INTRODUCTION

Since the early 1900s, human life expectancy has increased by approximately 30 years. This change has led to the identification of the so-called age-associated diseases and the establishment of the concept of inflamm-aging. Aging is associated with a profound immune imbalance characterized by metabolic reprogramming, reduced immune responses, and increased risk of infections. People with HIV (PWH) are now living a near-normal lifespan, attributed to the success of antiretroviral therapy (ART). Currently, the number of older PWH (ie, age ≥50 years) account for more than 50% of PWH in the United States. This increase in the number of aging PWH highlight the need for increased research efforts to understand the intersection of chronic HIV infection and aging.

The concept of precocious immune aging in HIV infection is supported by reports of higher risk of age-associated diseases at younger ages compared with their healthy peers. This increased risk has been linked to chronic immune activation and immune exhaustion that persists despite ART. Epigenetic studies have shown an approximate 5-year increase in the immune age of ART-treated virally suppressed PWH compared with healthy subjects. Suboptimal responses to vaccination, along with higher susceptibility to infections increase the risk of serious illnesses and hospitalizations in the elderly and PWH. Recently, our group has studied the mechanisms driving immune dysfunction in HIV and aging and characterized how HIV-associated precocious immune aging is different from biological aging in comparison to dynamics of innate response to vaccination in healthy control (HC). Using seasonal influenza vaccination as a probe for evaluating immune competence, we showed that young PWH achieve a lower serum antibody response compared with healthy...
young individuals, whereas older individuals showed comparable responses between HIV and HCs.13,14

The systems biology approach is now widely applied to several medical and/or biological questions,16 including identification of predictors of vaccine-induced antibody and T-cell responses.17–20 Application of systems biology to studies on aging is specially fitting, given the complex nature of aging.21 In this study, we evaluated peripheral blood mononuclear cells (PBMC) transcriptional profiles in relation to age and HIV status in a group of young and old HIV and HCs.

**METHODS**

**Study Participants**

A subset of 48 individuals with 24 HIV 24 HC representing the 2 binary categorical variables under investigation: (1) HIV status (positive or negative) and (2) age (<40 or ≥59 yrs), were selected from the original Flu Responses Of people in relation to Age and HIV (FLORAH) study13 and categorized as young HIV infected (YH, n =12), old HIV (OH, n =12), young healthy (YC, n =12), and old HC (OC, n =12). Characteristics of the cohort are reported in Table 1. Individuals were characterized as young (<40 years) and old (≥59 years) based on their age at study entry. Self-reported data on the comorbidities such as cardiovascular disease, stroke, diabetes, kidney disorders, bone loss, cancer, hypertension, and lipid levels did not differ between our HIV and HC groups. The risk factors such as coinfections (Hepatitis C Virus and Hepatitis B Virus), alcohol use, and tobacco use also did not differ between HIV and HC groups. All HIV-infected individuals were under viral control on ART for at least 1 year before study entry. This study was approved by the Institutional Review Boards of University of Miami and Miami Veteran Affairs Medical Center.

**RNA Sequencing**

Total RNA was extracted (Qiagen RNeasy plus mini kit) from cryopreserved PBMC and sequenced (Illumina HiSeq 2500; 125 bp, paired-end, 15 million reads/sample). Raw demultiplexed fastq paired-end read files were trimmed of adapters and filtered using the program skewer to remove any with an average phred quality score of less than 30 or a length of less than 36 bp. Trimmed reads were aligned to the Homo Sapiens NCBI reference genome assembly version GRCh38 using the HISAT2 and sorted using SAMtools. Aligned reads were counted and assigned to gene meta-features using the program featureCounts as part of the Subread package. Count files were assessed for quality control, normalized, and analyzed using an in-house pipeline using the edgeR generalized linear model and likelihood ratio testing method (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2796818/) for differential gene expression. The gene set variation analysis library was used across the samples with moderated t-statistics being applied by the package linear models for microarray data for testing differential pathway enrichment. Correlation between gene expression or pathway enrichment scores and age was evaluated by the Pearson correlation coefficient. The accession number for upload to the Gene Expression Omnibus and Sequence Read Archive public databases is GSE178670 (https://www.ncbi.nlm.nih.gov/geo/).

**Pathway Analysis**

Significant genes correlated with age were selected by Spearman correlation analysis ($P \leq 0.05$). The list of significant genes including Spearman correlation coefficient rho values and $P$ values was uploaded to Ingenuity Pathway Analysis software (IPA, Qiagen Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

**TABLE 1. Demographic Characteristics of Study Participants**

| HIV Negative (HC) | HIV Positive |
|-------------------|--------------|
| **Young**         | **Old**      | **Young** | **Old** |
| No. of individuals| 12           | 12        | 12       | 12       |
| Age, yr           |              |           |          |          |
| Median            | 33.5         | 64        | 31       | 61       |
| Range             | 23–39        | 61–71     | 19–39    | 60–71    |
| Sex               |              |           |          |          |
| Male/female       | 8/4          | 8/4       | 7/5      | 9/3      |
| Race, n           | 4/6/2        | 4/6/2     | 3/9/0    | 3/9/0    |
| Ethnicity, n      | 3/8          | 4/8       | 5/7      | 3/8      |
| Hispanic/non-Hispanic | 3/8    | 4/8       |          |          |
| HIV               |              |           |          |          |
| HIV viral load (copies/mL) | <50 | <50       |          |          |
| Absolute cell counts (cells/mm³) | 1846.6 ± 562.3 | 1749.3 ± 483.1 | 1804.5 ± 379.5 | 1425.6 ± 373.7 |
| CD3, mean ± SD    | 1189.7 ± 369.1 | 1080.94 ± 362.9 | 1254.9 ± 338.1 | 939.9 ± 319.3 |
| CD4, mean ± SD    | 836.7 ± 424.4 | 742.56 ± 181.6 | 895.6 ± 335.9 | 564.3 ± 303.8 |
| CD8, mean ± SD    | 510.5 ± 316.9 | 503.46 ± 304.05 | 381.6 ± 279.6 | 349.6 ± 323.0 |
| B cell (% of lymphocytes), mean ± SD | 9.7 ± 5.6 | 10.5 ± 7.2 | 8.6 ± 4.4 | 10.7 ± 6.0 |
Core analysis was performed using these genes to map to the ingenuity knowledge base (genes only). P value was calculated by the Fisher exact test. For upstream regulators and canonical pathway analysis, P values less than 0.05 was considered significant. Activation Z score was calculated to predict activation or inhibition of pathway/regulators. Orange and blue color indicates predicted pathway/regulators activation or predicted inhibition, respectively. Z score greater than 2 or less than −2 was considered to be significantly activated or inhibited.

For the superpathway approach, individual pathways identified by IPA that showed significant differences were grouped using an in-house database based on broad immune functions attributed to them. The list of pathways and the superpathway is reported in Supplementary Table 1 (see Supplemental Digital Content 1, http://links.lww.com/QAI/B760).

Predictive Model of Age and HIV Status Using Transcriptional Signatures

Individual genes were screened to identify their association with age by Spearman correlation. Significant genes (P ≤ 0.05) were further screened in IPA, and genes associated with immune-related pathways were selected. To build predictive models on outcome (ie, Age Group [Y vs O]), LASSO (least absolute shrinkage and selection operator) or Elastic Net Regression was applied to help reduce co-correlated genes using R package glmnet. Fit generalized linear model via penalized maximum likelihood at alpha parameter (0, 0.1, …1) and repeated-corrected 10-fold cross-validation was run for 500 times, and averaged accuracy was used to select optimal regularization parameter alpha. The resulting models from LambdaMin (the value of λ that gives minimum mean cross-validated error) were further assessed by bootstrapping 500 times. The initial model included only genes present more than 490 times.

Initial model was tuned to get best classification on outcome using R package caret. Stepwise regression was further used if singularity occurred. Repeated cross-validation was performed by 3 folds and 100 repeats, which model error was taken as the mean error from 100 repeats. As the sample size is small in this analysis, leave-one-out cross-validation was also performed to test the model performance against one data point at each iteration. Models with higher accuracy were further tuned on 4 machine learning algorithms (Generalized Linear Model, Random Forest, Naive Bayes, and k-nearest neighbors). Generalized Linear Model generated best prediction. For best model, Partial Least Squares Discriminant Analysis was performed using R mixOmics package to show its classification and discrimination ability.

RESULTS

Metabolism and Innate Immunity Pathways Associated With Age are Conserved in Both Healthy and HIV

To understand the effect of HIV infection and age on the transcriptome of immune cells, we performed RNA Seq using PBMC and analyzed differentially expressed genes (DEG) related to age in HC and HIV groups separately.18 We identified a higher number of genes correlating with age in HC compared with HIV (9662 vs 4,662, respectively). A total of 2924 genes were common between HIV and HC, accounting for 62.7% of all genes correlating with age in HIV. We then performed pathway analysis using age-associated DEGs to explore molecular pathways associated with age in HC and HIV. The correlation coefficients were used to calculate an activation score (see Methods). This analysis resulted in 26 pathways with significant association with age in HC and 12 pathways in HIV (Fig. 1).

Five pathways were significantly enriched and common for both groups, and they were all predicted to be activated with age: Glycogenesis I, glucoseogenesis, FcyR-mediated phagocytosis, production of nitric oxide and reactive oxygen species in macrophages, and natural killer (NK) signaling and Triggering receptors expressed on myeloid cells-1 signaling (Figs. 1, and 2A). Among the 7 age-associated pathways, unique for HIV were oxidative phosphorylation (metabolic process associated with the production of Adenosine triphosphate), death receptor signaling, and interferon (IFN) signaling, which were activated with age (Fig. 2A). In HC, we also identified pathways associated with T-cell function including Inducible co-stimulator-Inducible co-stimulator ligand signaling in T helper cells and protein kinase C signaling in T cells (Fig. 2A).

Interestingly, HIV exhibited similar age-associated molecular pathways seen in HC, but they had a weaker activation score than HC. For example, interleukin (IL)-15 signaling was identified in HIV but with a much lower activation score compared with HC. In some cases, the activation score was opposite between HC and HIV. This was the case for Inducible co-stimulator-Inducible co-stimulator ligand signaling in T helper cells and Eukaryotic Initiation Factor 2 signaling (Fig. 2A). Overall, these results identified 3 major points: (1) HIV showed weaker activation of age-related molecular differences compared with HC as a reference group; (2) age-related changes in metabolic pathways and innate immune activation are conserved in both HIV and HC; and (3) in HC, age is also associated with an increase in T-cell immune activation.

IFN Signaling Plays a Major Role in HIV Aging

Using IPA, we performed upstream regulator analysis to identify the potential master transcriptional regulators that can explain the observed age-associated gene expression patterns in our data sets for HC and HIV (Fig. 2B). We found 6 upstream regulators in HC, of which 3 upstream regulators are known to interact with each other: TP53 (gene that encodes the tumor suppressor protein p53), SREBF1 plays a role in regulation of the lipid metabolism, and NUPR1, a transcription regulator. Interestingly, in HIV, we found an upstream regulator pathway formed by IRF9, IRF1, and STAT1 that associated with IFN signaling. This result implies a potential larger role for IFN signaling with a feedback mechanism for the age-associated signature in HIV. The unique role of IFN in HIV was also confirmed by the comparison of upstream regulators of HC and HIV. In fact, this analysis showed that IRF1, 2, and 9 were found as upstream regulators only in HIV (Fig. 2C).
Precocious Immune Aging in HIV Can Be Detected at the Molecular Level

HIV-infected individuals have been shown to exhibit precocious immune aging. This phenomenon could explain the weaker age-associated molecular differences observed in the previous results. To understand whether HIV individuals exhibit accelerated immune aging at the molecular level, we performed a different analysis in which, instead of correlating the gene expression with age, we contrasted gene expression between young and old in both HC and HIV. This analysis identified 1377 genes that were differentially expressed between young and old HC participants (1367 upregulated in old and 10 in young, Fig. 3). In HIV, however, only 139 genes were differentially expressed (132 upregulated in old and 7 in young), of which 96 overlapped with DEGs in HC, confirming the presence of a common molecular signature of aging. To further characterize whether HIV was interfering with the aging-associated transcriptional profiles, we evaluated the statistical interaction of HIV infection and age on gene expression patterns. We identified 148 age-associated DEGs altered by the presence of HIV infection (data not shown). Taken together, these results demonstrated that in HIV individuals, precocious immune aging can also be observed in the PBMC transcriptome.

Transcriptional Signatures Predictive of Age Are Different for HC and HIV

Finally, we built a predictive model of age based on RNA Seq data for HC and HIV. To better identify genes with biological relevance, we selected a subset of age correlating genes based on their association with immune-related pathways from IPA database (Qiagen). With this approach, we reduced the number of genes from 9662 to 1110 for HC and from 4662 to 519 in HIV. The selected genes were then additionally filtered by LASSO or Elastic Net to reduce
co-correlating genes. Using a generalized linear model, we identified 3 genes (MST1R, JMJD6, and ANXA4) out of the starting 1110 genes identified in HC that were able to predict the age group (young or old) with an accuracy of 97% in HC. The same model showed an accuracy of only 70% when applied to HIV. The model was validated by repeated cross-validation as described in Methods. We then applied the same statistical approach to HIV. From the starting 519 genes that showed correlation with age, 6 were selected for the linear general model. These 6 genes were DOCK1, DBI, KLRG1, IGBP1, BPI, and PCDHB15. Using this model, we were able to predict the age groups for our cohort with an accuracy of 98% in HIV but only 61.9% in HC. Of note, no genes overlapped between HIV and HC in the final models despite there being 359 genes in common between HIV and HC from the select group.

In HC, old were characterized by higher expression of JMJD6 and MST1R (Fig. 4A). Both genes are involved in...
The number of DEG is reported inside the circle.

**DISCUSSION**

In this study, we applied a systems biology approach to investigate the differences in molecular/transcriptional profile induced by aging with and without concomitant HIV infection. We found evidence of precocious immune aging in HIV, where fewer DEGs between young and old were observed, but we also described a core of age-induced changes that were conserved in HC and HIV composed of metabolic and innate immune activation pathways. Overall, our data showed that at the transcriptional level, HIV differs from HC. However, the fact that we could observe pathways altered by age that were conserved in both HC and HIV suggests the possibility to identify therapeutic targets within these pathways able to target immune aging in both HC and HIV.

Precocious immune aging is the phenomenon by which HIV-infected individuals reach an immune status more similar to that of older HIV-negative individuals. Flow cytometry analysis from the FLORAH cohort showed that young HIV has a higher number of differences compared with their healthy peers than old HIV. Indeed, in the FLORAH cohort, we observed higher frequency of immune activated and potentially exhausted immune cells from the adaptive immune system such as double-negative B cells and effector CD4+ T cell. Interestingly, for innate immunity, this was not the case. In fact, for monocytes, OH and OC had higher frequencies of inflammatory monocytes compared with YH and YC, respectively.

In line with the hypothesis that young HIV exhibits precocious aging, we found that more gene transcripts associated with age in HC than HIV (9662 vs 4662). Similarly, we found that only 139 genes were differentially expressed between YH and OH compared with 1377 DEGs identified from the comparison between YC versus OC. By performing interaction analysis, we could also show that HIV has a direct effect on 148 age-related genes. Despite the reduced differences in gene transcription between age groups in HIV, pathway analysis showed a shared signature of aging in both HIV and HC associated with innate immunity and metabolism. For innate immunity, we found mainly pathway associated with NK and monocytes. These results confirmed the relation between innate immunity and age showed by Nakaya and colleagues, but it was interesting to find that the same pathways were also associated with age in HIV. It is also notable that despite 5 pathways being shared between HIV and HC, several others identified in HC showed a weaker activation Z score with age in HIV. These results are very likely the consequence of the lesser diversity in transcriptome between young and old PWH compared with that between young and old HC.

HIV and HC also showed activation of metabolism with age, particularly glycolysis and gluconeogenesis. Association of the glucose metabolism in aging has been shown in the HIV-uninfected population and this event is hypothesized to be due to the functional decline of the mitochondria in advanced age. Gluconeogenesis has been shown in different models to be increased due to the increased need for glucose induced by aging. On the other hand, senescent cells have been shown to switch to a glycolytic state, and several studies have shown a functional link between a more highly glycolytic state and senescent phenotypes. Glycolysis is not only associated with senescence but also with immune activation. Studies have shown that activated immune cells (e.g., dendritic cells, macrophages, T cells, and B cells) switch their metabolic state toward glycolysis to meet the energy needs of the activated status requirements. For this reason, the increase in glycolysis could also be the result of a more activated immune system. In fact, glycolysis is also found to be increased in HIV-infected individuals. Despite precocious immune aging, glycolysis is still associated with age in HIV reinforcing the importance of this pathway in the aging mechanism. These similarities between HC and HIV demonstrate that a common core of transcriptional differences persists in HIV-infected individuals despite the accelerated immune aging.

Only in HIV, we observed activation of the oxidative phosphorylation pathway. The activation of this pathway with age is an indication of a stronger metabolic disequilibrium associated with aging in HIV. In fact, this pathway is responsible for the production of radical oxygen species. Higher concentration of radical oxygen species was shown to
associate with mitochondrial dysfunction that in turn worsens the glucose metabolism. A significantly lower mitochondrial DNA and mitochondrial oxidative phosphorylation protein levels in PBMC were found in chronic HIV-infected individuals compared with the seronegative group. Mitochondrial dysregulation is also commonly observed in muscle tissues from HIV-infected individuals. Several factors such as direct mitochondrial toxicity of HIV, antiretrovirals,
comorbidities, and persistent inflammation may contribute to these metabolic abnormalities.

In our study, we also observed activation of death receptor signaling pathway in HIV. Death receptors belong to the tumor necrosis factor receptor superfamily. Signaling via death receptors contributes to the regulation of the adaptive immune response in different ways, most notably by triggering activation-induced cell death of T cells. Dysregulation of this pathway can lead to immune disorders and also impacts on tumorigenesis or immune response (eg, cytotoxicity).40,41

Another pathway that we found to be activated with age in HIV but not in HC was the IFN signaling pathway. IFNs are cytokines that have potent antiviral effects. They work as first line of defense against viral infections and have important roles in immunosurveillance.42 Induction and persistence of the IFN signature related to infection status, transmission outcomes, and viral burden was demonstrated in HIV infection and also during viral suppression with ART.43–45 but to our knowledge, no observation regarding the possible association of this pathway with the aging process in HIV has been reported. IFN signaling is considered one of the factors driving persistent immune activation described in HIV-infected individuals and is associated with development of immune dysfunction and non-AIDS morbidity.44,46 Moreover, we found IRF1, IRF9, and STAT1 among the upstream regulators in HIV; these genes are also part of the IFN signaling pathway suggesting the existence of a positive feedback mechanism of this pathway in HIV and aging. Overall, our observations suggest that aging worsens the type I IFN-related dysfunctions in HIV. The importance of the alterations in the IFN signaling pathways in HIV has significant relevance in the current understanding of the COVID-19 immunopathology as IFN potentiates the recruitment and activation of various immune cells. Thus, although a robust IFN response is required as a first line of defense against viral infections, systemic/uncontrolled or prolonged IFN production can lead to inflammatory diseases. It is therefore critical to understand the regulation of the IFN response in HIV and COVID-19 as age and HIV-associated alterations in IFN signaling pathways may further worsen the situation.

We reasoned that the activation of the IFN signaling pathway with age in HIV could also be behind the differences between HIV and HC observed in our age predicting model. In fact, old HIV, contrary to old HC, showed no genes associated with IL-12 signaling and monocyte activation; instead, they show upregulation of genes associated with B-cell activation and immune suppression of the innate immunity. In old HIV, type 1 IFN may compensate for the lack of IL-12 signaling effect by inducing immune exhaustion, production of immune suppressive cytokines (eg, IL-10) and impair B-cell immune response towards a short-lived antibody-secreting cell.47–49

Despite our results in HC are consistent with literature data, the limited number of individuals and underrepresentation of the middle age group still represent the 2 major limitations of our study. Also, the bulk RNA Seq approach does not provide resolution on specific immune cell types, which may differ between HC and HIV. In conclusion, the data presented here are one of the first attempts to apply systems biology to elucidate the combined effects of advanced age and chronic HIV infection on immune responses. We demonstrated that the presence of HIV infection reduces the transcriptional differences between YH and OH, confirming the idea of precocious immune aging and suggesting that this perturbation affects the immune transcriptome. However, a conserved set of genes comprised of metabolic and innate immunity transcriptional pathways associate with aging regardless of the HIV immune status.

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