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BRIEF REPORT

Gag p24 Is a Marker of Human Immunodeficiency Virus Expression in Tissues and Correlates With Immune Response

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We demonstrate that human immunodeficiency virus (HIV) gag p24 protein is more readily detected in gut and lymph node tissues than in blood $CD4^+$ T cells and correlates better with CD4 count during antiretroviral therapy (ART). Gut p24 levels also measurably decline with ART in natural controllers. During ART, gut p24 expression is more strongly associated both with HIV-specific $CD8^+$ T-cell frequency and plasma soluble CD14 levels than gut HIV RNA expression. This study supports using gag p24 as a marker of HIV expression in HIV^+ tissues to study effects of viral persistence and to monitor efficacy of treatment in HIV-based clearance studies.

Keywords. HIV; gag p24; lymph nodes; rectal biopsy; fine needle aspirates; biomarker; HIV persistence.

Most studies of human immunodeficiency virus (HIV) persistence in tissues have relied on detecting viral nucleic acid and immune cell subsets (eg, $CD4^+$ T cells) that harbor HIV, with poorer understanding of viral protein expression. Classical DNA-based polymerase chain reaction assays detect proviral DNA but fail to discriminate between intact and the extensive percentage of defective proviruses [1]. Enhanced approaches resolve these differences but cannot differentiate between transcriptionally silent and actively expressing infected cells [2]. Similarly, cell-associated HIV RNA (caRNA) measures transcriptionally active proviruses, but most detectable HIV transcripts fail to produce viral protein, which is required for adaptive immune response recognition and clearance [3, 4]. Conversely, some putatively "defective" non-replication-competent proviruses can still produce viral proteins and elicit immune responses [5]. As proteins are more likely than nucleic acid to be sensed by the immune system, we sought to quantify p24 in tissue and blood with our enhanced digital assay [6] and assess relationships between tissue p24 and markers of immune recovery, immune activation, and HIV-specific cytotoxic lymphocyte responses in treated people with HIV (PWH).

MATERIALS AND METHODS

Participants

Participants provided informed consent and underwent either flexible rectal sigmoidoscopy biopsy, inguinal lymph node fine needle aspiration (FNA), or excisional lymph node biopsy at the University of California, San Francisco and the University of Minnesota, under institutional review board approval. Rectal samples (~3 mm diameter) were obtained as described elsewhere [7]. While most biopsies were used in real time for immunologic or virologic studies [7], 2 pieces were flash frozen, embedded in optimal cutting temperature (OCT) compound, and stored at −80°C for future analysis. FNAs were collected and prepared [8]. Lymph nodes were excised, dissected into 2 pieces, and kept at −80°C [9]. For viremic vs aviremic studies, participants were grouped by HIV and antiretroviral therapy (ART) status (HIV^-, untreated PWH, and ART-suppressed PWH stratified by CD4 count (<350 and >500 cells/μL); peripheral blood mononuclear cells (PBMCs) were stored when available [7]. Longitudinal rectal samples from a prior study of ART in HIV controllers (plasma HIV RNA<2000 [viremic] or <40 copies/mL [elite] in absence of ART) were assessed [7]. All laboratory personnel were blinded to group status.

Cell Isolation From Rectal Tissue

Two cryopreserved OCT blocks were dissociated to single cells by enzymatic digestion and mechanical disruption using tumor dissociation kit-human and gentleMACS Dissociator (Miltenyi). As CD4-negative selection kits were unvalidated for tissues, $CD4^+$ T cells were isolated by positive selection

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using human CD4 kit (Miltenyi) and counted (1×10^5 to 2×10^6). Both CD4-positive and CD4-negative fractions were collected, when available, pelleted, lysed in 1% Triton x-100/0.5% casein/phosphate-buffered saline and 50% heat-inactivated fetal bovine serum at 4×10^6 cells/mL and frozen at −80°C.

CD4 T-Cell Isolation From PBMCs

$CD4^+$ T cells were isolated using negative selection [8], counted (7×10^5 to 3×10^6), lysed, and stored at −80°C until analysis.

Lymphocyte Isolations

Lymph node biopsies were dissociated to single cells (1×10^5 to 7×10^6) [9]. FNAs were collected, split, counted (1.5×10^5 to 6×10^6), and analyzed [8].

p24 Analysis

Lysates were centrifuged 10 minutes at 10 000*g*, 4°C, and incubated with uncoated M-280 streptavidin beads (ThermoFisher Scientific) containing 10 µg/mL mouse immunoglobulin G (GenScript). Following 3 hours rocking at 4°C, lysates were centrifuged and supernatants run on HD-1 Analyzer [6].

Immunologic Measurements

Markers of T-cell activation from cryopreserved PBMCs and lymph node FNAs were measured using flow cytometry as described previously [10, 11]. HIV Gag-specific T-cell responses were reported previously [1]. Total Gag-specific T-cell response was defined as the fraction of cells expressing either CD107a, interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin 2 (IL-2), and/or macrophage inflammatory protein 1β after 5 hours' incubation with Gag peptides (after subtracting nonstimulated controls). Plasma soluble CD14 (sCD14) levels were assessed by enzyme-linked immunosorbent assay [7].

Virologic Measurements

Plasma viral load and caRNA were quantitated [3] with lower limit of detection of 9 copies/mL. For the longitudinal study of HIV controllers, HIV RNA levels (5′ elongated transcripts) were previously assessed in rectal mononuclear cells [1].

Statistical Analysis

Total rectal tissue p24 levels were derived by normalizing p24 in $CD4^+$ and $CD4^-$ flow-through fractions to the proportion of $CD4^+$ vs $CD4^-$ cells among rectal mucosal mononuclear cells assessed by flow cytometry. Differences between groups were assessed with Wilcoxon rank-sum tests and correlations between continuous variables assessed with Spearman rank-order correlations. Longitudinal changes in p24 in HIV controllers initiating ART were assessed with linear mixed models, $\log_{10}$-transforming p24 to satisfy model assumptions.

RESULTS

Rectal gag p24 Levels Are Associated With ART Status and Peripheral Blood CD4 Count

We quantified viral gag p24 protein within rectal tissue and matched blood, when available, of viremic HIV^+ individuals (n = 10), ART-suppressed immunologic responders (n = 7; CD4 counts ≥500 cells/µL) and immunologic nonresponders (n = 7; CD4 counts ≤350 cells/µL), and uninfected participants (n = 5; Figure 1A; see Supplementary Table 1 for study participant characteristics) and assessed correlations with immunological markers. HIV gag p24 was more readily detected in HIV^+ rectal tissue above a nonspecific signal (Figure 1A) than in blood (Figure 1B), suggesting higher p24 expression in tissues at equivalent $CD4^+$ T-cell inputs. HIV gag p24 in tissue vs blood was moderately correlated among all HIV^+ participants when combined ($\rho = 0.54$, $P = .0169$) (Supplementary Figure 1), but not when restricted to ART-suppressed participants ($P = .35$; data not shown). As expected, viremic participants had higher median rectal and blood $CD4^+$ p24 than other groups (Figure 1A and 1B). Rectal tissue p24 also discriminated significantly between treatment status much better than blood $CD4^+$ T-cell p24 (Figure 1A and 1B). Interestingly, ART-suppressed immunological nonresponders had significantly higher median rectal p24 than immunologic responders ($P = .009$). Among ART-suppressed participants, higher rectal p24—but not blood $CD4^+$ p24—was associated with lower CD4 counts ($\rho = -0.69$, $P = .0059$) and CD4/CD8 ratios ($\rho = -0.48$, $P = .08$; Supplementary Figure 2). Among viremic participants, there was no evidence for a relationship between peripheral blood or rectal p24 and plasma HIV RNA levels ($P > 0.38$ for both; data not shown).

Rectal p24 Decreases in Viremic and Elite Controllers After ART and Correlates With HIV-Specific T-Cell Responses and sCD14

We examined whether rectal mononuclear cell p24 was detectable in viremic (n = 5) and elite (n = 6) controllers and whether changes occurred following ART initiation. Prior to ART, median plasma HIV RNA level was 111 copies/mL in viremic controllers (range, 44–514 copies/mL) and <40 copies/mL in elite controllers. After a median of 6 weeks of ART, rectal cell lysate p24 declined in both controller groups by a mean 66% in the $CD4^+$ T-cell fraction (Figure 1C, $P = .044$) and 43% in the $CD4^-$ flow-through cell fraction (Figure 1D, $P = .006$), which was retained in this study for measurement. We next assessed the relationship between rectal p24 and previously measured rectal tissue caRNA levels, inflammation biomarkers (sCD14, interleukin 6, D-dimer, soluble tumor necrosis factor receptors 1 and 2, and C-reactive

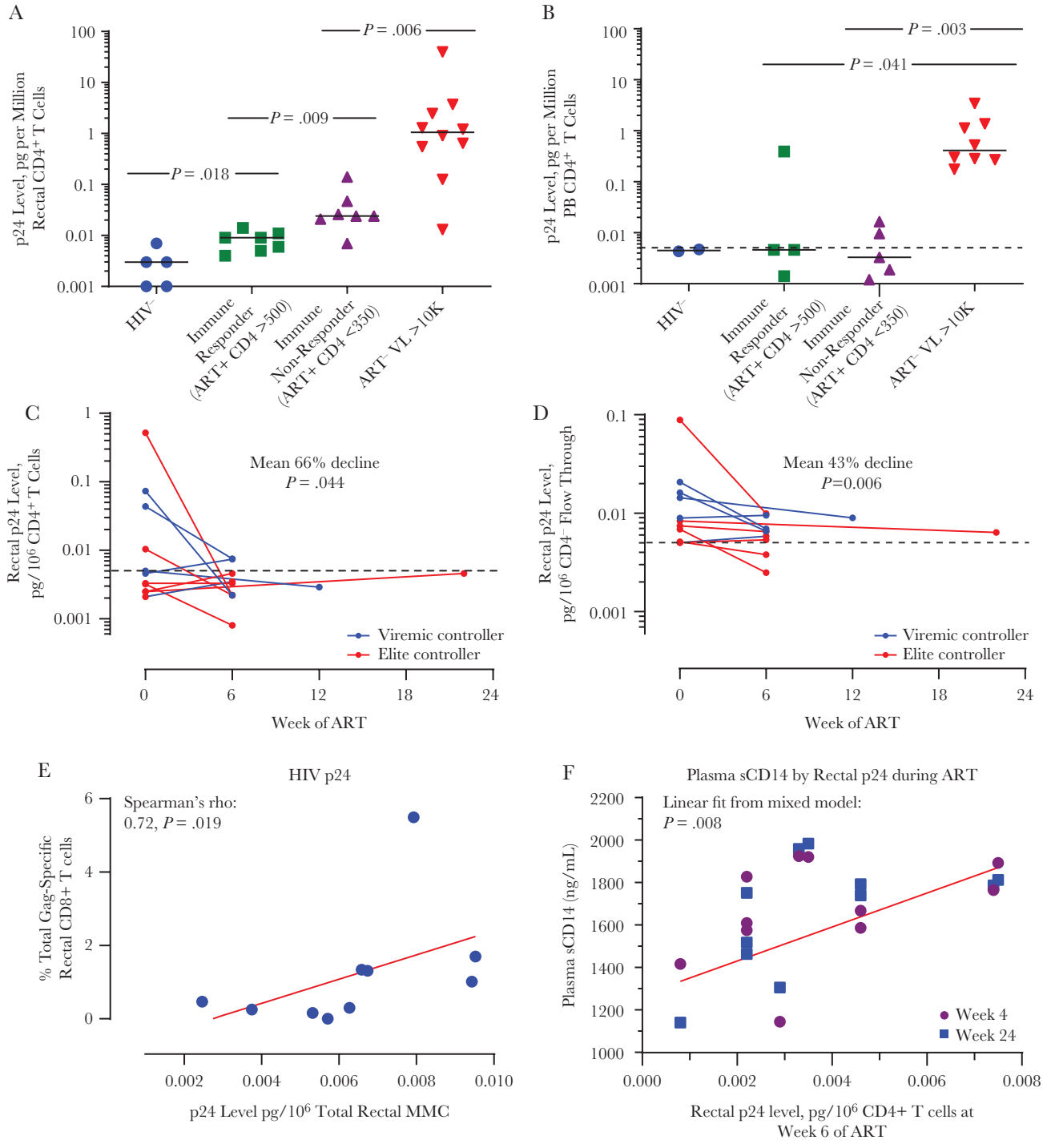

Figure 1. Rectal p24 levels are strongly linked to immune status in treated human immunodeficiency virus (HIV). *A*, p24 in isolated rectal CD4$^+$ T cells from HIV-negative, viremic, and aviremic (immune responder and immune nonresponder) participants. *B*, p24 in peripheral blood CD4$^+$ T cells matched with the rectal samples from the same participants. Dashed line represents assay limit of detection for blood CD4$^+$ T-cell matrix). *C*, Rectal p24 levels decrease after antiretroviral therapy (ART) initiation in both viremic and elite controllers in isolated CD4$^+$ T cells and CD4-negative flow-through cells (*D*). Rectal HIV p24 protein correlated with total Gag-specific CD8$^+$ T-cell responses (*E*) and plasma soluble CD14 levels (*F*) during ART. Abbreviations: ART, antiretroviral therapy; HIV, human immunodeficiency virus; MMC, mucosal mononuclear cells; PB, peripheral blood; sCD14, soluble CD14; VL, viral load.

protein) and HIV-specific T-cell responses from our previous study [1]. HIV-specific T-cell responses included total Gag-specific CD4$^+$ and CD8$^+$ responses (% expressing IFN-γ, IL-2, TNF-α, and/or CD107a). Previously obtained flow cytometry was used to assess frequencies of CD4$^+$ cells (among all mucosal mononuclear cells [1]) to derive total

p24 among all cells. Although there was no evidence for a relationship between rectal p24 in $CD4^+$ T cells and HIV caRNA (5′ elongated transcripts) levels within rectal $CD4^+$ T cells, rectal p24 levels in the CD4-negative flow-through tended to be positively associated with rectal HIV caRNA in rectal $CD4^+$ T cells across timepoints in a linear mixed model (Supplementary Figure 2*A*; $P = .01$). There was no evidence for a relationship between either tissue measure of viral burden and HIV-specific T-cell responses prior to ART (data not shown). Nevertheless, during ART, higher rectal p24 was strongly associated with greater total Gag-specific $CD8^+$ T-cell responses in rectal tissue ($\rho = 0.72$, $P = .019$; Figure 1E), and was strongest among $CD8^+$ T cells expressing the degranulation marker CD107a ($\rho = 0.74$, $P = .014$; data not shown). Higher rectal p24 was also associated with greater frequencies of Gag-specific $CD107a^+$ $CD4^+$ T cells in rectal tissue ($\rho = 0.74$, $P = .037$) as well as Gag-specific $CD107a^+$ and IFN-γ^+ $CD8^+$ T cells in peripheral blood ($\rho = 0.81$ and 0.71, $P = .005$ and $P = .021$, respectively [1]). Higher rectal p24 levels were also associated with higher plasma sCD14 during ART ($P = .008$; Figure 1F). While there were strong and consistent associations between rectal p24, plasma sCD14, and HIV-specific T-cell responses in rectal tissue and blood, there was no evidence for a relationship between rectal HIV RNA levels and plasma sCD14 levels ($P = .78$), or total HIV-specific $CD8^+$ T-cell responses in rectal tissue ($P = .78$; Supplementary Figure 2*B*) or peripheral blood ($P = .70$).

HIV p24 Protein Persists in Lymph Nodes Despite ART

p24 was also assessed in lymph node biopsies from 10 additional well-suppressed participants (viral load <20 copies/mL). p24 varied across participants with some samples displaying high p24 while others at levels bordering matrix background (~0.009 pg/million cells under these assay conditions for lymph nodes; Figure 2A). Whether the variation was due to differences between participants or differences between lymph nodes within individual participants was unclear and should be explored in future studies. Nevertheless, the presence of high p24 levels in lymph nodes of some fully suppressed donors is striking and highlights ongoing viral expression in lymphoid tissues while on ART.

Measurable p24 Protein in Lymph Node FNAs

Since p24 protein was detected in whole lymph node biopsies, we assessed whether p24 could be detected from less invasive FNA. Split lymph node FNA samples from viremic (n = 2) and ART-suppressed (n = 7) participants were evaluated as single-cell suspensions. High p24 levels were detected in FNAs from the 2 viremic participants (Figure 2B) and above the limit of detection (0.005 pg/million cells for FNA matrix) in all 7 ART-suppressed participants (Figure 2B), with similar p24 range seen in whole lymph nodes. Higher p24 was also associated with greater frequencies of $PD1^+$ $CD4^+$ T cells (Supplementary Figure 3*A*), $CXCR5^+$ $PD1^+$ T cells (Supplementary Figure 3*B*) and T follicular helper (Tfh) cells (Supplementary Figure 3*C*), as measured by flow cytometry in the split sample in which sufficient material was available for analyses by both readouts.

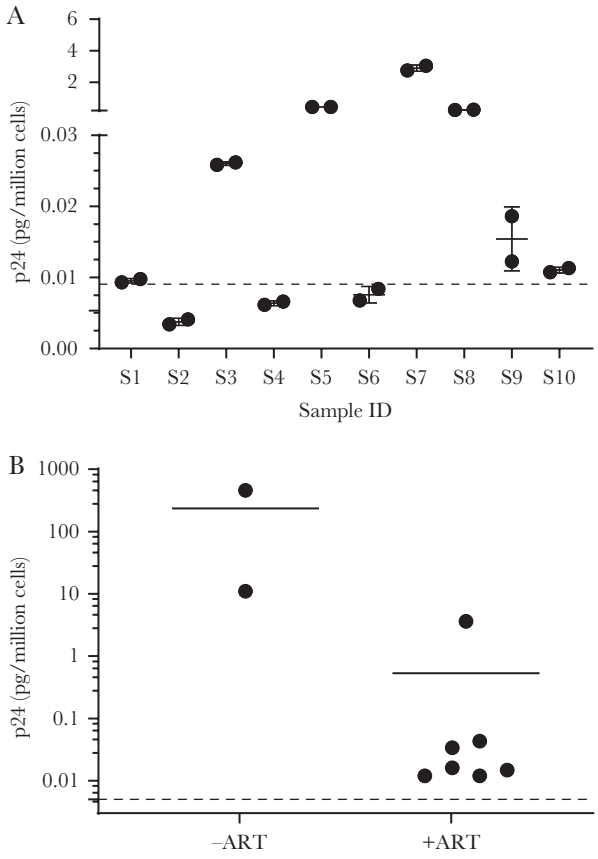

Figure 2. Human immunodeficiency virus (HIV) p24 protein persists in lymph nodes despite suppressive antiretroviral therapy (ART). *A*, Quantitation of p24 protein in excisional lymph node biopsies obtained from ART-suppressed participants. *B*, Quantitation of p24 protein in lymph node fine needle aspirate samples from viremic untreated (-ART) and ART-suppressed (+ART) participants with HIV. Dashed line represents assay limit of detection.

DISCUSSION

Systemic immune activation and detectable HIV-specific T-cell responses persist in many PWH despite prolonged ART-mediated viral suppression [12, 13]. One possible explanation is the continuous expression of HIV antigen or virus in lymphoid tissues despite ART. To explore this, we measured HIV gag p24 protein in rectal and lymph node tissues of PWH and found that p24 is more readily detected in tissues than in blood $CD4^+$ T cells. We also found that rectal p24 levels, and not viral RNA levels, discriminated between treatment and immunologic status better than blood p24. It is noteworthy that p24 was detectable in CD4-negative fraction of rectal tissue, consistent with either CD4 downregulation in productively infected cells and/or HIV infection in other cell types, an area of future study.

Our tissue-based p24 assay was sufficiently sensitive to measure ART-mediated declines in HIV controllers and showed that higher rectal p24—but not HIV RNA—was associated with higher frequencies of mucosal HIV-specific $CD8^+$ T cells and plasma sCD14 levels. This suggests that tissue p24 levels are more closely linked to the subset of infected cells that are visible to the immune system and that HIV protein expression is more strongly linked to immune activation than viral RNA. Indeed, our studies in lymph node FNAs also hint at relationships between higher p24 and greater frequencies of $PD1^+$ $CD4^+$ T cells, $CXCR5^+$ $PD1^+$ T cells, and Tfh cells, consistent with the upregulation of immune checkpoint proteins upon viral activation of T cells and the association with productive infection in Tfh cellular compartments [14].

Ongoing interactions between viral protein expression and the immune system have many implications for mechanisms of persistence and therapeutic approaches. Indeed, tissue p24 expression during ART may contribute to the expansion of HIV-specific T cells. However, persistent antigen exposure may also drive immune dysfunction and insufficient effector cell responses, or cells may harbor intrinsic mechanisms which render them resistant to cytotoxic T lymphocyte cell kill [15]. Interestingly, pre-ART rectal p24 was not associated with frequencies of HIV-specific $CD8^+$ T cells in HIV controllers. It is possible that HIV-specific $CD8^+$ T cells may be the primary effector cells responsible for clearing infected cells in the absence of ART (and reducing p24), counterbalancing the effect of rectal p24 on expanding these cells.

In conclusion, tissue p24 levels appear to be more strongly linked to immunologic status than blood $CD4^+$ T-cell p24 levels or tissue-based HIV RNA levels. Measuring HIV protein expression in tissues will likely be important in future HIV cure studies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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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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