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BRIEF REPORT

Interleukin 18 (IL-18) and IL-3 in Extracellular Vesicles: Biomarkers for Durable Elite Control of HIV-1

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Plasma extracellular vesicle (EV)-associated cytokines were quantified in people with HIV (PWH) with different virological control status, including elite controllers (EC) who maintain persistent control (PC) or not (TC). Cytokine signatures and pathways were determined for each group. Median EV-associated cytokine levels were higher among PWH than HIV-uninfected. EC showed the highest levels of EV-associated cytokines among PWH with PC levels higher than TC levels. IL-18 levels best distinguished PWH from uninfected controls, and EC from ART-treated, and IL-3 distinguished PC from TC. The role of EV-cytokines in intercellular communication and endogenous control of HIV expression should be investigated further.

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Elite controllers (EC) spontaneously control human immunodeficiency virus-1 (HIV-1) replication without antiretroviral therapy (ART), representing an exceptional model of functional cure. There are currently no reliable biomarkers for predicting EC, nor for distinguishing which EC will be transient controllers (TC) who ultimately lose HIV-1 replication control over time or persistent controllers (PC) who maintain it [1–3].

Previous small studies have found differences in viral (genetic diversity, frequency of viral blips) and host (HIV-1–specific T-cell responses, indices of immune activation) factors between TC and PC [1, 4–6]. However, broader characterization in larger and well-defined cohorts is warranted to address questions such as which EC should be treated with ART and to define distinguishing biomarkers.

Cytokines are traditionally measured as soluble factors in plasma, but cytokines are also encapsulated within and embedded on the surface of plasma extracellular vesicles (EVs) [7, 8]. In this context, we performed a detailed evaluation of 39 cytokines associated with plasma EVs to determine if EV-associated cytokine abundance and diversity could distinguish well-characterized cohorts of people with HIV (PWH) with different viroimmunological status.

METHODS

Sample Selection and Preparation

Frozen plasma samples were obtained from 30 participants without HIV and 30 PWH in each of 3 groups: (1) ART-naive; (2) ART-treated with nondetectable viremia for at least 1 year; and (3) EC with <50 HIV-RNA copies/mL in the absence of ART for a median of 14.4 years (detailed demographics in Supplementary Table 1). EC included 15 TC who lost virological control (defined as at least 2 consecutive viral load measurements above the detection limit in 1 year) and 15 PC who sustained viral suppression [1, 9]. Plasma samples for TC were obtained a median of 1.38 years (interquartile range, 0.95–2.88 years) before the loss of control. The study was approved by the regional Ethic Committee (CAEIG), an external scientific and ethics committee of the HIV Biobank (Spanish HIV/AIDS Research Network). Platelet-poor plasma (PPP) was prepared by centrifuging 500 µL plasma at 3000*g* for 15 minutes at 4°C. PPP was processed using ExoQuick Ultra for plasma (SystemBio) according to the manufacturer's instructions, to obtain EVs.

Cytokine Measurement

Two in-house multiplexed bead-based assays were developed to measure 39 markers using a previously published assay

that measured 33 cytokines [7] and another assay to measure an additional 6 markers (Supplementary Methods).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS) 19.0 (IBM) and the packages *randomForest, pROC, rpart,* and *gplots* of R software (http://cran.r-project.org). A $P < .05$ was considered nominally significant (Supplementary Methods).

Ingenuity Pathway Analysis

Comparison analyses on cytokine data (fold change compared to controls, with ± 1.25 cutoff) for each PWH subgroup were performed in ingenuity pathway analysis (IPA) software (Qiagen Bioinformatics). IPA identified canonical signaling pathways, causal networks, and biological functions based on *P* value of overlap using Fisher exact test, with Benjamini-Hochberg multiple hypothesis correction ($P < .01$ considered significant) or Z-scores to infer activation (positive z-score) or inhibition (negative z-score), z-score ≥ 2 indicating significance. My Pathway analysis connected significant canonical pathways and biological functions with key cytokines based on the IPA Knowledge Base.

RESULTS

Elevated EV-Associated Cytokine Levels Among PWH, Especially PC

We measured 39 cytokines associated with EVs isolated from PPP from the study populations described in Supplementary Table 1. Overall, the relative median levels of EV cytokines were 1.33-fold higher for the PWH population than for the uninfected control group. EC had higher levels of EV cytokines than did the ART treated (1.11-fold higher) and ART naive (1.32-fold higher). Within EC, the levels of cytokines were 1.36-fold higher for PC than for TC. Figure 1 shows a fold change heat map of the relative concentrations of EV cytokines for each group (see Supplementary Figure 1 for heat map with standardized means).

Identification of Specific Cytokine Biomarkers for EC and PC

Random forest and principal component analysis (PCA) identified potential EV cytokine signatures for the study groups while area under the curve (AUC) represents the probability of each cytokine to correctly distinguish the study group to which each individual belongs. Overall, higher EV interleukin 18 (IL-18) best distinguished between PWH and uninfected controls with median levels of 1.15 versus 0.26 pg/mL having an AUC of 0.74 by receiver operating characteristic (ROC) analysis. Thus, there is a 74% chance that IL-18 levels distinguish PWH and controls. Sensitivity was 68.9% and specificity 76.7%.

Higher levels of EV IL-18 also distinguished EC from ART-treated persons with suppressed viremia displaying an AUC of 0.942 and a sensitivity of 73.3% and specificity of 100%. EC also had higher EV IL-18 levels than did the ART naive with a ROC analysis displaying AUC of 0.746 with a sensitivity of 73.3% and specificity of 66.7%. Higher levels of EV IL-3 and EV tumor necrosis factor-related apoptosis inducing ligand (TRAIL) were best at discriminating TC and PC phenotypes with an ROC analysis showing an AUC of 0.824 and 0.814, a sensitivity of 73.3% and 86.7%, and a specificity of the 86.7% and 73.3%, respectively. The figures for all the cytokines identified as signatures for each study group are shown in Supplementary Table 2. See Supplementary Figures 2–5 for random forest, PCA, and ROC curves.

Algorithms designed using Decision Trees analysis showed that among individuals with suppressed viremia (EC and ART treated), 100% of participants with EV IL-18 levels equal to or above 2.23 pg/mL were correctly classified as EC. Within the EC groups, 91% with interferon-γ (IFN-λ) levels equal or greater than 81.41 pg/mL (n = 10) were correctly classified as PC (Supplementary Figure 6).

IPA Analysis of Cytokine Expression by PWH Groups

Comparison analyses examined differential protein expression between EC and ART treated, and between PC and TC, to identify canonical pathways, putative causal networks, and biological functions. In general, all predictions were more significant for EC than ART treated, and more for PC than TC. Top canonical pathways included IL-17 signaling and virus-induced inflammatory pathways (Supplementary Figure 7A). Causal network analysis identified molecules involved in inflammation as potential upstream regulators, particularly nuclear factor κB (NFκB; Supplementary Figure 7B). Biological function analysis predicted more significant antiviral activity, general immune responses, and specific immune cell and endothelial cell responses for EC than for ART treated, and more for PC than TC. (Supplementary Figures 8 and 9). One exception was innate lymphoid cells (ILC), which were significant for TC but not PC.

The IPA Knowledge Base demonstrated the role of IL-18 (distinguishing EC from ART treated) and IL-3 and TRAIL (distinguishing PC from TC) in significant canonical pathways and biological functions. IL-18 was notably associated with natural killer (NK) cell, T-cell, and ILC functions (Figure 2*A*), which were more significant in EC. IL-3 and TRAIL were significantly associated with myeloid, especially dendritic cell (DC), and B-cell functions, which were more significant in PC (Figure 2*B*).

DISCUSSION

Our study revealed significantly higher levels of plasma EV-associated cytokines among PWH with different virologic control status than among uninfected individuals. EV-association of cytokines may provide advantages, including protection from the action of antagonists, homeostatic systems, or enzymes that limit their biological function, and inclusion of

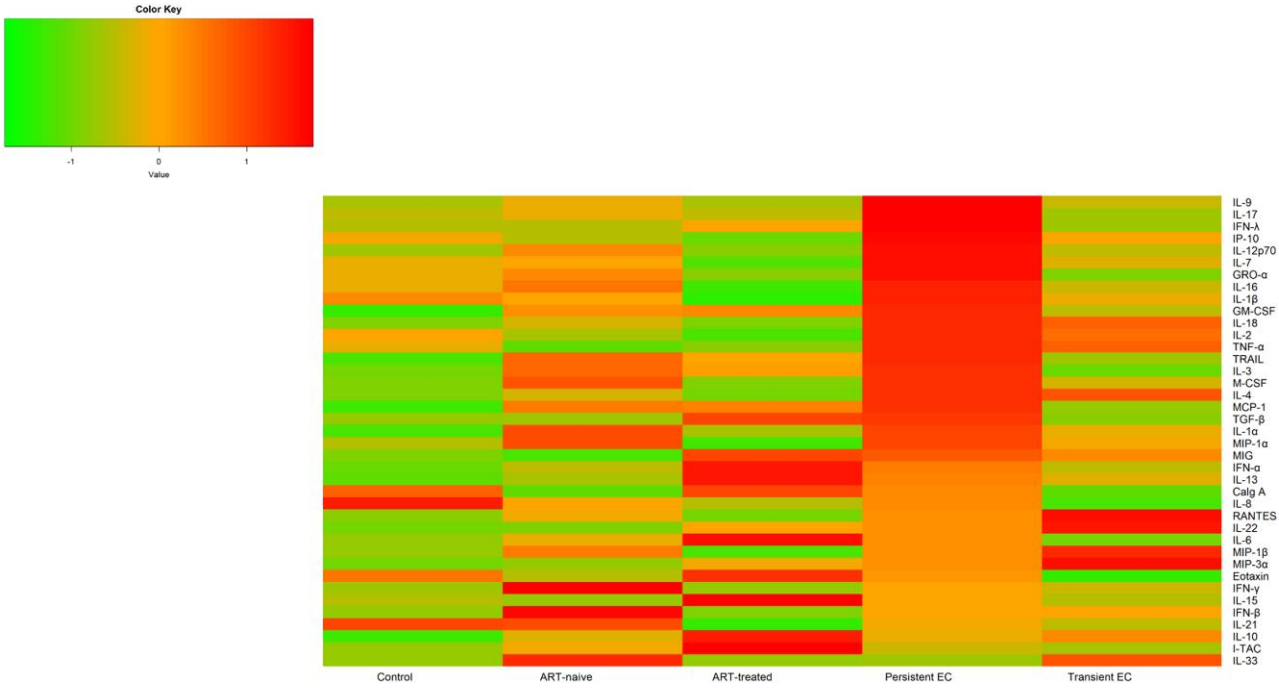

Figure 1. Heap map of the relative cytokine levels associated with extracellular vesicles. The levels of cytokines for each study group are represented as the standardized mean of each group as a comparator for each cytokine. Abbreviations: ART, antiretroviral therapy; EC, elite controller.

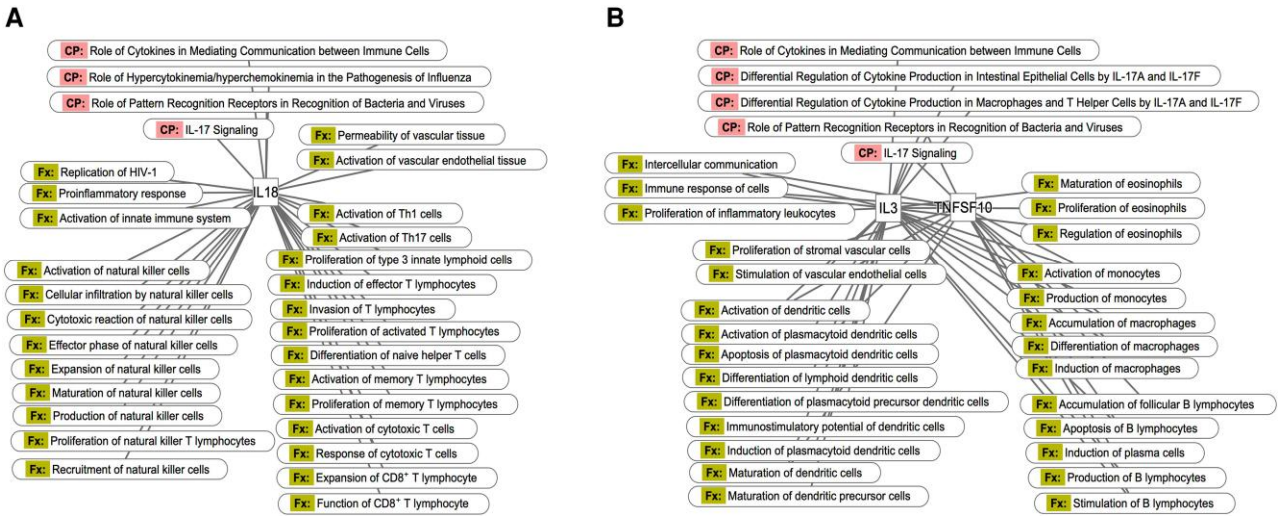

Figure 2. Pathways analysis of key distinguishing cytokines: IL-18 and IL-3/TRAIL. My Pathway analysis in ingenuity pathway analysis was used to link (*A*) IL-18, the cytokine best distinguishing elite controllers from antiretroviral therapy treated, and (*B*) IL-3/TRAIL, the cytokines best distinguishing persistent from transient controllers, to top canonical pathways and downstream biological functions. Abbreviations: CP, canonical pathways; Fx, biological functions; IL, interleukin; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

surface receptors that direct cytokines to particular cells or allow them to targeted transportation to distant sites without dilution. EV association of proteins may also confer different or stronger effects compared to soluble proteins, particularly because EVs may carry multiple components (proteins, lipids, nucleic acids) capable of influencing target cells [10, 11].

Among PWH, EC showed the highest levels of EV-associated cytokines, and PC more than TC. Traditionally, only soluble cytokines are measured in plasma, and only a few small studies have compared the inflammatory profiles of TC and PC [1, 12]. Further analyses identified higher levels of EV IL-18 as a biomarker for EC, and higher EV IL-3 and TRAIL best discriminated

PC from TC. IPA analysis of EV-associated cytokine expression provided predictions consistent with viral immune defenses and among PWH, but these associations were much more significant for EC than ART treated, and among EC, more significant for PC than TC. IL-18 was linked with biological functions of NK, T-cell subsets, and ILC and IL-3/TRAIL with DC and B-cell functions. Soluble levels of IL-18 and TRAIL have been reported to be increased in the circulation of PWH, but less is known for IL-3, a molecule that supports plasmacytoid DC (pDC) survival, enabling the early innate immune response to HIV infection [13]. Interestingly, it has been observed that pDC from controllers produced higher amounts of type I IFN in response to HIV, likewise higher capacity to induce a reduction of HIV replication and TRAIL-induced apoptosis of HIV-infected $CD4^+$ T-cells than pDC from viremic patients [14, 15].

This study has some limitations. Despite well-characterized cohorts with different virologic status, the sample size for each group is small, limiting our statistical power. Cytokine concentrations measured by Luminex may differ from those obtained by clinical-use enzyme-linked immunosorbent assay (ELISAs), reducing the clinical applicability of our identified cutoff values. In future studies, inclusion of additional cytokines and signaling molecules would enhance biological interpretation of the data. Finally, the cytokine patterns observed represent a correlation, and may not necessarily contribute causally to the persistent elite control.

In conclusion, higher plasma levels of EV-associated cytokines, specifically IL-18, IL-3, and TRAIL, may represent a new signature for durable ART-free natural control of HIV replication. Protected transport of cytokines in EVs might represent a sophisticated mechanism to deliver targeted signals to host cells selectively maintaining local environments of activation and inflammation that control HIV replication. The balance between endogenous control of HIV replication and the potential long-term harmful health consequences for the EC population should be further evaluated.

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

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