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MAJOR ARTICLE

IL-32 Drives the Differentiation of Cardiotropic CD4$^+$ T Cells Carrying HIV DNA in People With HIV

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Interleukin 32 (IL-32) is a potent multi-isoform proinflammatory cytokine, which is upregulated in people with HIV (PWH) and is associated with cardiovascular disease (CVD) risk. However, the impact of IL-32 isoforms on CD4 T-cell cardiotropism, a mechanism potentially contributing to heart inflammation, remains unknown. Here we show that IL-32 isoforms β and γ induce the generation of CCR4$^+$CXCR3$^+$ double positive (DP) memory CD4 T-cell subpopulation expressing the tyrosine kinase receptor c-Met, a phenotype associated with heart-homing of T cells. Our ex vivo studies on PWH show that the frequency of DP CD4 T cells is significantly higher in individuals with, compared to individuals without, subclinical atherosclerosis and that DP cells from antiretroviral-naive and treated individuals are highly enriched with HIV DNA. Together, these data demonstrate that IL-32 isoforms have the potential to induce heart-homing of HIV-infected CD4 T cells, which may further aggravate heart inflammation and CVD in PWH.

Keywords. HIV; cardiovascular disease; IL-32; inflammation; heart-homing; CD4 T cells.

Access to antiretroviral therapy (ART) has dramatically reduced AIDS-associated deaths in people with human immunodeficiency virus (PWH). However, long-term viral suppression by ART fails to normalize chronic inflammation and systemic immune activation in PWH [1, 2]. Chronic upregulation of multiple proinflammatory cytokines in this population leads to increased risk for the development of non-AIDS comorbidities such as cardiovascular diseases (CVDs) through a number of mechanisms that include immune cell activation, endothelium dysfunction, and upregulation of inflammatory cytokines and chemokines involved in leukocyte migration into the vessel wall [1, 3–9]. We have recently described the persistent upregulation of the multi-isoform proinflammatory cytokine interleukin 32 (IL-32) in PWH, even under ART [10, 11]. Moreover, we observed that IL-32 isoforms are differentially expressed in this population and that specific isoforms could induce HIV transcription from latently infected primary CD4$^+$ T cells, which may foster the chronic inflammation [10]. IL-32 is expressed in multiple distinct isoforms generated by alternative splicing (α, β, γ, D, ϵ, θ, δ, ζ, η, and small/sm) [12, 13]. These isoforms are potent inducers of other inflammatory cytokines involved in the development of CVD such as tumor necrosis factor-α (TNF-α), IL-1β, IL-8, and IL-6 [13–22]. We further showed that distinct IL-32 isoforms are upregulated in both men and women with HIV and that these isoforms were associated with subclinical atherosclerosis in coronary and carotid arteries, respectively [23, 24].

However, how IL-32 isoforms contribute to heart inflammation and development of atherosclerosis remains unclear. Given the fundamental role of CD4 T cells in shaping the adaptive immune responses in HIV infection, being the major targets for HIV and main HIV reservoirs [25], in addition to their potential to migrate and deliver HIV to the heart, it was highly relevant to investigate the role of IL-32 on CD4 T-cell functions as linked to the pathogenesis of CVD. In this regard, it was recently shown that T cells have the potential to migrate to the heart through mechanisms involving the expression of the chemokine receptors CCR4 and CXCR3 and the interaction between the tyrosine-protein kinase c-Met and hepatocyte growth factor (HGF) expressed by heart [26, 27]. Therefore, in this study, we aimed to investigate the impact of the IL-32 isoforms on primary CD4 T cells by focusing on the expression of chemokines and chemokine receptors to gain insight into the potential

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mechanisms underlying heart-homing of T cells and their link with CVD.

METHODS

Peripheral Blood Mononuclear Cells and Plasma Samples

Peripheral blood mononuclear cells (PBMCs) and plasma were collected from PWH and HIV-negative participants from the Canadian HIV and Aging Cohort Study (CHACS). Subclinical atherosclerosis in the cohort was defined by the presence of atherosclerotic plaque in coronary arteries, measured using a cardiac computed tomography scan with injection of contrast media as we previously described [23, 28].

Cell Purification and Stimulation

$CD4^+$ T cells were isolated from total PBMCs by double-negative selection using EasySep Human CD4-T Enrichment kits (StemCell). Purified $CD4^+$ T cells or PBMCs were stimulated at 37°C with 5% CO_2 in 24-well plates in the presence/absence of 500 ng/mL recombinant IL-32α, IL-32β, or IL-32γ (R&D Systems) for 12 hours (for total RNA sequencing and quantitative reverse transcription polymerase chain reaction [RT-PCR]), 72 hours for chemokine measures by enzyme-linked immunosorbent assay (ELISA), or 5 days for phenotypic analysis with flow cytometry.

RNA Sequencing and Quantitative RT-PCR

Total cellular RNA was extracted using the RNeasy kit (Qiagen) as per the manufacturer's instructions. RNA transcription and gene expression profiling was carried out on the microarray platform of the Genome Quebec Innovation Center (Montreal, QC, Canada) using the NovaSeq6000 S2 PE100-50 M. Quantification of chemokines gene expression was carried out as previously described [10, 29–33] and detailed in Supplementary Methods.

Quantification of Soluble Proteins

IL-32, CXCL1, IL-8/CXCL8 (R&D Systems), CCL1, CCL22, and CCL24 (RayBiotech), and HGF (Abcam) were quantified in plasma from PWH and HIV-negative controls and supernatants of IL-32–stimulated CD4 T cells by ELISA as per the supplier's instructions.

In Vitro Transmigration of $CD4^+$ T Cells

Primary CD4 T cells were resuspended in serum-free Roswell Park Memorial Institute (RPMI) medium with or without 100 ng/mL of the CCR4 antagonist (2-[1,4'-bipiperidin]-1'-yl-*N*-cycloheptyl-6,7-dimethoxy-4-quinazolinamine, dihydrochloride; Cayman Chemicals) and seeded in the upper chamber of transwell plate (3.0 μm pore size; Costar) then incubated for 10 minutes at 37°C, 5% CO_2. The bottom chamber contained 500 μL of the 72 hours supernatant of $CD4^+$ T cells stimulated with either of IL-32 isoforms. T cells were then allowed to migrate for 12 hours before being collected from the bottom chamber and counted using a hemocytometer. The 12 hours migration time was selected based on qualification assays in which 6 hours was not sufficient for cell migration.

Antibodies and Flow Cytometry

Antibodies against the following receptors were used in the study: human CD3, CD4, CD8, CCR4, CXCR3, CD45RA, CD27, CCR7, CCR5 Abs from BD Biosciences, and c-Met from Novus Biologicals. Stained cells were analyzed on a BD LSR II (BD Bioscience) or LSR Fortessa (BD Bioscience).

Quantification of Integrated HIV DNA

HIV integrated DNA was quantified in lysates of primary CD4 T cells, isolated from ART-treated and treatment-naive participants, using ultrasensitive nested real-time PCR (10^5 cells/test in triplicates; detection limit, 3 HIV DNA copies), as we previously described [25, 34, 35].

Study Approval

Studies involving human participants were approved by the Institutional Review Board of Centre Hospitalier de l'Université de Montréal (ethical approval No. CE.11.063). Written informed consent was obtained from all study participants.

RESULTS

IL-32 Isoforms Differentially Induce the Expression of Distinct Chemokines in CD4 T Cells

The impact of IL-32α, IL-32β, and IL-32γ (the only available IL-32 recombinant isoforms) on primary CD4 T-cell transcriptome was tested by performing RNA sequencing on total RNA extracted from 12 hours IL-32–stimulated cells. We performed systematic differential gene expression analysis for the significantly ($P < .05$) modulated genes with a cutoff value of 1.3-fold change. The RNA sequencing data showed that individual IL-32 isoforms have distinct impact on $CD4^+$ T-cell gene transcription with IL-32α, IL-32β, and IL-32γ deriving the modulation of expression of 1080, 522, and 604 genes, respectively (Figure 1*A*, left). Only 39 genes were commonly modulated by the 3 IL-32 isoforms, including upregulated genes such as major histocompatibility complex class II and L1 cell adhesion molecules. Interestingly, IL-32β and IL-32γ, but not IL-32α, shared a common feature of upregulating gene expression of several chemokines among the top 100 significantly modulated genes, including CCL-22, CCL-24, and CCL-1, in addition to CXCL-1 in IL-32γ and CXCL-8 in IL-32β-stimulated conditions, respectively (Figure 1*A*, right and Table 1). These data were further validated by RT-qPCR of IL-32–stimulated $CD4^+$ T cells. As shown in Figure 1*B*, IL-32γ significantly upregulated the expression of CCL-22, CCL-24, CCL-1, and CXCL-1, and also CXCL-8 ($P < .0001$, $P = .0014$, $P = .0004$, $P = .0118$, and $P = .0007$, respectively). Similarly, IL-32β induced gene expression of CCL-22, CCL-24, CCL-1, and CXCL-8 but not CXCL-1 ($P = .0001$, $P = .0006$, $P = .0012$, and $P = .0059$,

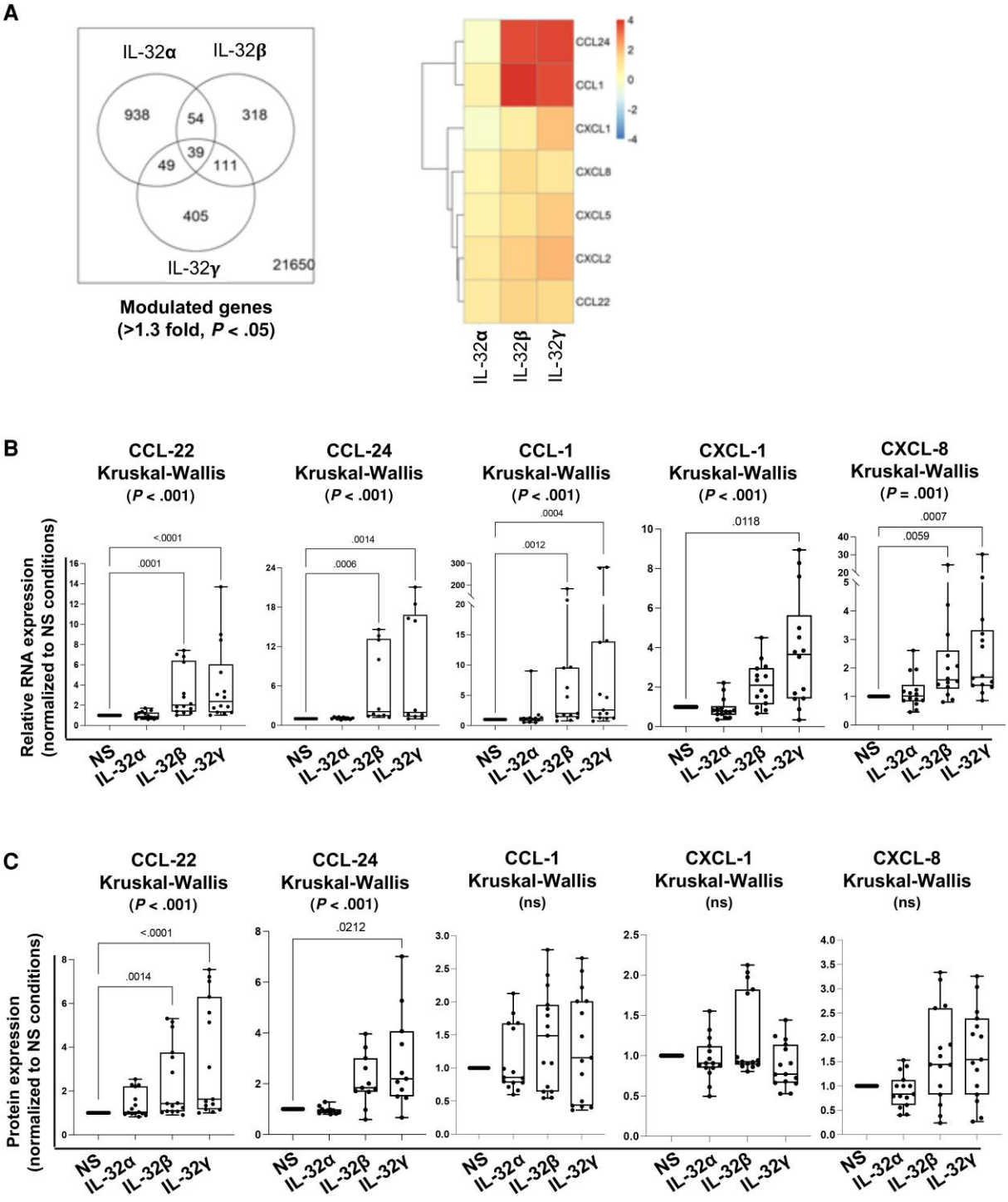

Figure 1. Total RNA sequencing analysis and in vitro validation of IL-32–modulated genes in CD4$^+$ T cells. *A*, Left, Venn diagram of differentially modulated genes identified by RNA sequencing in IL-32 isoform-stimulated (α, β, γ, 500 ng/mL) human primary CD4$^+$ T cells (12 hours stimulation, n = 5) compared to nonstimulated cells. The 21650 number on the figure represents the significantly and non-significantly modulated genes by IL-32 isoforms related to the non-stimulated conditions. Numbers of the significantly modulated genes with a cut-off value of 1.3 fold are shown inside the circles. Right, heat map of significantly upregulated chemokines in IL-32 isoform-stimulated CD4 T cells relative to nonstimulated cells showing the differential expression of selected chemokines expressed by CD4 T cells in response to IL-32α, β, and γ isoforms. *B*, Quantitative RT-PCR for the expression of chemokines CCL22, CXCL1, CXCL8 (n = 14), CCL1 (n = 13), and CCL24 (n = 10) in CD4$^+$ T cells stimulated with IL-32 isoforms (500 ng/mL) for 12 hours (shown as the fold change of stimulated relative to nonstimulated cells). *C*, Protein expression of CCL-22, CXCL1, CXCL8, CCL1 (n = 15), and CCL24 (n = 11) in the supernatant of IL-32–stimulated CD4$^+$ T cells for 72 hours measured by ELISA (shown as the fold change of stimulated relative to nonstimulated cells). Data in B and C are presented with box-plots with whiskers showing minimum to maximum values (line within boxes is plotted at the median). Nonparametric Kruskal-Wallis test and Dunn subtests were used to assess the significance of differences in chemokine expression between IL-32α, IL-32β, or IL-32γ stimulated CD4$^+$ T cells and controls. Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin; ns, not significant; NS, nonstimulated; RT-PCR, reverse transcription polymerase chain reaction.

Table 1. Upregulated Chemokines Among the Top 100 Upregulated Genes by Individual IL-32 Isoform in CD4 T Cells

Isoform	Description	HGNC Symbol	Fold Change	*P* Value
IL-32γ	C-C motif chemokine ligand 24	CCL24	13.46	.003
	C-C motif chemokine ligand 1	CCL1	12.59	.023
	C-X-C motif chemokine ligand 2	CXCL2	3.98	.031
	C-X-C motif chemokine ligand 1	CXCL1	3.56	.018
	C-X-C motif chemokine ligand 5	CXCL5	3.29	.026
	C-C motif chemokine ligand 22	CCL22	2.80	.034
IL-32β	C-C motif chemokine ligand 1	CCL1	12.59	.013
	C-C motif chemokine ligand 24	CCL24	13.46	.004
	C-C motif chemokine ligand 22	CCL22	2.80	.028
	C-X-C motif chemokine ligand 8	CXCL8	2.10	.029

Abbreviations: HGNC, HUGO Gene Nomenclature Committee; IL, interleukin.

respectively). We further quantified these chemokines by ELISA in the supernatants of IL-32–stimulated cells (72 hours). A strong donor-to-donor variability in CD4 chemokine responses to IL-32 stimulation was observed, with CXCL-8, CXCL-1, and CCL-1 proteins showing a tendency for upregulation by both IL-32β and IL-32γ without reaching statistical significance. However, upregulation of CCL-22 protein in response to both IL-32β and IL-32γ and CCL-24 in response to IL-32γ, was significantly consistent ($P = .014$, $P < .0001$, and $P = .0212$, respectively; Figure 1*C*). IL-32α did not impact the protein expression of any of the different chemokines. Under conditions combining the 3 IL-32 isoforms together with ratios corresponding to their RNA expression level [10], CCL-22 protein expression was lower compared to either IL-32β– or IL-32γ–activated condition, likely due to the anti-inflammatory effect of IL-32α as we have previously shown [10] (Supplementary Figure 1). Together, our data demonstrated that IL-32β and IL-32γ upregulate the expression of several chemokines in CD4 T cells, notably CCL-22 and CCL-24 at both RNA and protein levels. Given the chemoattractant functions of CCL-22 on T cells [36], the IL-32–mediated upregulation of this chemokine may likely contribute to the recruitment of cells expressing the CCL-22 receptor CCR4 to inflammatory sites where IL-32 is persistently expressed, as is found within the atherosclerotic tissues in people with coronary artery disease [37].

Upregulation of CCL22 in PWH Receiving ART

We further investigated the expression of CCL-22 and CCL-24, together with CXCL-1, CXCL-8, and IL-32 total proteins, in plasma from ART-treated PWH (n = 26) and compared their levels to HIV-negative controls (n = 34) (Supplementary Table 1). Our quantification assays showed that only CCL-22 together with IL-32 were significantly higher in ART-treated PWH compared to controls ($P = .0144$ and $P = .0022$, respectively; Figure 2*A*), with CXCL-8 and CXCL-1 levels showing a tendency for upregulation without reaching statistical significance. These results suggested that the IL-32/CCL-22 axis is upregulated in HIV infection and is likely involved in leukocyte migration. To test whether CCL-22 levels induced by IL-32 in vitro can attract T cells, we performed a transmigration assay in which primary CD4 T cells were seeded in the upper chamber of a transwell and supernatant from the IL-32–stimulated and nonstimulated conditions were placed in the lower chamber. Following 12 hours' incubation, migrated CD4 T cells were counted in the lower chamber. As depicted in Figure 2*B* (middle and right), the migrated cell fraction was higher when supernatants from IL-32β– and IL-32γ–stimulated cells were used compared to supernatants from nonstimulated control or the IL-32α–stimulated cells ($P = .027$ and $P = .0083$ for IL-32β and IL-32γ, respectively). The use of CCL-22 receptor CCR4 antagonist significantly diminished CD4 T-cell migration in both IL-32β and IL-32γ conditions, which further confirms the specificity of the transmigration of CD4 T cells through the CCL-22/CCR4 axis. The CCR4 antagonist significantly inhibited CD4 T-cell migration in positive controls using recombinant CCL-22 (Supplementary Figure 2). Notably, CCR4 antagonist did not impact the CD4 viability (Supplementary Figure 3). Together, these data suggest a role for IL-32 isoforms in activating the chemoattraction of $CCR4^+$ CD4 T cells through CCL-22.

IL-32 Increases the Population Expressing CCR4, CXCR3, and c-Met in $CD45RA^-$ Memory CD4 T-Cell Subset In Vitro

The migration pattern of cardiotropic CD4 T cells was recently shown to be mediated by the expression of a unique homing receptor signature including CCR4, CXCR3, and c-Met [26, 27]. These studies showed that c-Met (a tyrosine-protein kinase that binds to the HGF expressed by heart) preferentially instructs cardiotropic memory T-cell recruitment, whereas CCR4 and CXCR3 receptors sustain T-cell recruitment [26, 27]. Given the impact of IL-32 on the CCR4 ligand CCL-22 and the induction of CCR4-specific CD4 T-cell migration, we decided to to investigate the impact of IL-32 isoforms on the expression of the cardiotropic receptors including CCR4 in vitro. Hence, we stimulated PBMCs from HIV-negative controls with IL-32 isoforms (500 ng/mL for 5 days) and examined the cellular levels of c-Met, CCR4, and CXCR3 by flow cytometry (Figure 3*A*). We observed that IL-32γ, and to a lower extent IL-32β, increased the frequency of cells expressing both CCR4 and CXCR3 on a double-positive (DP) population ($CCR4^+CXCR3^+$) within the $CD4^+CD45RA^-$ memory T-cell subset (Figure 3*B*). In addition, IL-32γ, but not IL-32α or IL-32β increased the cell population expressing c-Met under stimulated compared to nonstimulated conditions (Figure 3*C*).

We further analyzed the expression of c-Met on $CD45RA^-$ memory CD4 T-cell subpopulations separated by CCR4 and CXCR3 expression (DP, $CCR4^+CXCR3^+$; single positive [SP] CCR4, $CCR4^+CXCR3^-$; SP CXCR3, $CCR4^-CXCR3^+$; and

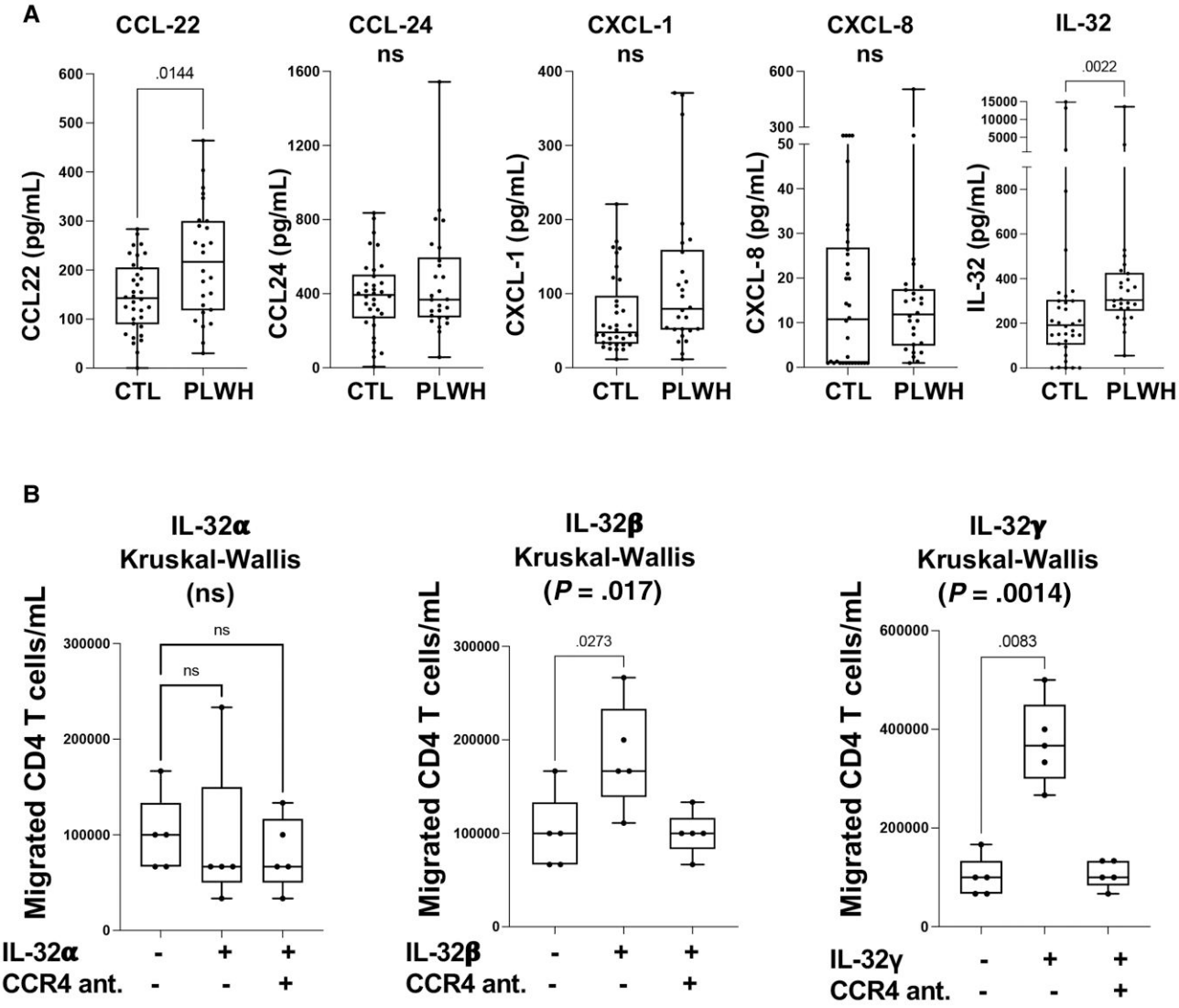

Figure 2. Expression of IL-32–modulated chemokines in PWH compared to HIV-negative controls and transmigration of human primary CD4 T cells towards IL-32–induced CCL-22. *A*, Plasma levels of CCL-22, CCL-24, CXCL-1, CXCL-8 in addition to total IL-32 proteins measured by ELISA from antiretroviral therapy-treated PWH (n = 24) and HIV-negative CTL participants (n = 34). Data analyzed with the nonparametric Mann-Whitney test to assess significance ($P < .05$). *B*, Trans-well assay for human primary CD4 T-cell migration in response to IL-32–induced CCL-22. CD4 T cells isolated by negative selection from PBMCs of CTL participants were used in the migration assay with/without CCR4 antagonist (n = 5). Following 12 hours of incubation at 37°C, 5% CO_2, CD4 T cells migrated towards the IL-32–induced CCL-22 were counted in the lower chamber. Data in A and B are presented with box-plots with whiskers showing minimum to maximum values (line within boxes is plotted at the median). Nonparametric Kruskal-Wallis test and Dunn subtests were used to assess the significant differences between the stimulation conditions. Abbreviations: CCR4 ant., CCR4 antagonist; CTL, control; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; IL, interleukin; ns, not significant; PBMC, peripheral blood mononuclear cell; PWH, people with HIV.

double negative [DN], $CCR4^{-}CXCR3^{-}$) from IL-32–stimulated and nonstimulated control cells (n = 11). Data presented in Figure 3*D* show that DP memory $CD4^{+}$ T cells expressed the highest level of c-Met (determined by the mean fluorescence intensity) in IL-32–stimulated and nonstimulated conditions (Figure 3*D*). Together, our results suggested that IL-32γ and to a lesser extent IL-32β increase the proportion of memory CD4 T cells with the heart-homing triple positive phenotype ($CCR4^{+}CXCR3^{+}$c-Met^{+}).

Increased Frequency of $CCR4^{+}CXCR3^{+}$ DP CD4 T Cells With Heart-Homing Phenotype in ART-Treated PWH With Subclinical Atherosclerosis

Given the impact of IL-32 isoforms on the expression of heart-homing receptors combined with the chronic upregulation of IL-32 in PWH, as we previously showed [10, 23, 24], we aimed to investigate whether the heart-homing $CCR4^{+}CXCR3^{+}$ DP CD4 T cells expressing c-Met are enriched in PWH and whether they are associated with subclinical atherosclerosis. To this end, we performed ex vivo phenotypic

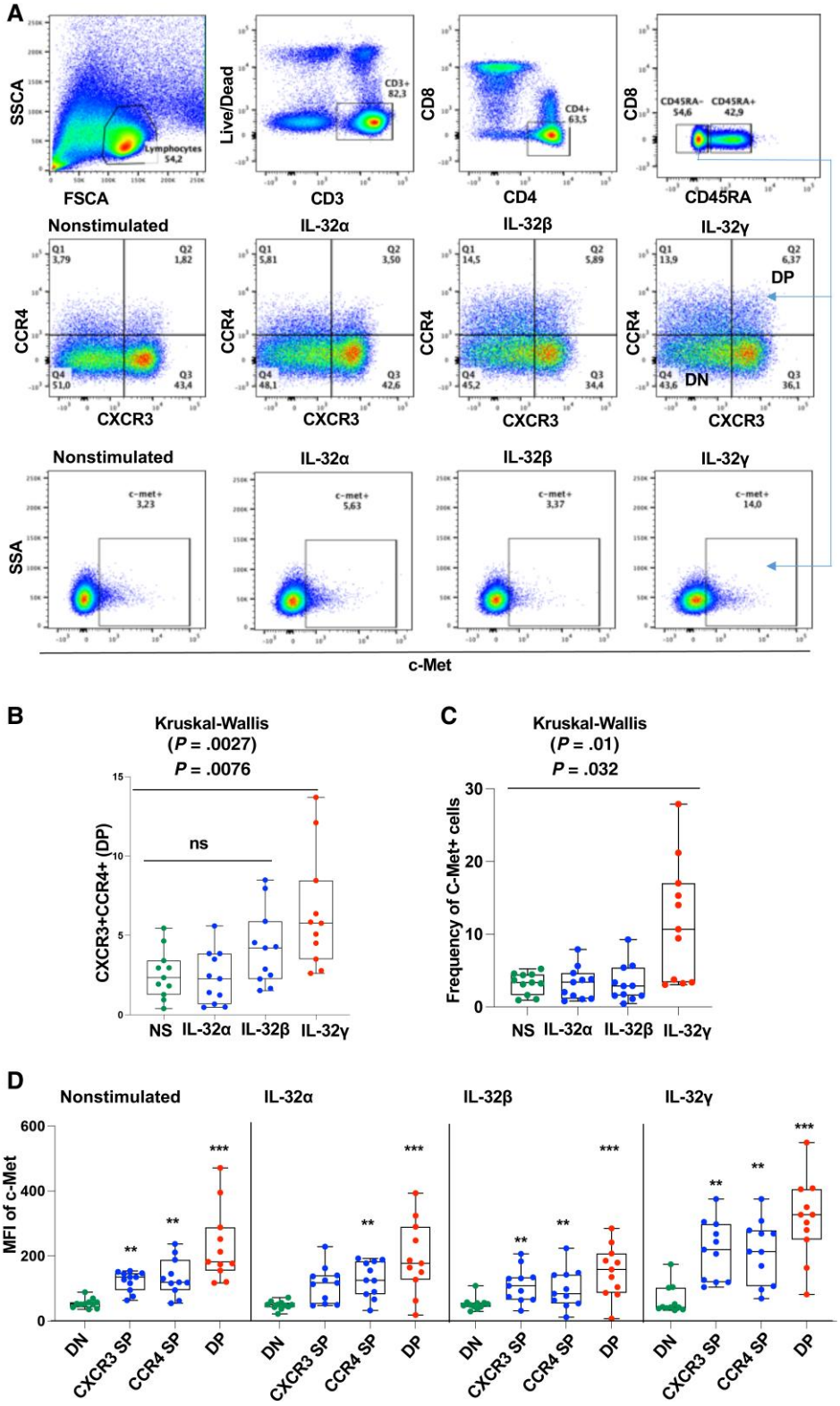

Figure 3. Upregulation of CCR4, CXCR3, and c-Met on memory CD4 T cells in response to IL-32 stimulation in vitro. *A*, Representative flow cytometry dot blots analysis showing the gating strategy on $CXCR3^{+}CCR4^{+}$ DP and $c\text{-}Met^{+}$ memory CD4 T cells. PBMCs from uninfected controls (n = 11) were stimulated with IL-32 isoforms α, β, and γ (500 ng/mL) for 5 days and stained with CD3, CD4, CD8, CD45RA, CCR4, CXCR3, and c-Met antibodies, then analyzed by flow cytometry. *B*, Analysis of the frequency of DP cells in IL-32–stimulated conditions. *C*, Analysis of the frequency of $c\text{-}Met^{+}$ memory CD4 T cells in IL-32–stimulated conditions. *D*, Expression of $c\text{-}Met^{+}$, determined by the MFI, on the memory $CD4^{+}$ T-cell subsets DP, DN, CCR4 SP, and CXCR3 SP from IL-32–stimulated and nonstimulated cells. Data in B, C and D are presented with box-plots with whiskers showing minimum to maximum values (line within boxes is plotted at the median). Data analyzed with the nonparametric test Kruskal-Wallis and Dunn subtest. Abbreviations: DN, double negative; DP, double positive; IL, interleukin; MFI, mean fluorescence intensity; ns, not significant; NS, nonstimulated; PBMC, peripheral blood mononuclear cell; SP, single positive; SSC, side scatter.

Table 2. Demographic and Clinical Parameters of Study Participants

Variable	Control, Plaque Negative	Control, Plaque Positive	*P* Value	PWH, Plaque Negative	PWH, Plaque Positive	*P* Value	PWH vs Controls *P* Value
No. of participants	10	10		10	10		
Age, y	51.3 ± 5.6	59.8 ± 8.5	.014	53.8 ± 2.7	53.4 ± 6.8	NS	NS
Predicted 10-y Framingham risk score	9.3 ± 4.4	11.7 ± 4.4	NS	10.4 ± 4.5	7.9 ± 2.7	NS	NS
Duration of ART, y	NAP	NAP		13.3 ± 7.5	13 ± 8.7	NS	
Viral load, log_{10} copies/mL	NAP	NAP		1.9 ± 0.9	2.2 ± 1.3	NS	
Nadir CD4 count, cells/mm^3	NAP	NAP		153 ± 122	267 ± 162	NS	
CD4 count, cells/mm^3	NA	NA		527 ± 205	618 ± 241	NS	
CD4/CD8 ratio	NA	NA		0.83 ± 0.45	0.84 ± 0.53	NS	NS
D-dimer, mg/L	0.249 ± 0.049	0.352 ± 0.144	NS	0.288 ± 0.104	0.316 ± 0.174	NS	NS
hsCRP, mg/L	NA	NA		7.71 ± 7.63	5.89 ± 6.23	NS	NS
LDL-C, mmol/L	3.34 ± 0.73	3.07 ± 0.52	NS	2.64 ± 0.43	3.16 ± 0.93	NS	NS
HDL-C, mmol/L	1.33 ± 0.27	1.48 ± 0.33	NS	1.13 ± 0.31	1.19 ± 0.38	NS	NS

Data are mean ± SD.

Abbreviations: ART, antiretroviral therapy; hsCRP, high-sensitivity C-reactive protein, HDL, high-density lipoprotein; LDL, low-density lipoprotein; NAP, not applicable; NA, not available; NS, not significant; PWH, people with human immunodeficiency virus.

analysis on PBMCs from both PWH and HIV-negative controls with or without subclinical atherosclerosis that we refer to here as CVD (CVD^+ and CVD^-, respectively). Tested populations included n = 10 per group as follows: PWH CVD^+, PWH CVD^-, control CVD^+, and control CVD^-. As shown in Table 2, these 4 groups were comparable in terms of traditional risk factors, such as Framingham risk score, cholesterol level, and D-dimer. By first comparing the frequency of DP CD4 T cells (gated on memory $CD45RA^-$ subset; Figure 4*A*) in total population of PWH (n = 20) versus controls (n = 20), we observed that frequencies of the DP cells were significantly higher in PWH ($P = .0052$; Figure 4*A*, lower left). When PWH were stratified by the presence or absence of subclinical CVD, we observed a significant enrichment of DP cells in blood from PWH with, compared to their counterparts without, subclinical CVD ($P = .0089$; Figure 4*A*, lower middle). No significant differences were observed in frequency of DP cells from HIV-negative controls with or without subclinical CVD (Figure 4*A*, lower right). Because c-Met signal instructs memory T cells for heart-homing [26], we then assessed the frequency of $c\text{-}Met^+$ cells on memory CD4 T cells from both PWH and HIV-negative controls. As shown in Figure 4*B*, $CD45RA^-$ memory CD4 subpopulation from PWH (n = 20) contained a higher frequency of $c\text{-}Met^+$ cells compared to controls (n = 20, $P = .0008$; Figure 4*B*, lower left). These $c\text{-}Met^+$ cells contained significantly higher frequency of DP $CCR4^+CXCR3^+$ cells from PWH with subclinical CVD compared to their counterparts without CVD ($P = .0185$; Figure 4*B*, lower middle). This phenotype was specific for PWH because it was not observed in the control group (Figure 4*B*, lower right). Finally, we aimed to investigate whether the c-Met ligand HGF was also increased in PWH. HGF was quantified by ELISA in plasma from PWH (n = 36) and controls (n = 24) (Supplementary Table 2). Significantly higher levels of plasma HGF were observed in PWH compared to controls ($P = .048$; Figure 4*C*). However, HGF levels were not significantly different when either PWH or control participants were stratified by subclinical CVD status (Supplementary Figure 4). Together, our data suggested that the increased frequency of $CCR4^+CXCR3^+$ double positive memory CD4 T-cell population expressing the heart-homing signature with c-Met is specific to PWH with subclinical CVD.

Central Memory Double Positive $CCR4^+CXCR3^+$ T Cells Are Enriched With Integrated HIV-DNA In Vivo

By further analyzing the distribution of memory subsets within the DP $CCR4^+CXCR3^+$ population we observed that these cells were significantly enriched with the central memory T_{CM} cells ($CD45RA^-CD27^+CCR7^+$) compared to either the $CCR4^-CXCR3^-$ DN, $CXCR3^+$ SP, or the $CCR4^+$ SP cells ($P = .0068$, $P = .0005$, and $P = .0171$, respectively; Figure 5*A*, middle). This distribution was not restricted to PWH as it was also observed in cells from HIV-negative controls (Figure 5*A*, right). Because memory CD4 T cells are known to be preferential targets for HIV infection in vivo [38], with the central memory CD4 T-cell subset harboring the main HIV reservoir in ART-treated PWH [25], we sought to determine if the DP $CCR4^+CXCR3^+$ T_{CM} cells contain HIV DNA. We first studied the differential expression of the HIV coreceptor CCR5 on the 4 CCR4/CXCR3 memory $CD4^+$ T-cell subsets on cells from the same participants as in Figure 5*A* . Our data showed that CCR5 expression was significantly higher in the DP compared to either the DN or CXCR3 SP cells in both PWH ($P = .0001$ and $P = .0017$, respectively; Figure 5*B*, middle) and HIV-negative controls (Figure 5*B*, right). We then investigated the presence of HIV DNA in the DP population using cells isolated from ART-naive, viremic PWH (n = 5, average CD4 count 557 (SD178) cells/mm^3 and average viral load

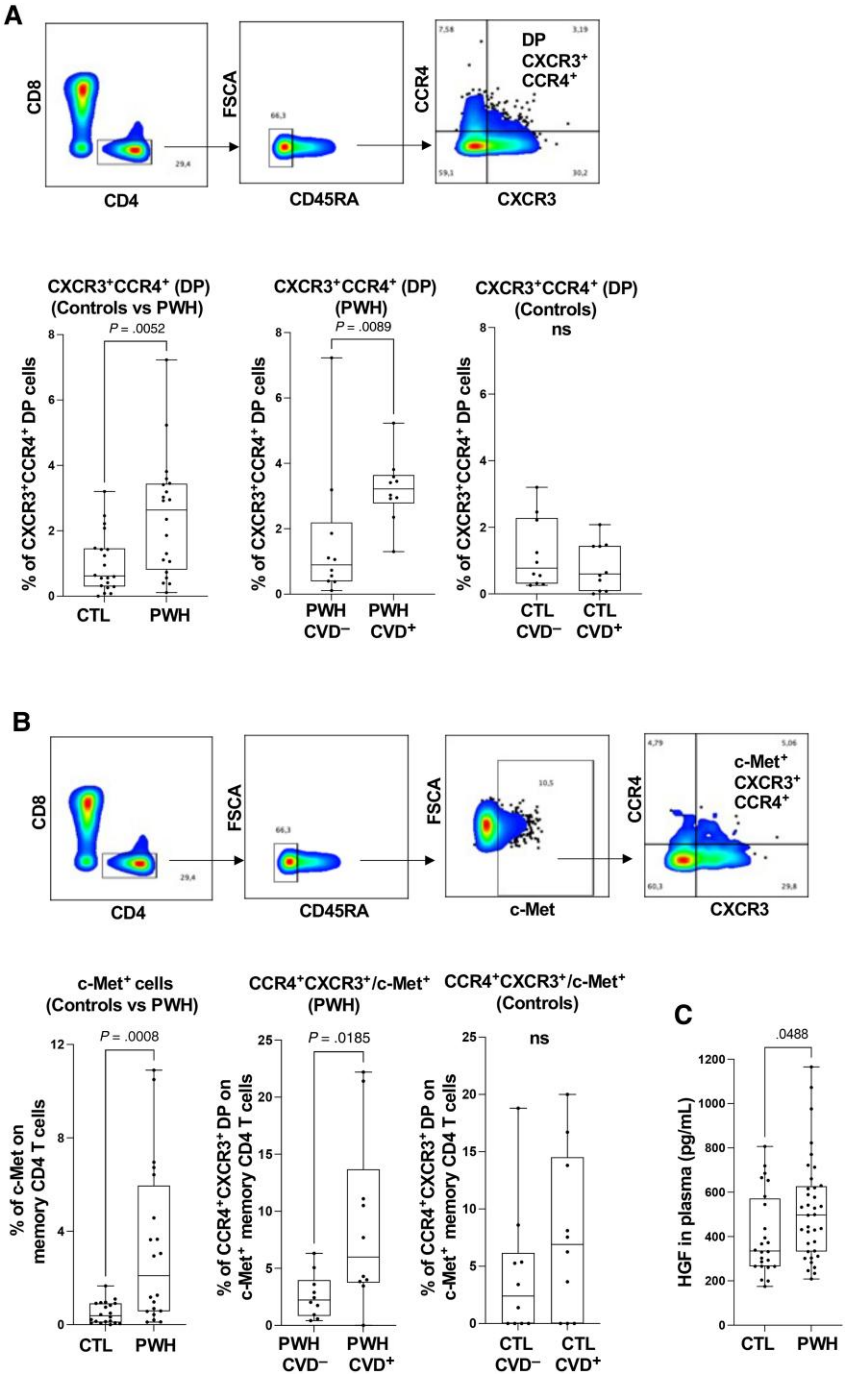

Figure 4. Phenotypic characterization of memory CD4 T cells with CCR4, CXCR3, and c-Met from PWH and HIV-negative controls with/without coronary artery subclinical CVD (n = 10/subgroup). *A*, Upper, gating strategy to identify memory ($CD4^+CD45RA^-$) subsets based on the expression of CXCR3, CCR4, and c-Met ($CCR4^+CXCR3^+$ DP, $CCR4^-CXCR3^-$ DN, $CCR4^+CXCR3^-$ CCR4 SP, and $CCR4^-CXCR3^+$ CXCR3 SP). Lower left, the frequency of memory DP CD4 T cells in the total population of PWH (n = 20) compared to controls (n = 20). Middle, the frequency of memory DP CD4 T cells in PWH with (CVD^+, n = 10) compared to their counterparts without (CVD^-, n = 10) subclinical CVD. Left, the frequency of memory DP CD4 T cells in control participants with (n = 10) compared to their counterparts without (n = 10) subclinical CVD. *B*, Upper, gating strategy on memory CD4 T cells with c-Met expression. Lower left, c-Met expression on memory CD4 T cells from the total population of PWH (n = 20) compared controls (n = 20). Middle, frequency of DP population on c-Met^+ memory CD4 T cells from PWH with (CVD^+) or without (CVD^-) subclinical CVD. Right, frequency of DP population on c-Met^+ memory CD4 T cells from controls with and without subclinical CVD. *C*, Comparison between the plasma levels of HGF from PWH (n = 36) and controls (n = 24). Data in A and C are presented with box-plots with whiskers showing minimum to maximum values (line within boxes is plotted at the median). Data analyzed with the nonparametric Mann-Whitney test. Abbreviations: CTL, control; CVD, cardiovascular disease; DN, double negative; DP, double positive; FSC, forward scatter; HGF, hepatocyte growth factor; HIV, human immunodeficiency virus; ns, not significant; PWH, people with HIV; SP, single positive.

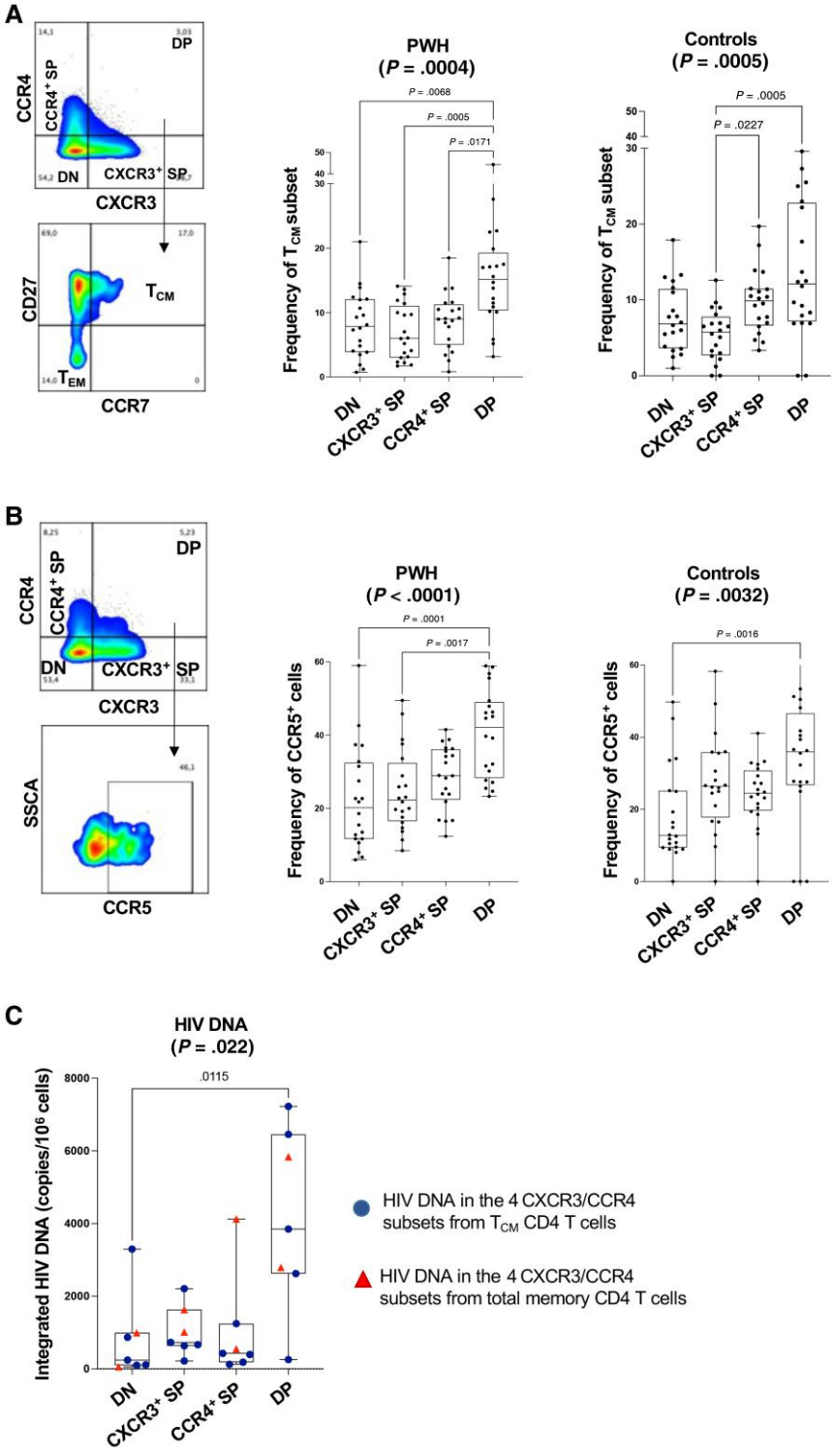

Figure 5. Susceptibility of central memory $CCR4^+CXCR3^+$ CD4 T cells to HIV infection in vivo. *A*, Left, gating strategy on central memory T_{CM} cells from the DP ($CXCR3^+CCR4^+$) cells. Right, bar graphs showing enrichment of central memory subset within the $CCR4^+CXCR3^+$ DP, DN, CCR4 SP, and CXCR3 SP CD4 T cells from PWH and HIV-negative controls (n = 20/group). *B*, Left, gating strategy on $CCR5^+$ cells from the DP ($CXCR3^+CCR4^+$) cells. Right, frequency of $CCR5^+$ cells on the DP, DN, CCR4 SP, and CXCR3 SP cells from the same participants as in (*A*). *C*, Levels of integrated HIV DNA in DP, DN, CCR4 SP, and CXCR3 SP from CD4 T_{CM} cells (data from n = 5 different antiretroviral therapy-naive PWH) and DN, CCR4 SP, and CXCR3 SP from total memory CD4 T cells (n = 2 from the same PWH). HIV DNA was quantified by real-time nested PCR. Each symbol represents mean values of triplicate wells from individual donors. Data in A, B and C are presented with box-plots with whiskers showing minimum to maximum values (line within boxes is plotted at the median). *A–C*, Data analyzed with the nonparametric test Kruskal-Wallis and Dunn subtest. Abbreviations: DN, double negative; DP, double positive; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; PWH, people with HIV; SP, single positive; $T_{CM,}$ central memory.

109 257 (SD 80 226) copies/mL). Of note, CCR5 expression was also higher on DP cells from these ART-naive participants (Supplementary Figure 5). Our data showed that the DP cells were highly enriched with integrated HIV DNA (n = 5 gated on T_{CM}, and n = 2 gated on CD45RA$^-$ memory cells, $P = .0115$; Figure 5C). Similar results were also obtained from CD45RA$^-$ memory CD4 T cells from ART-treated participants (undetectable viral load), where HIV integrated DNA was enriched in the DP cells from 2 of 3 participants (Supplementary Figure 6). Together, our data suggested that cells with the heart-homing phenotype CCR4$^+$CXCR3$^+$ (cells expressing the highest level of c-Met) are preferential targets for HIV infection in vivo.

DISCUSSION

In this study, we investigated the differential impact of IL-32 isoforms on the activation and potential implication of CD4 T cells in the pathogenesis of CVD. Multiple studies have shown that CD4 T cells with effector memory and proinflammatory phenotypes are enriched in the atherosclerotic lesions of people with clinically symptomatic atherosclerosis [39], which suggests a potential role of activated CD4 T cells in the pathogenesis of CVD. Here, we demonstrated that IL-32, a proinflammatory cytokine known to be upregulated in the atherosclerotic lesions of people with coronary artery disease [37], induces the expression of chemokine candidates representing critical mediators of leukocyte homing to the sites of inflammation such as CXCL-8, CCL-22, CXCL1, CCL1, and CCL-24 by CD4 T cells. This function was limited to IL-32β and γ, which is consistent with our recent observations on the role of these inflammatory isoforms in the activation and chemokine expression by other cell types such as coronary artery endothelial cells, which mainly produce CCL-2 and CXCL-8 in response to IL-32 isoforms [40]. However, in the current study, when testing the protein expression for the IL-32–modulated chemokines in CD4 T cells, only CCL-22 and CCL-24 were expressed whereas candidates such as CXCL-1, CXCL-8, and CCL-1 were not significantly upregulated. This was not surprising given the impact of posttranscriptional modifications on the chemokines' RNA turnover, stability, and translation into total proteins (reviewed by Fan et al [41]). Furthermore, combination of IL-32α with IL-32β and γ decreased the CCL-22 expression, likely due to the IL-32α–induced IL-10 expression as we have previously shown [10]. Of note, our ex vivo measurements on chemokines in plasma from the study participants demonstrated that CCL-22 was also significantly higher in PWH compared to HIV-negative controls. CCL-22 together with CCL-17 represent the chemokine ligands for CCR4, which is expressed by different CD4 T-cell subsets. The fact that IL-32 is highly expressed in the atherosclerotic lesions in people with coronary artery disease [37] suggests that IL-32–mediated upregulation of CCL-22 may induce the specific migration of CCR4$^+$ cells to the lesion sites. Meanwhile, CCR4 is expressed by cells with marked susceptibility to HIV infection such as Th17 and Th1/Th17 cells with the CCR4$^+$CCR6$^+$ or CXCR3$^+$CCR6$^+$ phenotypes, respectively, as we have previously shown [34, 35, 42, 43]. Expression of these chemokines receptors on these cells suggests their homing and recruitment to anatomic sites of HIV replication such as gut and lymph nodes. However, these chemokine receptors may also guide T cells for heart-homing because Th17 cells have been reported to be present in atherosclerotic lesions [44]. This is supported by recent data showing that the coexpression of both CCR4 and CXCR3 together with c-Met and the HIV coreceptor CCR5 instruct T-cell homing to the heart myocardium [26, 27]. Furthermore, c-Met$^+$ memory T cells were recently shown to be associated with cardiac inflammation [45]. Indeed, our results showed that memory CCR4$^+$CXCR3$^+$ CD4 T cells with c-Met, a phenotype that is also induced by IL-32, are enriched in PWH with coronary artery disease. However, it remains unclear how these heart-homing T cells may contribute to the coronary artery atherosclerosis. One of the possible mechanisms might be related to the potential of these cells to deliver and disseminate HIV to the heart and arterial tissues, which may further contribute to local inflammation and atherosclerosis. In line with this hypothesis, we demonstrate that these cells are highly permissive to HIV infection in vivo, as confirmed by their enrichment with HIV DNA from ART-naive and ART-treated individuals.

Heart-recruitment of the CCR4$^+$CXCR3$^+$c-Met$^+$ CD4 T cells was suggested to be mediated through the engagement of c-Met by the heart-produced HGF during antigen-priming of T cells in draining lymph nodes, followed by the migration of T cells to the antigen-rich sites [26]. However, it remains unclear which antigens within the heart tissues may drive the migration of these cells. While we did not characterize the antigenic specificity of the CCR4$^+$CXCR3$^+$c-Met$^+$ CD4 T cells in the current work, which represents a study limitation, our group has previously reported the relatively high expression of CXCR3 and CCR4 on HIV-specific CD4 T cells [46]. Therefore, one would expect heart tissues to be enriched with HIV antigens. However, this needs to be investigated and future studies are planned to address this question in detail. Another important limitation of the current study is that the phenotypic characterization of cells expressing CCR4, CXCR3, and c-Met as well as the HIV quantification assays were all done on men but not women because our study was carried out on participants from the CHACS cohort, which is mainly recruiting men with HIV [23, 28]. Future studies will also investigate these phenotypes in women with HIV.

In conclusion, the current study demonstrates a new function for the inflammatory cytokine IL-32, in mediating heart-homing on CCR4$^+$ HIV-infected CD4 T cells. This mechanism, if taking place in vivo, may contribute to heart inflammation and the development of CVD. Our data thus highlight the importance of the

IL-32/CCR4 axis and suggest the potential targeting of IL-32 to limit the development of CVD in PWH.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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