

# A comparative phase 1 clinical trial to identify anti-infective mechanisms of vitamin D in people with HIV infection

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**Objectives:** To determine if there is a biological mechanism that explains the association between HIV disease progression and increased mortality with low circulating vitamin D levels; specifically, to determine if restoring vitamin D levels induced T-cell functional changes important for antiviral immunity.

**Design:** This was a pilot, open-label, three-arm prospective phase 1 study.

**Methods:** We recruited 28 patients with low plasma vitamin D (<50 nmol/l 25-hydroxyvitamin D<sub>3</sub>), comprising 17 HIV+ patients (11 on HAART, six treatment-naïve) and 11 healthy controls, who received a single dose of 200 000 IU oral cholecalciferol. Advanced T-cell flow cytometry methods measured CD4<sup>+</sup> T-cell function associated with viral control in blood samples at baseline and 1-month after vitamin D supplementation.

**Results:** One month of vitamin D supplementation restored plasma levels to sufficiency (>75 nmol/l) in 27 of 28 patients, with no safety issues. The most striking change was in HIV+ HAART+ patients, where increased frequencies of antigen-specific T cells expressing macrophage inflammatory protein (MIP)-1 $\beta$  – an important anti-HIV blocking chemokine – were observed, with a concomitant increase in plasma MIP-1 $\beta$ , both of which correlated significantly with vitamin D levels. In addition, plasma cathelicidin – a vitamin D response gene with broad antimicrobial activity – was enhanced.

**Conclusion:** Vitamin D supplementation modulates disease-relevant T-cell functions in HIV-infected patients, and may represent a useful adjunct to HAART therapy.

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## Introduction

Excess mortality and morbidity in people with HIV infection (PWHIV) have been markedly reduced with the introduction of HAART, but remain above the level of the general population. A number of immunological factors may be responsible for this deficit, including an

increased pro-inflammatory state and incomplete restoration of CD4<sup>+</sup> T-cell subset composition and function by HAART treatment [1].

Vitamin D has well documented immunomodulatory effects, including enhancement of antimicrobial defense pathways [2]. It is therefore of potential significance that

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associations of vitamin D deficiency with increased morbidity and mortality in PWHIV have been documented (see [3]). The potential global health gain from a reduction in excess morbidity is a strong rationale for exploring the possible role of vitamin D in PWHIV. Vitamin D has a significant impact on many aspects of immune function through signalling via the ubiquitously expressed vitamin D receptor (VDR). Consequently, vitamin D deficiency, which is highly prevalent globally, is associated with many diseases with underlying immune pathology, including cancer, autoimmune diseases, respiratory and cardiovascular disease, and infections [4]. The enzymes associated with the generation of the active form of vitamin D –  $1\alpha,25$ -Dihydroxyvitamin D – and the inactivation of this form, are expressed in many cell types, including dendritic cells, macrophages, and epithelial cells, suggesting a role for modulation of immunity locally. Vitamin D regulates directly, or indirectly, more than 200 genes (>1% of the human genome) [2,5], largely through specific vitamin D response element (VDRE) genomic binding sites. A well recognized impact of vitamin D is to promote innate antimicrobial defense mechanisms, including up-regulating cathelicidin [2,5], which may underpin the many reported associations of low vitamin D status and risk of infections, particularly respiratory (see [6–8]). In terms of adaptive immunity, vitamin D can promote resolution of inflammation through expansion of regulatory T cells (Tregs) [7], increased anti-inflammatory cytokine expression (e.g. IL-10), down-regulation of key pro-inflammatory cytokines, and through regulation of T-cell signalling [8,9].

These findings underpin the considerable recent interest in exploring vitamin D as a supplementary immunomodulatory therapy in the treatment of infectious diseases associated with chronic inflammation, with evidence of improved clinical outcomes for tuberculosis [10] and respiratory infections [6–8]. Vitamin D deficiency is common in HIV-infected individuals [11,12], where more than 80% of patients have low levels (<30 ng/ml) [13]. Many antiretroviral drugs are themselves implicated in impairing vitamin D synthesis [14,15], potentially contributing to deficiency, which is associated with more rapid disease progression, AIDS events, and higher mortality [3]. Although the association of vitamin D levels with CD4<sup>+</sup> cell counts is inconsistent (see [16]), vitamin D deficiency has been linked with decreased odds of CD4<sup>+</sup> recovery on HAART [17].

These considerations prompted us to conduct a longitudinal pilot study to assess the potential immunological benefits of administering high-dose vitamin D to HIV-infected patients. Induction of Treg frequencies, of recognized importance in curbing excess immune activation in HIV infection [18], served as the primary study endpoint. Secondary parameters included dampening of T-cell CD38<sup>+</sup> expression, a marker of excess

immune activation [19], the induction of antiviral effector T-cell recall responses, and anti-infective secreted cathelicidin (LL37) [20,21]. This study provides novel insights on these specific immunomodulatory effects of vitamin D in HIV infection.

## Methods

### Clinical methods

#### *Setting and volunteers*

Three groups of volunteers were selected: patients with HIV infection who were naïve to therapy and were unlikely to require it during the study duration; patients with HIV infection who were stable on long-term HAART for at least 6 months, with an undetectable viral load; and healthy controls who were uninfected. HIV-infected patients were recruited from the outpatient clinic of St Thomas' Hospital, London. Healthy volunteers (controls) were recruited from King's College, London, who responded to a brief circular e-mail invitation. All patients gave fully signed informed consent. Volunteers were eligible for screening if they had a plasma level of 25-OH vitamin D that was 20 ng/ml or less ( $\leq 50$  nmol/l). Strict exclusion criteria included: diagnosis of chronic diseases that might interfere with the interpretation of the steady results, including current infection with hepatitis B or C, or inflammatory conditions such as rheumatoid arthritis, or were on, or had recently taken, systemic anti-inflammatory medications such as corticosteroids. HIV-uninfected volunteers had a 2-min OraQuick mouth swab HIV test to confirm HIV antibody-negative status. Women from all groups had a urine pregnancy test to ensure they were not pregnant. No additional exclusion criteria were applied as vitamin D is known to impact across age, sex, and ethnicity, and therefore our patient groups were diverse.

This was a proof-of-principle investigation designed to determine whether there were potential biological mechanisms that would explain the preferential outcomes of people with HIV, who have a higher level of plasma vitamin D. We planned for between 6 and 12 volunteers to be recruited into each of the three groups. The endpoints were decided prior to study commencement. The primary endpoint was change in CD4<sup>+</sup>CD25<sup>+</sup> T-regulatory cell frequencies. The key secondary endpoints were the expression of predefined cytokines, specifically CCL5 [macrophage inflammatory protein (MIP)-1 $\beta$ ], interferon (IFN) $\gamma$ , interleukin (IL)-2, CD107a, and cellular markers of immune activation, specifically CD38<sup>+</sup> cells, and plasma 25-OH vitamin D levels. The trial was a phase 1 comparative, open-label study, and patients were selected in a non-randomized fashion according to whether they met the entry criteria for one of the three groups. All volunteers received vitamin D, and the comparative elements were healthy controls

**Table 1. Demographic disposition and baseline status.**

Parameter	HAART	Naïve	HC
Number of participants	11	6	11
Male sex number (percentage)	9 (81%)	4 (67%)	3 (27%)
Age in years median (min–max)	41 (35–49)	36 (32–45)	29 (23–43)
Ethnicity			
Black/African number (percentage)	2 (18%)	5 (83%)	3 (27%)
Indian number (percentage)	1 (9%)	–	–
Oriental number (percentage)	1 (9%)	–	1 (9%)
Philipino number (percentage)	–	–	1 (9%)
White/Caucasian number (percentage)	7 (63%)	1 (17%)	5 (45%)
White/other number (percentage)	–	–	1 (9%)
Viral load median (min–max)	<20 (<20–8121)	124022 (1010–247035)	–
Frequency of CD4 <sup>+</sup> T cells baseline median (min–max)	40.6 (24–54.7)	45 (28.9–66.8)	64.6 (47–71.5)
Frequency of CD4 <sup>+</sup> T cells 4 weeks median (min–max)	36.5 (26.2–55.5)	44.9 (23.9–48.7)	68.1 (34.2–72.1)
Nadir absolute CD4 <sup>+</sup> cell count $\mu$ l median: mean (min–max)	278:251 (32–504)	349:309 (151–372)	–
Duration of HIV infection in years median (min–max)	9.5 (1–19)	4.5 (0.5–12)	–
Duration on HAART in years median (min–max)	4 (1–14)	–	–
Vitamin D level baseline median (min–max) (nmol/l)	36 (20–62)	34 (23–43)	32 (20–49)
Vitamin D level 4 weeks median (min–max) (nmol/l)	75 (25–151)	113 (73–138)	92 (63–174)

HC, healthy control.

versus HIV-infected patients, and HIV-infected patients on HAART versus HAART-naïve patients.

The study was independently and rigorously monitored by the Guys and St Thomas' Research and Development Department, in agreement with the Declaration of Helsinki, and was approved by the relevant University and National Health Service (NHS) ethics committees (Ethics ref: 11/LO/1573 Ethics committee: NRES Committee London-Westminster). The trial was registered with the European Clinical Trials Register EudraCT Number: 2010–023122–21 (Table 1).

#### Sample collection and vitamin D administration and monitoring

Patients were observed to take 200 000 IU cholecalciferol within ten 20 000-IU gelatin capsules. Study procedures, at baseline and week 4 after vitamin D supplementation, included safety bloods for biochemistry, haematology, and a directed physical examination. Thirty-six millilitres of peripheral blood was collected into sodium heparin tubes (BD Biosciences, Oxford, UK) 2 ml in EDTA tubes (BD Biosciences), and 3 ml in Tempus tubes (Applied Biosystems, Cheshire, UK). Samples were analysed for HIV-1 viral load and lymphocyte subsets, including CD4<sup>+</sup> cell counts. Plasma vitamin D levels were estimated using the ARCHITECT 25-OH vitamin D assay (Abbott Diagnostics PLC, Abbott Park, Illinois, USA), a chemiluminescent microparticle immunoassay for the quantitative determination of 25-hydroxyvitamin D in human serum and plasma.

#### Laboratory methods

##### Antibodies for immunostaining

Detailed information about antibodies for immunostaining is listed in sTable 1 (<http://links.lww.com/QAD/A681>).

##### Whole blood immunostaining

Whole heparin blood (125  $\mu$ l) was stained with following antibodies listed in sTable 1 (<http://links.lww.com/QAD/A681>): CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD38<sup>+</sup>, and CD39<sup>+</sup>. Cells were lysed (10 $\times$  Lysing buffer; BD Biosciences), fixed and acquired on a BD Fortessa flow cytometer using FACSDiva 6.1 software (BD Biosciences).

##### Intra-cytoplasmic cytokine staining

Frozen and thawed peripheral blood mononuclear cells (PBMCs) (1  $\times$  10<sup>6</sup> cells/ml) were incubated for 16 h with cytomegalovirus (CMV)-pp65 peptide pool (1  $\mu$ g/ml, JPT Peptide Technologies GmbH), HIV gag peptide pool (2  $\mu$ g/ml), or Staphylococcal enterotoxin B (1  $\mu$ g/ml, Sigma-Aldrich, Dorset, UK) and CD107 fluorescein isothiocyanate (BD Biosciences), and CD28<sup>+</sup> and CD49d (1  $\mu$ g/ml each, both BD Biosciences) with the addition of Brefeldin A (10  $\mu$ g/ml; Sigma-Aldrich) and Monensin (2  $\mu$ mol/l; Biolegend, London, UK) for the last 14 h. Cells were stained for CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and a viability dye (Invitrogen, Paisley, UK), lysed, permeabilized (10 $\times$  lysing buffer, 10 $\times$  permeabilizing solution 2, both BD Biosciences) and stained intracellularly for IFN $\gamma$ , IL-2, and MIP-1 $\beta$ . For Treg/IL17 immunostaining, SEB-activated PBMCs (as above) were stained for CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD25<sup>+</sup>, and a viability dye (Invitrogen), lysed, permeabilized (FoxP3 Staining Kit, eBioscience, Hatfield, UK), and stained intracellularly for FoxP3 and IL-17. For phospho extracellular signal-regulated kinases (pERK) staining, PBMCs were first stained for CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and a viability dye (Invitrogen). After staining, cells were activated with PMA (1 ng/ml; Sigma-Aldrich) for 10 min. After fixing and permeabilization (Cytotfix buffer and Phosflow Perm Buffer III; BD Biosciences), cells were stained intracellularly with anti-ERK1/2 (pT202/pY204) (30 min, 4°C). For all conditions, cells were fixed and acquired on

a BD Fortessa flow cytometer using FACSDiva 6.1 software (BD Biosciences).

#### Flow cytometry data analysis

Data analysis was performed using FlowJo software (Treestar, Ashland, Oregon, USA). First, a lymphocyte gate, singlet gate (FSC-A versus FSC-H), live/dead gate was used to identify living lymphocytes. We then gated for CD3<sup>+</sup> T cells on CD4<sup>+</sup> and CD8<sup>+</sup>, and combined these gates with the Boolean operator 'or' to obtain the CD3<sup>+</sup> T-cell population. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were gated on a CD8<sup>+</sup> versus CD4<sup>+</sup> plot. CD4<sup>+</sup> and CD8<sup>+</sup> T cells positive for the markers of interest (IFN $\gamma$ , CD107, IL-2, or MIP; IL-17, CD25<sup>+</sup> and FoxP3; CD38<sup>+</sup> and CD39<sup>+</sup>; pERK) were separately identified by plotting each activation marker against CD4<sup>+</sup> or CD8<sup>+</sup>, respectively. To analyse responding T cells in detail, Boolean gating (see [22] for description) [Boolean algebraic operations (and, not, or) combined with standard gating techniques] was used to identify each subset of T cells, producing IFN $\gamma$ , CD107, IL-2, or MIP-1 $\beta$  alone or in any combination, resulting in  $n^2 - 1$  (here 15) different subsets of activated T cells.

#### Cytokine ELISA

Plasma concentrations of MIP-1 $\beta$  were measured by Quantikine ELISA kit (R&D Systems, Abingdon, UK). An additional 45 plasma factors was measured by a multiplex bead-based ELISA (ProcartaPlex Multiplex Immunoassay, Affymetrix, USA).

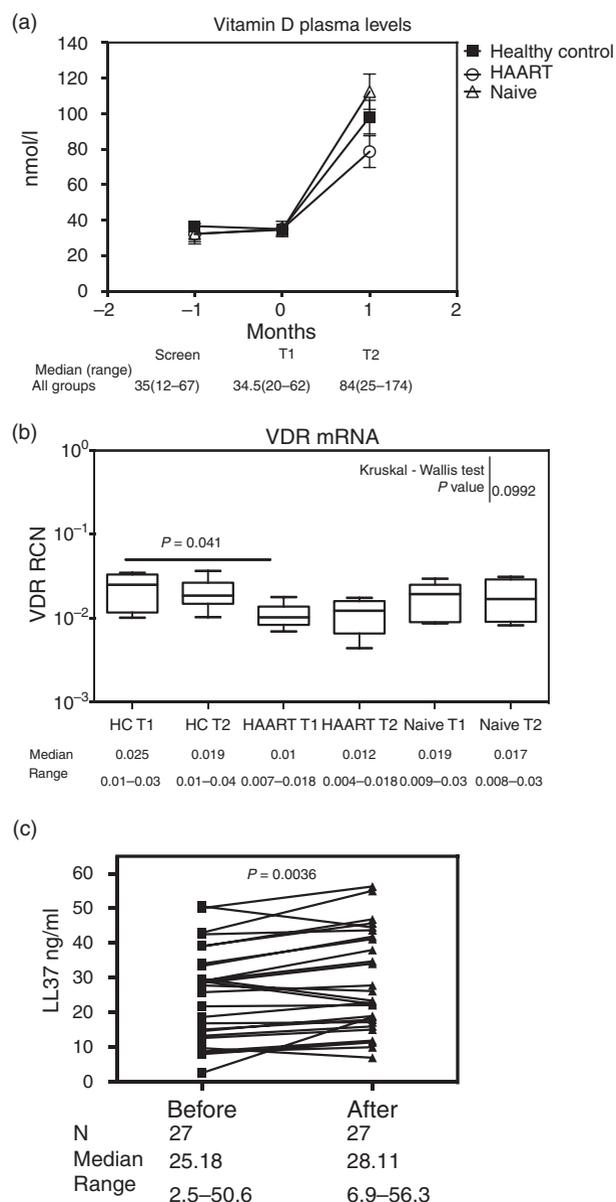
#### Statistics

Statistical analysis was performed using SPSS 19 (SPSS Inc., Chicago, Illinois, USA). Normal distribution was tested using the Kolmogorov–Smirnov test, and most data were determined to be non-normally distributed. Accordingly, Mann–Whitney *U* test was used to test for significance between groups, and Wilcoxon signed-rank test was used for significance between related samples.

## Results

### Vitamin D supplementation results in restoration of sufficiency with concomitant induction of the key vitamin D response gene LL37

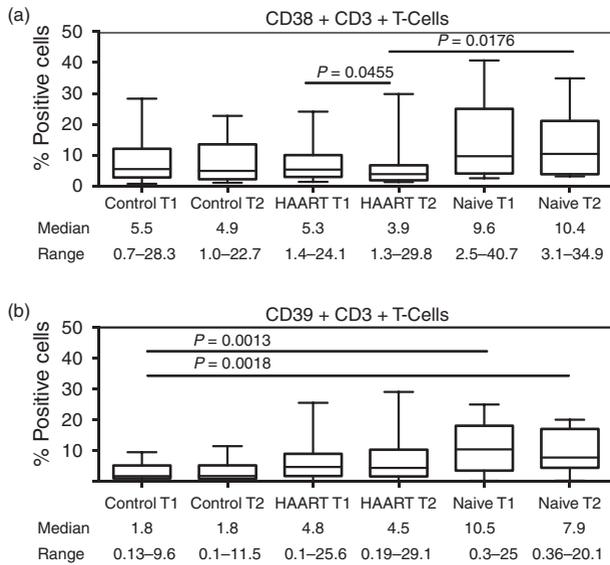
Supplementation with 200 000 IU cholecalciferol restored circulating 25-hydroxyvitamin D to sufficiency levels (>75 nmol/l) at 4 weeks (T2) in all three groups recruited, HIV+ naive (Naive), HIV+ HAART (HAART), and healthy control (Table 1 and Fig. 1a), with the exception of one HAART patient. As the benefits of supplementation are critically linked to VDR expression, VDR mRNA was compared before (T1) versus 4 weeks after vitamin D (T2). No significant changes were noted across groups, although a trend for lower VDR expression in HAART samples compared to



**Fig. 1. Vitamin D levels and vitamin D receptor expression.**

(a) Mean (+SE) vitamin D levels in plasma from HC (square), HAART (circle) and Naive patients (triangle) are shown at screening (—1), before vitamin D supplementation (T1), and 4 weeks (T2) after supplementation. (b) Median RCN (relative copy number) of VDR mRNA levels relative to GAPDH in each group at T1 and T2 time points is shown. (c) LL37 level in all patients tested at T1 versus T2 is shown. *P* value determined by Wilcoxon-paired *t* test. HC, healthy control; SE, standard error; VDR, vitamin D receptor.

healthy control was observed (Fig. 1b). No changes in mRNA expression of the heterodimeric partners of VDR, RXRB, and RXRA were observed (data not shown). Plasma levels of the antimicrobial peptide LL37 also increased significantly post-supplementation when all samples across all clinical groups were considered (Fig. 1c).



**Fig. 2. Vitamin D dampens T-cell CD38<sup>+</sup> expression.** Box and Whisker plot shows the median frequencies of CD38<sup>+</sup> (a) and CD39<sup>+</sup> (b) in CD3<sup>+</sup> T cells. *P* value determined by one-way ANOVA and Wilcoxon matched paired test, or Mann–Whitney *U* test used to confirm differences between two time points within a group (T1 versus T2) or between group differences, respectively. ANOVA, analysis of variance.

### Vitamin D modestly reduces CD38<sup>+</sup> T-cell frequency in HIV-infected patients on HAART

Specific effects on T-cell function were next assessed. Increased frequencies of activated CD38<sup>+</sup> T cells are recognized as a strong correlate of disease [19], whilst CD39<sup>+</sup> represents a further T-cell activation marker also detected on a subset of Tregs [18]. Accordingly, the frequency of CD38<sup>+</sup> CD4<sup>+</sup> T cells positively correlated with viral load ( $R=0.555$ ,  $P<0.032$  all patients) and inversely with total CD4<sup>+</sup> T-cell number ( $R=-0.726$ ,  $P<0.001$ ). CD38<sup>+</sup> CD8<sup>+</sup> T cells also correlated with plasma viral load ( $R=0.787$ ,  $P=0.001$ ) as previously published (see [19]), and at baseline, Naïve patients had a higher frequency of CD38<sup>+</sup> and CD39<sup>+</sup> CD8<sup>+</sup> T cells than healthy control or HAART patients (Fig. 2, sFig. 1, <http://links.lww.com/QAD/A680> for gating strategy). Vitamin D dampened the marginally elevated CD38<sup>+</sup> noted in the HAART group (Fig. 2a), but failed to reduce the very high levels of CD38<sup>+</sup> T cells in Naïve patients, nor did it impact CD39<sup>+</sup> (Fig. 2b). Additionally, the frequency of basal CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs and PMA/ionomycin stimulated IL-17<sup>+</sup> T cells, were also not significantly altered (sFig 2m, n, <http://links.lww.com/QAD/A680>).

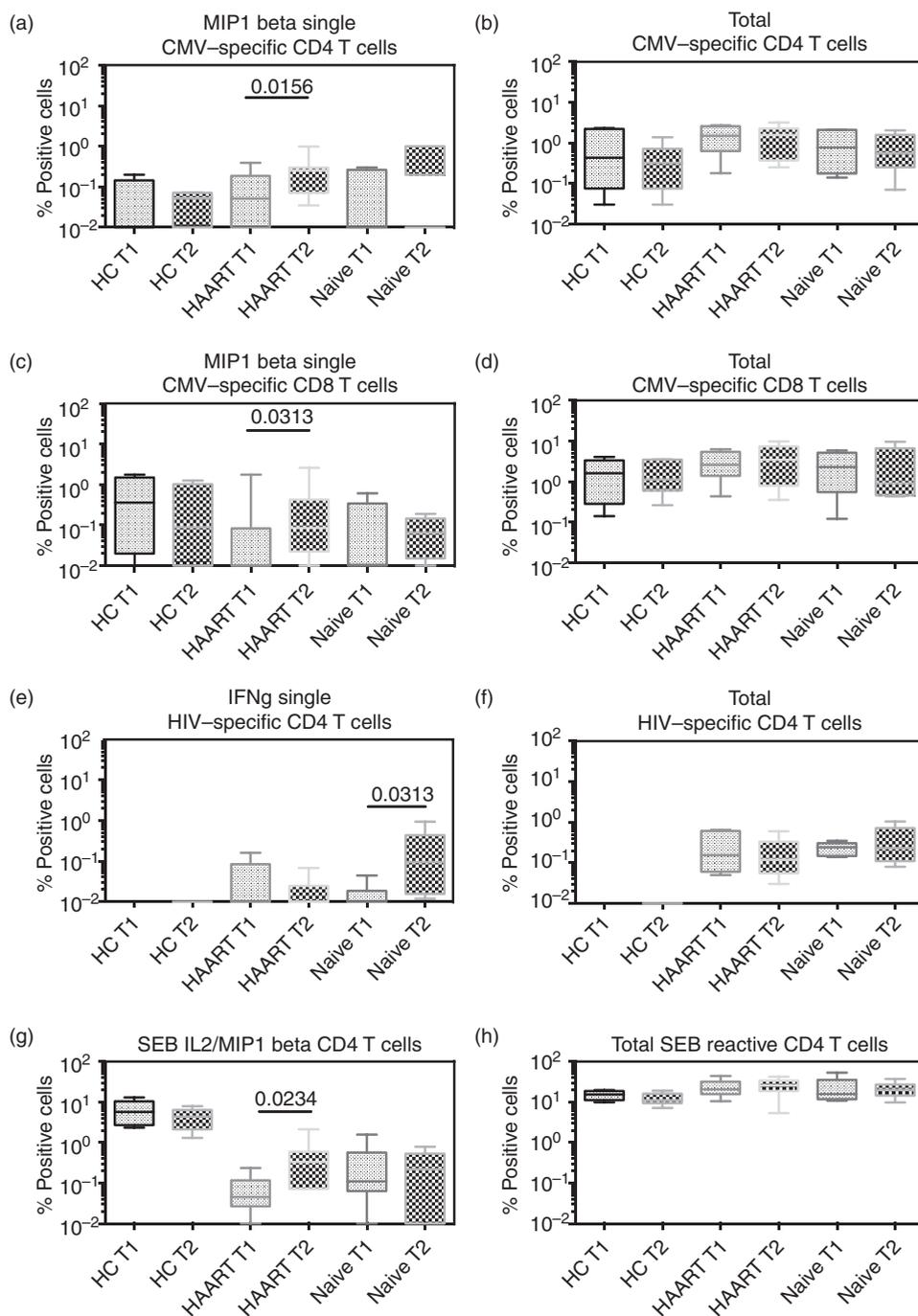
### Vitamin D significantly impacts the quality of the T-cell response and increases MIP-1β+ T-cell frequency in HAART patients

Advanced flow cytometry was used to assess the impact of vitamin D on CD4<sup>+</sup> and CD8<sup>+</sup> T-cell recall responses. The effector molecules IFNγ, IL-2, MIP-1β, and CD107

were measured simultaneously after antigen-specific stimulation with HIV-Gag and CMV-pp65 peptides or polyclonal stimulation with SEB. Boolean gating was used to determine 15 different subsets of T cells producing all possible combinations of these effector molecules [22] (see sFig 3, <http://links.lww.com/QAD/A680>, for gating strategy and sFig 4a and b, <http://links.lww.com/QAD/A680>, respectively, for frequencies of all CD4<sup>+</sup> and CD8<sup>+</sup> T-cell subsets measured). The single most striking observation was in MIP-1β expression within the HIV+ HAART group where the frequency of CMV-specific CD4<sup>+</sup> and CD8<sup>+</sup> MIP-1β single+ T cells, as well as MIP-1β/IL-2-expressing cells after SEB stimulation (Fig. 3a, c, g respectively) was modestly but significantly increased post-vitamin D supplementation. This MIP-1β response was not seen in HIV+ Naïve patients, although the frequency of CD4<sup>+</sup> T cells producing only IFNγ after HIV-Gag stimulation increased in this group (Fig. 3e). Increased frequencies of MIP-1β+ CMV-specific T cells in HAART were reflected by an overall increase in single MIP-1β+ cells and the MIP-1β/IL-2 subset following SEB stimulation, which was significantly lower in both the HAART and Naïve compared to the healthy control (Fig. 3g and sFig 4a, b, <http://links.lww.com/QAD/A680>). Whilst vitamin D altered these specific subsets, the corresponding frequency of total specific T cells expressing all four cytokines did not (Fig. 4b, d, f, h); these data highlight yet again the subtle, context-dependent T-cell changes induced by vitamin D. Importantly, induction of effector responses was also reflected in significant increase in proximal signalling following T-cell activation shown by increased pERK expression post-vitamin D supplementation, from a subset of available samples from the healthy control and HAART patients (sFig 5, <http://links.lww.com/QAD/A680>).

### Vitamin D levels correlate with MIP-1β+ CD4<sup>+</sup> T-cell frequency and soluble plasma MIP-1β

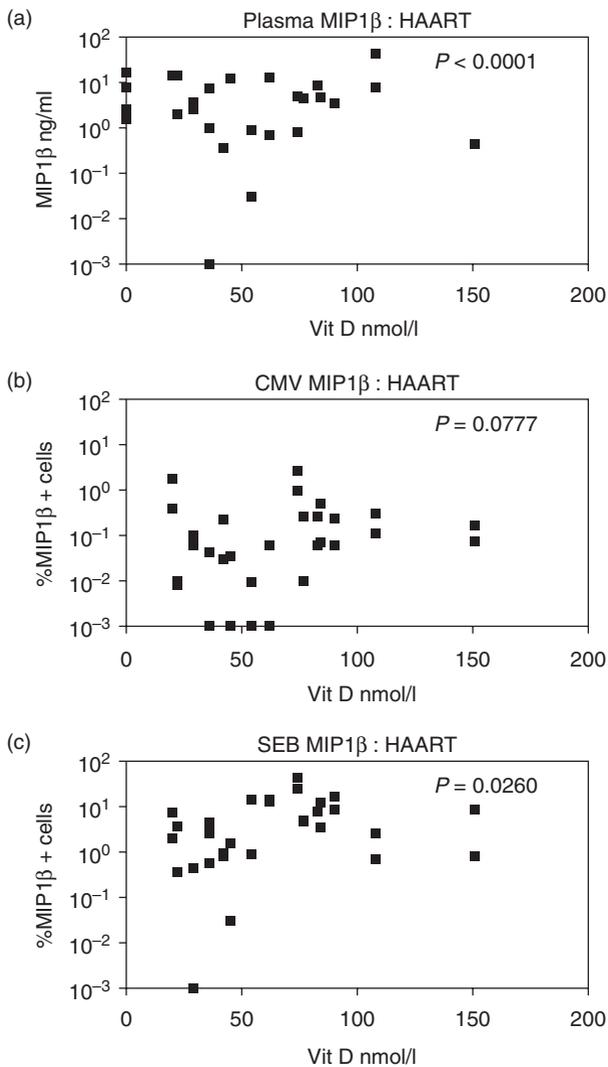
As MIP-1β is a key anti-HIV chemokine (see [23]), we assessed if the marked increase in MIP-1β T-cell frequencies (Fig. 3) was reflected in increased circulating MIP-1β using a highly sensitive specific ELISA. Consistent with the T-cell data, only HAART+ patients demonstrated elevated plasma MIP-1β (Fig. 4a–c) (non-significant data for healthy control and Naïve not shown). Importantly, total plasma MIP-1β in HAART correlated strongly ( $P<0.001$ ) with 25-hydroxyvitamin D3 levels (Fig. 4a); additionally, total SEB-stimulated MIP-1β CD4<sup>+</sup> T-cell frequencies of this group also correlated with vitamin D levels ( $P=0.026$ ; Fig. 4c). A multiplex bead ELISA was then used to determine if additional chemokines beyond MIP-1β were regulated. Interestingly, eight cytokines [IL-1, IL-6, IL-7, IL-23, IL-31, IFNα, tumour necrosis factor (TNF)α, and TNFβ] (sTable 3, <http://links.lww.com/QAD/A683>), all chemokines (sTable 3, <http://links.lww.com/QAD/A683>),



**Fig. 3. Vitamin D induces functional changes to CD4<sup>+</sup> T-cell recall responses.** Box and Whisker plots show median frequencies of T-cell subsets identified to significantly differ at 4 weeks after (T2) relative to prior supplementation (T1). Changes to MIP-1 $\beta$  single CMV-CD4<sup>+</sup> (a), MIP-1 $\beta$  single CMV-CD8<sup>+</sup> (c), IFN $\gamma$  single HIV-CD4<sup>+</sup> (e), and MIP- $\beta$ , IL-2 double-positive SEB-CD4<sup>+</sup> (g) were noted in HAART patients (a, c, e, g). The corresponding total number of CMV-CD4<sup>+</sup>, CMV-CD8<sup>+</sup>, HIV-CD4<sup>+</sup>, and SEB-CD4<sup>+</sup>, which expresses any combination of all parameters studied: IFN $\gamma$ , IL-2, CD107a, MIP-1 $\beta$  at T2 versus T1 is also shown (b, d, f, h). *P* values determined by Wilcoxon matched paired test in T2 versus T1 in each clinical group are shown. IFN, interferon; IL, interleukin.

and growth factors (sTable 3, <http://links.lww.com/QAD/A683>) tested were significantly lower in HIV+ patients versus healthy control at baseline (see sTable 2, <http://links.lww.com/QAD/A682> for *P* values). Most of

these factors were not altered by vitamin D supplementation, with the exception of epidermal growth factor, which was significantly up-regulated in the Naive group and MIP-1 $\beta$  in the HAART group (Fig. 4).



**Fig. 4. Vitamin D levels correlate with plasma MIP-1 $\beta$  and MIP-1 $\beta$ + CD4<sup>+</sup> T-cell responses in HAART+ patients.** Scatter graphs show correlation between vitamin D levels and plasma MIP-1 $\beta$  concentration in nine HAART patients (a), frequency of CD4<sup>+</sup> cells secreting only MIP-1 $\beta$  specific to CMV in eight HAART patients (b), or following SEB stimulation in eight HAART patients (c). In each case, each group includes data from T1 (pre-vitamin D) and T2 (post-vitamin D) with two independent tests for each function against mean vitamin D levels being shown. *P* value determined by Spearman's *r*.

## Discussion

The present study demonstrates how the correction of vitamin D deficiency, using single, high-dose supplementation, affects the immune system of patients with and without HIV infection. The most clinically significant and novel findings in respect of anti-HIV immunity were the increased frequency of MIP-1 $\beta$  T cells and the increased plasma MIP-1 $\beta$  in HAART+ HIV+ patients 1 month after supplementation. Furthermore, each of these parameters correlated with plasma

25-hydroxyvitamin D levels. In concert with our findings of a modest effect of vitamin D on reducing immune activation and increasing circulation of the anti-infective molecule, LL37/cathelicidin, this suggests a potentially beneficial anti-HIV effect. Most of the effects were pronounced in patients who were HIV-infected and on HAART. A recent study demonstrated an association between low plasma vitamin D and increased morbidity and mortality in people with HIV infection, the majority of who were on HAART [3]; the present findings provide a novel and plausible biological mechanism underpinning these associations.

MIP-1 $\beta$  is a key anti-HIV factor as it blocks HIV infection through direct competition with the virus for binding to its cell surface receptor, CCR5, and MIP-1 $\beta$ -secreting CD4<sup>+</sup> T cells are poorly infected by HIV (see [23,24]). Significantly, a CCR5 blocker, which mimics the effect of MIP-1 $\beta$ , maraviroc, is an effective component of the HAART regimens [25]. There is, therefore, considerable interest in finding ways to increase soluble MIP-1 $\beta$  levels and to expand MIP-1 $\beta$ + CD4<sup>+</sup> T cells. HAART itself promotes CD4<sup>+</sup> T-cell recovery and effectively suppresses viral load, but within the CD4<sup>+</sup> T-cell compartment, not all subsets of T cells are equally restored (see [19]). In this context, the capacity of vitamin D to enhance MIP-1 $\beta$ + CD4<sup>+</sup> T cells and increase soluble MIP-1 $\beta$  in patients on HAART may be clinically significant, suggesting the potential of vitamin D as an adjunct to HAART.

The expansion of MIP-1 $\beta$ + CMV-specific and IFN $\gamma$ + HIV-specific T cells following supplementation in infected patients is likely due to the recognized ongoing stimulation of T cells specific to these antigens in HIV-infected compared to uninfected patients, and implies that vitamin D induces the expansion of T cells that have previously been exposed to antigen or pre-committed antigen-experienced T cells rather than inducing de-novo responses. Pertinent to this notion are studies that highlight CMV-specific T cells to be predominantly MIP-1 $\beta$ <sup>hi</sup>, and HIV-specific T cells to be MIP-1 $\beta$ <sup>lo</sup>/IFN $\gamma$ <sup>hi</sup> [23]. Our observations of enhanced T-effector cell responses and pERK mobilization are consistent with the known effects of vitamin D on T-cell activation and T-cell receptor signalling [8,9]. Beyond T-effector cell regulation, VDR knockout mice studies show vitamin D to be critical in dampening chronic T-cell activation [26], and others and we have shown this to be mediated, at least partly, by promoting the expansion of Foxp3+ Treg numbers (see [7]). This study, however, failed to find an effect of vitamin D on Treg number; potential reasons may be the small cohorts studied, the requirement for longer periods of vitamin D exposure, and that most patients were not profoundly vitamin D-deficient (see Table 1). These data imply that the impact of vitamin D is context-dependent, impacting each clinical group in a unique manner.

The antimicrobial mechanisms of vitamin D have been studied extensively in tuberculosis, where the well documented enhancement of antimicrobial peptide production and autophagy are of likely importance [21,27]. However, antiviral mechanisms also exist as indicated by observational studies in HIV, respiratory virus, and other infections [20,21]. Cathelicidin is reported to possess antiviral activity, including anti-HIV-1 activity [20,21], and a vitamin D-mediated autophagic response that inhibits HIV-1 was recently reported [27]. These functions, together with the newly described capacity to enhance MIP-1 $\beta$ , may at least in part explain observational reports of beneficial associations between vitamin D and HIV-1 disease.

Even in the era of HAART, HIV remains one of the greatest causes of increased morbidity and mortality worldwide. Any therapies that might alleviate this, directly or as an adjunct to HAART or immune therapies, would mitigate the disease burden. Vitamin D might offer one such strategy, with an excellent safety profile, being easy to administer, well tolerated, cheap, and with recognized additional health benefits. This detailed mechanistic study was designed to determine potential major new observations regarding vitamin D supplementation in HIV infection. As vitamin D is known to broadly regulate immunity to infection across age, sex, and ethnicity, this small proof-of-concept study did not control for these parameters, although we accept that these limitations need to be addressed in a larger clinical trial. The findings from our study require further mechanistic investigation and the potential clinical effects in HIV-infected patients need examining in suitably powered clinical studies.

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B.P. conceived the project; B.P. designed the clinical trial, which was conducted by B.P., M.-A.B., N.P.; A.V., R.L., and C.H. were instrumental in experimental design; A.V. supervised all laboratory work and data analysis; R.L., S.K., and B.R. conducted experiments. A.V., B.P., R.L., and C.H. wrote the manuscript.

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## Conflicts of interest

There are no conflicts of interest.

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