, et al. Immune exhaustion occurs concomitantly with immune activation and decrease in regulatory T cells in viremic chronically HIV-1-infected patients. *J Acquir Immune Defic Syndr.* 2010;54:447–454.
40. Matsui T, Nakao Y, Koizumi T, et al. 1,25-Dihydroxyvitamin D3 regulates proliferation of activated T-lymphocyte subsets. *Life Sci.* 1985; 37:95–101.
41. Badenhoop K, Bogdanou D, Chung L, et al. The effect of vitamin D therapy on peripheral HLA-DR expressing T cell subpopulations in patients with autoimmune Addison's disease: a randomized controlled Trial : vitamin D metabolism & action. *ENDO Meet*. 2014.
42. Quarona V, Zaccarello G, Chillemi A, et al. CD38 and CD157: a long journey from activation markers to multifunctional molecules. *Cytometry B Clin Cytom.* 2013;84B:207–217.
43. Sandoval-Montes C, Santos-Argumedo L. CD38 is expressed selectively during the activation of a subset of mature T cells with reduced proliferation but improved potential to produce cytokines. *J Leukoc Biol.* 2005;77:513–521.
44. Sconocchia G, Titus JA, Mazzoni A, et al. CD38 triggers cytotoxic responses in activated human natural killer cells. *Blood.* 1999;94:3864–3871.
45. Kang SW, Kim SH, Lee N, et al. 1,25-Dihyroxyvitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved noncoding sequence region. *J Immunol.* 2012;188:5276–5282.
46. Blair PA, Noreña LY, Flores-Borja F, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*. 2010;32:129–140.
47. Bahri R, Bollinger A, Bollinger T, et al. Ectonucleotidase CD38 demarcates regulatory, memory-like CD8+ T cells with IFN-γ-mediated suppressor activities. *PLoS One*. 2012;7:e45234.
48. Canning MO, Grotenhuis K, de Wit H, et al. 1-alpha,25-Dihydroxyvitamin D3 (1,25(OH)(2)D(3)) hampers the maturation of fully active immature dendritic cells from monocytes. *Eur J Endocrinol.* 2001;145:351–357.
49. Stoeckler JD, Stoeckler HA, Kouttab N, et al. 1alpha,25-Dihydroxyvitamin D3 modulates CD38 expression on human lymphocytes. *J Immunol.* 1996;157:4908–4917.
50. Chen S, Sims GP, Chen XX, et al. Modulatory effects of 1,25-Dihydroxyvitamin D3 on human B cell Differentiation. *J Immunol.* 2007;179:1634–1647.
51. Savarino A. Human CD38 interferes with HIV-1 fusion through a sequence homologous to the V3 loop of the viral envelope glycoprotein gp120. *FASEB J.* 2003. doi:10.1096/fj.02-0512fje.
52. Savarino A, Bottarel F, Calosso L, et al. Effects of the human CD38 glycoprotein on the early stages of the HIV-1 replication cycle. *FASEB J.* 1999;13:2265–2276.
53. Bensi T, Mele F, Ferretti M, et al. Evaluation of the antiretroviral effects of a PEG-conjugated peptide derived from human CD38. *Expert Opin Ther Targets.* 2009;13:141–152.
54. Lou Y-R, Molnár F, Peräkylä M, et al. 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand. *J Steroid Biochem Mol Biol.* 2010;118:162–170.
55. Wu-Wong RJ, Nakane M, Yung-Wu C, et al. Different effects of calcidiol and calcitriol on regulating vitamin D receptor target gene expression in human vascular smooth muscle cells. *J Cardiovasc Dis.* 2013;1:15–20.
56. Neogi U, Shet A, Sahoo PN, et al. Human APOBEC3G-mediated hypermutation is associated with antiretroviral therapy failure in HIV-1 subtype C-infected individuals. *J Int AIDS Soc.* 2013;16:18472.
57. Drannik AG, Nag K, Yao XD, et al. Anti-HIV-1 activity of elafin is more potent than its precursor's, trappin-2, in genital epithelial cells. *J Virol.* 2012;86:4599–4610.
58. Campbell GR, Spector SA. Toll-like receptor 8 ligands activate a vitamin D mediated autophagic response that inhibits human immunodeficiency virus type 1. *PLoS Pathog.* 2012;8:e1003017.
59. Miller Sanders C, Cruse JM, Lewis RE. Toll-like receptor and chemokine receptor expression in HIV-infected T lymphocyte subsets. *Exp Mol Pathol.* 2010;88:26–31.
60. Duarte RF, Chen FE, Lowdell MW, et al. Functional impairment of human T-lymphocytes following PHA-induced expansion and retroviral transduction: implications for gene therapy. *Gene Ther.* 2002;9: 1359–1368.