Multiparametric Bioinformatics Distinguish the CD4/CD8 Ratio as a Suitable Laboratory Predictor of Combined T Cell Pathogenesis in HIV Infection

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Multiparametric Bioinformatics Distinguish the CD4/CD8 Ratio as a Suitable Laboratory Predictor of Combined T Cell Pathogenesis in HIV Infection

Marcus Buggert,* Juliet Frederiksen,† Kajsa Noyan,* Jenny Svärd,‡ Bahilonia Barqasho,* Anders Sönnerborg,*‡ Ole Lund,‡ Piotr Nowak,**‡ and Annika C. Karlsson*

HIV disease progression is characterized by numerous pathological changes of the cellular immune system. Still, the CD4 cell count and viral load represent the laboratory parameters that are most commonly used in the clinic to determine the disease progression. In this study, we conducted an interdisciplinary investigation to determine which laboratory parameters (viral load, CD4 count, CD8 count, CD4 %, CD8 %, CD4/CD8) are most strongly associated with pathological changes of the immune system. Multiparametric flow cytometry was used to assess markers of CD4+ and CD8+ T cell activation (CD38, HLA-DR), exhaustion (PD-1, Tim-3), senescence (CD28, CD57), and memory differentiation (CD45RO, CD27) in a cohort of 47 untreated HIV-infected individuals. Using bioinformatical methods, we identified 139 unique populations, representing the “combined T cell pathogenesis,” which significantly differed between the HIV-infected individuals and healthy control subjects. CD38, HLA-DR, and PD-1 were particularly expressed within these unique T cell populations. The CD4/CD8 ratio was correlated with more pathological T cell populations (n = 10) and had a significantly higher average correlation coefficient than any other laboratory parameters. We also reduced the dimensionalities of the 139-unique populations by Z-transformations and principal component analysis, which still identified the CD4/CD8 ratio as the preeminent surrogate of combined T cell pathogenesis. Importantly, the CD4/CD8 ratio at baseline was shown to be significantly associated with CD4 recovery 2 y after therapy initiation. These results indicate that the CD4/CD8 ratio would be a suitable laboratory predictor in future clinical and therapeutic settings to monitor pathological T cell events in HIV infection. The Journal of Immunology, 2014, 192: 2099–2108.

remedious efforts have been made to understand how the immune system deteriorates subsequent to chronic HIV type 1 infection (1). HIV tropism to CD4+ cells truly represents a fundamental cause of the initial destruction of effector memory Th cell immunity. However, AIDS development is a more complex situation wherein T cell homeostasis fails to replenish the initial loss of memory CD4+ T cells. The failure of CD4+ T cell regeneration is thought to be a consequence of multiple inflammatory factors that together contribute to major pathological T cell repertoire changes.

Pathological changes of the cellular immune system in HIV infection include T cell immune activation, exhaustion, senescence, memory abnormalities, and many other dysfunctional events. Although HIV affects most immune system compartments (reviewed in Refs. 2, 3), these changes largely affect CD4+ and CD8+ T cells. As early as 1989, Giorgi et al. (4) showed that HLA-DR and CD38 expression correlated with CD4+ T cell losses and AIDS development. Currently, these markers are widely used to assess T cell immune activation status. Numerous studies have shown that immune activation is an underlying mediator of immunodeficiency during HIV infections (5, 6) and is a better predictor of disease progression than HIV viremia itself (7–9). Antiretroviral therapy (ART) administration reduces immune activation (10–12), but despite several years of successful ART, the treated subjects rarely achieve similar levels of activation, compared with age- and sex-matched healthy individuals. In conjunction with immune activation, elevated inhibitory (exhaustion) receptor expression has been linked to HIV disease progression (13). The most studied marker of T cell exhaustion is PD-1, where several studies have found increased PD-1 expression on T cells after HIV infection (14, 15).

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Abbreviations used in this article: ART, antiretroviral therapy; MFI, median fluorescence intensity; PC, principle component; PCA, principal component analysis; VL, viral load.
levels of CD57 (20). Previous studies have shown that both untreated and treated HIV-infected individuals have highly senescent T cell repertoires (CD28" CD57") that resemble those found in much older healthy control populations (21). In agreement with an increased immunosenescent state, T cell differentiation is severely skewed, such that fewer naive (CD45RO) and memory CD4+ and CD8+ T cell activation, exhaustion, and senescence markers have not been conducted and correlated with the currently measured routine laboratory parameters. We conducted a cross-sectional study on 47 untreated HIV-infected subjects to identify which routine laboratory parameter was a suitable predictor of combined T cell pathogenesis in HIV infection.

Materials and Methods

Ethical statement

The Regional Ethical Review Board (Stockholm, Sweden; Dnr 2009-1485-31-3) approved the study where all participants were provided with written and oral information about the study. Written informed consent of all study subjects in accordance with the Declaration of Helsinki was documented.

Study subjects

An observational cohort of 47 HIV-infected individuals was recruited from the Outpatient HIV Clinic at Karolinska University Hospital Huddinge (Stockholm, Sweden). The patient clinical characteristics are detailed in Table I. All patient samples were collected from treatment naive subjects, except for three individuals with AIDS-defining illnesses who shortly before study inclusion initiated ART (mean 10 wk, range 2–16 wk). All VL measurements from these three subjects were excluded from the statistical analysis. An age- (median 39 y) and sex-matched (62% male) healthy control group of 21 individuals was recruited to compare all of the immunological markers.

Ab reagents

The same flow-cytometry panel, including 12 different parameters, was tested on all HIV-infected and healthy control subjects to avoid intraindividual and interindividual differences of the flow analysis. The following Abs were used: anti-CD3 allophycocyanin-H7 (clone SK7), anti-CD14 V500 (Clone M5E2), anti-CD19 V500 (Clone B43), BD Biosciences; anti-CD45RO ECD (clone UCHL1; Beckman Coulter); anti-CD4 BV650 (Clone OKT4), anti-CD8 BV650 (Clone OKT8), anti-CD4 BV421 (clone eH12.2H7), anti-CD27 BV785 (clone O323), anti-CD28 PerCP-Cy5.5 (clone CD28.2), anti-CD38 allophycocyanin (clone HFT2), anti-CD57 FITC (clone HCD57; Biologen); anti–HLA-DR PE-Cy7 (clone LN3; eBioscience); anti–HLA-DQ Qd565 (Clone 3B5; Life Technologies); and anti–Tim-3 PE (clone 344823; R&D Systems). LIVE/DEAD Aqua amine dye (Life Technologies) was used to discriminate dead cells and debris.

Cells and flow cytometric staining

The methods for preparation and staining of cells have previously been described in detail (25). In brief, PBMCs were isolated from whole blood by Ficoll-Paque (GE Healthcare) density gradient centrifugation and cryopreserved in FBS (Life Technologies) containing 10% DMSO. At the time of flow cytometry stainings, PBMCs were thawed, washed, and rested overnight at 37°C in RPMI 1640 medium (Amedia [Sigma Aldrich]) containing 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin, and 10 mM HEPES (Life Technologies). PBMCs were counted the following day using a Nucleocounter (ChemoMetec A/S) and plated to a concentration of 1.5 × 10^6 cells/well in V-bottom plates. Cells were washed and stained with all Ab reagents for 30 min at 20°C. PBMCs were washed and resuspended in PBS containing 1% paraformaldehyde. All flow cytometric analyses were conducted within 2 h after fixation.

Flow cytometric analyses

PBMCs were analyzed on a standardized 18-color LSR Fortessa (BD Biosciences) where minimally 600,000 total events were collected per run. Ab capture beads (BD Biosciences) were used for data compensation after separate stainings with all Abs used in the panel. FlowJo 8.8.7 (Tree Star) were used for flow cytometric gating analyses. All manual gatings were based on fluorescence minus one gating strategies as previously described (26).

Statistics

Experimental variables between two groups of individuals were analyzed using Mann–Whitney U test and Wilcoxon matched-pairs rank test. Correlations were assessed using nonparametric Spearman rank tests. Bonferroni corrections were applied to all cases where multiple testing was performed. The Kolmogorov–Smirnov test was used to test the null hypothesis that the two groups of samples were drawn from the same distribution, where a large p value suggests that the groups were drawn from the same distribution. Heat maps, a method commonly used to visualize a large matrix of numbers, were used during the analysis to get an illustrative overview of the data. Unsupervised hierarchical clustering was used in conjunction with heat map to find patterns in data in an unbiased fashion. The distance measure used for the hierarchical clustering was the dissimilarity index \( d_{ij} = 1 - corr(p_i, p_j) \), where \( p_i \) and \( p_j \) are the population frequency vectors for subjects \( i \) and \( j \), respectively, in the study.

The marker frequencies were analyzed using a linear combination of the column-wise Z-score transformed values:

\[
Z_{ij} = \frac{x_{ij} - \bar{x}_i}{SD_j},
\]

where \( i \) represents the row index, \( j \) represents the column index, \( n \) represents the total number of columns, \( x_{ij} \) is the average for column \( j \), \( SD_j \) is the SD of column \( j \), and \( x_i \) is the population frequency for row (individual) \( i \) and column (population) \( j \). The large data consisting of population frequencies from the Boolean combinations were analyzed using Z-score transformation and principal component analysis (PCA). PCA is an unsupervised statistical method for reducing data dimensionality whereas retaining the vital variation in fewer informative variables. The top two principle components (PCs) were plotted to visualize the trends such as clusters and outliers revealed by PCA.

In summary, statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software) and R environment (27). Permutation tests were analyzed using the data analysis program SPICE version 5.2009, provided by Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health (28).

Results

Profound signs of immunopathological changes in HIV-infected individuals

We first investigated whether a selected set of immune activation (HLA-DR, CD38), exhaustion (PD-1, Tim-3), senescence (CD28, CD57), and memory differentiation (CD45RO, CD27) markers were indeed differential in HIV-infected individuals (Table I), compared with healthy control subjects (1). The HIV-infected individuals showed increased signs of CD4+ and CD8+ T cell activation, exhaustion, senescence, and abnormal expression of memory markers (Supplemental Table I). In brief, the frequencies and median fluorescence intensities (MFIs) of all individual markers, as well as widely studied double-marker combinations (CD38+HLA-DR+ and CD57+CD28-), were compared between the groups with Mann–Whitney U tests, followed by corrections for multiple comparisons (Bonferroni adjustments). Significant differences between the groups were prominent, primarily for CD8+ T cells, which showed differences in the frequencies of all markers except CD45RO. In the analysis, the greatest significant difference was observed for CD38+HLA-DR+ and CD8+ T cells (\( p = 8.05 \times 10^{-5} \)). Nevertheless, CD4+ T cells had significantly elevated frequencies of CD38, CD57,
Distinct T cell populations delineate HIV+ subjects and healthy changes in HIV-infected individuals. Some markers represent highly abnormal immunopathological differences between the groups. Overall, these results confirm that these in-
jects were used to compare the immunological markers between the HIV-infected and healthy control subjects. Using the PCA plots, we observed clear differences between the groups, with the healthy control subjects clustering into a tight group (Fig. 1B). A Kolmogorov–Smirnov analysis was performed to test whether the HIV-infected and healthy control subjects were derived from the same distributions. In particular, the PC1 scores for the two groups were shown to be drawn from different popula-
tions (p = 2.22 × 10^-14), where the AIDS patients generally had a higher PC1 score. In addition, the PC2 score had a signifi-
cantly diverse distribution (p = 5.05 × 10^-8).

All 256 Boolean combinations for CD4+ and CD8+ T cells were con-
verted into the populations that differed between the groups, be-
nificantly diverse distribution (p = 5.05 × 10^-8).

Table I. Patient characteristics

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<td>9</td>
<td>880</td>
<td>65</td>
<td>114</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Yes, initiation of ART: median 5 wk (interquartile range 1–11) after sample collection.

**HIV RNA are in copies/ml.

*ART initiation at 2–16 wk before sample collection.

HLA-DR, PD-1, and Tim-3 expression, of which the PD-1 MFI consti-
tuted the greatest significant difference (p = 5.51 × 10^-8) be-
tween the groups. Overall, these results confirm that these in-
dividual markers represent highly abnormal immunopathological changes in HIV-infected individuals.

**Distinct T cell populations delineate HIV+ subjects and healthy control subjects**

Multiparametric analyses of flow-cytometry data allowed us to com-
bine all of the immunological markers into 256 distinct (Boolean) CD4+ and CD8+ T cell populations for comparisons between the HIV-infected and healthy control groups (Fig. 1A).

A PCA of the multiparametric flow-cytometry results enabled distinctions between HIV-infected individuals and healthy sub-
jects. Using the PCA plots, we observed clear differences between the groups, with the healthy control subjects clustering into a tight group (Fig. 1B). A Kolmogorov–Smirnov analysis was performed to test whether the HIV-infected and healthy control subjects were

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specifically, an early-differentiated (CD45RO+CD27+CD28+CD572) cell population that expressed CD38, HLA-DR, and PD-1 was the most significantly different between the groups ($p = 2.02 \times 10^{-10}$).

In conjunction with this finding, all of the eight highly discriminative CD8$^+$ T cell populations expressed CD38, HLA-DR, and PD-1, but not Tim-3, on different memory T cell populations.

Unsupervised hierarchical clustering (using a Spearman rank to measure similarity) was performed on all 139 distinct T cell populations that differed significantly between the HIV-infected and healthy control subjects. A dendrogram is shown in the heat map wherein the 139 significant populations are included (Fig. 1C). The frequency distribution of the Boolean populations is shown in Supplemental Fig. 1. A majority of the population frequencies are found to be between 0.001 and 1%. The majority of rare populations that occur in the HIV-infected individuals are shown to be absent in the control individuals. Similarly to the PCA analysis, the hierarchical clustering showed the expected results; specifically, the healthy control subjects clustered together, whereas the
HIV-infected individuals divided into several distinct clusters. Even if the HIV-infected individuals were distributed into several clusters, these clusters were linked to routine laboratory parameters that have previously been linked to HIV disease progression, including the CD4/CD8 ratio (data not shown).

Collectively, these results provide evidence that the specific combination of markers could be used to delineate HIV-infected subjects and healthy control subjects into different clusters. Furthermore, these 139 significant populations will be referred to as "pathological T cell populations."

**The CD4/CD8 ratio shows a superior correlation to individual markers of T cell pathogenesis**

Historically, most of the immunopathological changes quantified in HIV-infected individuals have been correlated with either the VL or CD4 count. We wanted to test the validity of those associations by comparing whether the individual cell-surface markers of immune activation, exhaustion, senescence, and memory differentiation were more strongly associated with the VL or CD4 count, compared with other routine laboratory parameters (CD4 %, CD8 count, CD8 %, and CD4/CD8 ratio). Following the Bonferroni corrections, the VL showed three significant correlations with activation markers (CD38, HLA-DR), whereas the CD4 count showed an additional correlation with the exhaustion marker PD-1. The same was true for CD8 %, which also showed three correlations with similar markers. The CD8 count was the poorest predictor of T cell pathogenesis, with only one significant correlation. However, both the CD4/CD8 ratio and the CD4 % each showed six independent correlations. Highly activated (CD38+/HLA-DR+) and senescent (CD28−CD57+) CD4+ and CD8+ T cells were also added into the multiple correlation analyses, but the highest number of correlations continued to be observed for the CD4/CD8 ratio (n = 8) and CD4 % (n = 8; Table II). All significant correlations are provided in Supplemental Table II.

To determine whether the CD4/CD8 ratio was a better surrogate of T cell pathological changes than the other laboratory parameters, the average of the Spearman correlation coefficients (r) was calculated for immunological markers that were significantly associated with at least one laboratory parameter (Table II). The CD4/CD8 ratio was shown to have the highest average correlation coefficient (r average = 0.596, SD ± 0.108). After pairwise comparisons (paired t tests) of the laboratory markers, the CD4/CD8 ratio was found to have a significantly higher average correlation coefficient than the CD4 count (p = 0.0059). The correlation coefficient of the CD4 count was not significantly higher than that of any other laboratory parameter (VL, CD4 %, CD8 count, CD8 %, CD4/CD8 ratio). A visualization of the superior CD4/CD8 ratio correlation with T cell pathological changes is shown in Fig. 2, which depicts the CD4 count, CD4 %, VL, and CD4/CD8 ratio versus the MFI of PD-1 on CD4+ T cells and the frequency of the HLA-DR+CD38+ combination on CD4+ and CD8+ T cells. The linkage between the CD4/CD8 ratio and CD4 % to markers of T cell pathogenesis was not surprising because these are not independent factors, and a very close relationship between these laboratory parameters was discovered (r = 0.97, p = 1.3 × 10−20). Other routine laboratory markers were also significantly associated with each other, but not to the same extent as the CD4/CD8 ratio and CD4 % (data not shown).

**Frequency of pathological T cell populations primarily correlates with the CD4/CD8 ratio**

Because the CD4/CD8 ratio and CD4 % displayed the strongest correlations with multiple individual T cell pathogenesis markers, we were interested to determine whether this finding was driven by specific combinations of markers. In correlation analyses of the 139 significant Boolean combinations, the CD4/CD8 ratio and CD4 % were again more often correlated with numerous T cell populations both before (n = 67 and n = 64) and after Bonferroni adjustments (n = 10 and n = 8), compared with the VL, CD4 count, CD8 count, or CD8 % (Table III). The most profound significant correlation was found between the CD4/CD8 ratio and an early-differentiated CD4+ T cell population that expressed activation and exhaustion markers (CD4+CD27−CD45RO+CD28−CD38−CD57−HLA-DR−PD-1−Tim-3−). In addition, the most significantly different population between the HIV-infected and healthy subjects (CD8+ T cells that expressed CD27, CD45RO, CD38, HLA-DR, and PD-1) only significantly correlated with the CD4 % (r = −0.56, p = 0.039). The VL, CD8 count, and CD8 % barely correlated with any of the pathological T cell populations after the Bonferroni adjustments (Table III). All significant correlations are provided in Supplemental Table III.

The average Spearman correlation coefficient for each routine laboratory parameter was again calculated with unique combinations of the significant markers for all laboratory parameters. These Spearman correlation coefficients were then used in a pairwise comparison with paired t tests (Table III). Again, the CD4/CD8 ratio showed a significantly stronger average correlation coefficient to T cell immunopathogenesis, compared with the CD4 count (p = 0.0006) and all other laboratory parameters (including CD4 %; p = 0.027). Also, after the Bonferroni correction, the CD4/CD8 ratio average correlation coefficient was significantly higher than those of all other laboratory parameters, except CD4 %. These analyses support the CD4/CD8 ratio as the strongest predictor of T cell immunopathogenesis among the routine laboratory parameters investigated in this study.

**Z-transformations and PCA combinations of immunopathological markers predominantly correlate with the CD4/CD8 ratio**

The measurement of numerous T cell surface markers might be a way to study the different immunopathological events that occur during HIV infection. Indeed, the CD4/CD8 ratio was highly correlated with multiple markers, but in an attempt to create a "T cell pathology score," we Z-transformed each immunological

<p>| Table II. Correlations of all routine laboratory parameters to individual and double combinations of all immunological markers |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>No. of Significant Correlations</strong></th>
<th><strong>No. of Significant Correlations (Bonferroni Corrected)</strong></th>
<th><strong>Average r Coefficient</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 count</td>
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<tr>
<td>CD4 %</td>
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<td>8</td>
</tr>
<tr>
<td>CD8 count</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>CD8 %</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>VL</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>16</td>
<td>8</td>
</tr>
</tbody>
</table>

*Nonparametric Spearman correlation coefficient.
marker to standardize the data such that the markers were comparable. Thereafter, we summed the $Z$-scores for all markers for each patient, thereby reducing the dimensionality. These scores were then correlated with each of the laboratory parameters (Table IV).

Using this approach, we performed linear combinations, and these repeatedly showed that the CD4/CD8 ratio ($r = 0.68, p = 2.7 \times 10^{-6}$) and CD4 % ($r = -0.69, p = 4.08 \times 10^{-5}$) were the two laboratory parameters that best correlated with the pathology scores for all T cell surface markers on an individual marker level. All other studied laboratory parameters were also significantly associated with the scores, using this approach. The same principle was used for the combinations of immunological markers. Similar to the previous analysis, only the significantly different T cell populations between the HIV-infected and healthy control subjects were included in the pathology score calculations. The CD4/CD8 ratio ($r = -0.74, p = 1.55 \times 10^{-7}$) and CD4 % ($r = -0.72, p = 9.91 \times 10^{-8}$) displayed the strongest correlations with the score, whereas the CD8 count did not show any significant correlation with the pathology score (Table IV).

Finally, a PCA analysis based on the 139 T cell populations defined as immunopathological was conducted for all HIV-infected individuals. In the PCA plot, a clear trend was evident, whereby individuals with lower CD4/CD8 ratios seemed to be skewed toward higher PC1 scores (Fig. 3A). To test this hypothesis, we correlated all laboratory parameters with PC1. The CD4/CD8 ratio ($r = -0.71, p = 5.79 \times 10^{-7}$) and CD4 % ($r = -0.70, p = 2.24 \times 10^{-5}$) correlated better with the PC1 score than did the CD4 count ($r = -0.58, p = 9.95 \times 10^{-5}$; Fig. 3B).

**CD4/CD8 ratio and T cell pathology scores predict the absolute CD4+ T cell reconstitution during ART**

We next sought to determine whether the CD4/CD8 ratio and the combined scores of T cell pathology could predict the level of CD4+ T cell reconstitution during 2 y on ART. A total of 30 subjects initiated ART after sample collection, of which two individuals were excluded from these analyses because of infrequent clinical information after ART introduction. The samples analyzed from the remaining 28 subjects were collected at a median of 5 wk (interquartile range 1–11) before ART initiation. Three subjects changed ART regimen during the 2-y interval, but neither of these or any of the other study subjects experienced virological failure during ART.

Surprisingly, we found that the CD4 count (Fig. 4A) at baseline did not correlate with the absolute CD4+ T cell recovery 2 y after ART initiation ($r = 0.27, p = 0.17$). Instead, the CD4/CD8 ratio (Fig. 4B) displayed a significant correlation with the absolute CD4+ T cell recovery during ART ($r = 0.44, p = 0.02$). In ac-

---

**Table III. Correlations of all routine laboratory parameters to all pathological T cell populations**

<table>
<thead>
<tr>
<th></th>
<th>No. of Significant Correlations</th>
<th>No. of Significant Correlations (Bonferroni Corrected)</th>
<th>Average $r$ Coefficient* $(\pm SD)$</th>
</tr>
</thead>
<tbody>
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<td>CD4 count</td>
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<td>4</td>
<td>0.50 $(\pm 0.14)$</td>
</tr>
<tr>
<td>CD4 %</td>
<td>64</td>
<td>8</td>
<td>0.57 $(\pm 0.10)$</td>
</tr>
<tr>
<td>CD8 count</td>
<td>38</td>
<td>1</td>
<td>0.41 $(\pm 0.10)$</td>
</tr>
<tr>
<td>CD8 %</td>
<td>45</td>
<td>2</td>
<td>0.48 $(\pm 0.10)$</td>
</tr>
<tr>
<td>VL</td>
<td>43</td>
<td>3</td>
<td>0.41 $(\pm 0.17)$</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>67</td>
<td>10</td>
<td>0.59 $(\pm 0.10)$</td>
</tr>
</tbody>
</table>

*Nonparametric Spearman correlation coefficient.
cordance with the previous observations, the T cell pathology scores, Z-scores (Fig. 4C), and PC1 scores (Fig. 4D) also showed significant associations at baseline with the absolute CD4 recovery after therapy initiation. The significance of T cell pathogenesis seems to have been driven by HLA-DR (Fig. 4E), PD-1 (Fig. 4F), and Tim-3 (Fig. 4G) expression on CD4+ T cells, as all of these

Table IV. Correlations of routine laboratory parameters to Z-scores of individual markers and all pathological T cell populations

<table>
<thead>
<tr>
<th>Individual Markers</th>
<th>Immunopathological Populations</th>
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<tr>
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<td>( R ) Coefficient</td>
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<td>CD4 count</td>
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<td>CD8 count</td>
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<tr>
<td>CD4/CD8 ratio</td>
<td>-0.74</td>
</tr>
</tbody>
</table>

*Nonparametric Spearman correlation coefficient.

FIGURE 3. Correlations between the combined principal component scores and routine laboratory markers. (A) All pathological T cell populations (\( n = 139 \)) were combined using PCA, generating a PC1 and PC2 score for each HIV-infected subject. Subjects with a CD4/CD8 ratio <0.3 (low), CD4/CD8 ratio = 0.3–0.5 (int), and CD4/CD8 ratio >0.5 (high) are dedicated with diverse colors in the PCA plot. (B) The combined PC1 scores from all immunopathological populations were correlated with all routine laboratory markers using Spearman nonparametric tests (followed by Bonferroni corrections).
factors were inversely correlated with the absolute CD4 recovery after ART. We also conducted slope analysis of the CD4 recovery after ART, but neither of the variables mentioned earlier were correlated at baseline with this outcome (data not shown).

Together, these different analyses conclude that, compared with the CD4 count and VL, the CD4/CD8 ratio is a better predictor of CD4 recovery at ART initiation and the immunopathological T cell changes that occur during untreated chronic HIV infection.

Discussion
A normal, healthy state is characterized by low-grade immune activation, which gradually fuels the aging process of the immune system over time. HIV accelerates this process by inducing a persistent and sustained inflammatory state that eventually deteriorates the immune system. This immunopathogenesis is multifaceted, but most studies have focused on specific pathological markers (e.g., CD45RO) and rarely on combinations of numerous markers. Multiplexed methods such as multiparametric flow cytometry are important to elucidate the comprehensive patterns of healthy versus altered immune systems (29, 30). Here, such an approach was used by combining bioinformatics analysis with statistical methods to organize individual immunopathological markers into distinct clusters of T cell populations (n = 512), and thereafter identify unique immunopathological populations (n = 139) that would distinguish HIV-infected subjects from healthy control subjects. Using this method, we wished to evaluate which laboratory marker(s) represents the preeminent surrogate of combined T cell pathogenesis in HIV-infected patients. We demonstrated that, compared with the CD4 count, the CD4/CD8 ratio was a better predictor of the combined T cell pathogenesis that occurs in HIV infection.

Numerous studies have demonstrated that HIV-infected individuals have elevated immune activation, exhaustion, and senescence levels in diverse memory T cell compartments (1, 19). These results were confirmed in this study, wherein the CD38+HLA-DR+ CD8+ T cell population was shown to be most significantly different between the HIV-infected individuals and healthy subjects. This finding is not surprising, because CD38+HLA-DR+ CD8+ T cells have previously been identified as one of the best correlates of HIV disease progression (7). More surprisingly, however, was that the PD-1 MFI was the most significantly different marker between the HIV-infected subjects and healthy control subjects in terms of the CD4+ T cells. Previous studies have shown that PD-1 is expressed on T cells in healthy human adults and is vital for memory T cell differentiation (31, 32). The concept that memory differentiation and PD-1 are linked to each other was supported by the fact that in this study, the frequencies of PD-1+ and CD45RO+ CD4+ T cells were highly associated with each other in both healthy (r = 0.90, p < 0.0001) and HIV-infected subjects (r = 0.88, p < 0.0001). However, the PD-1 MFI on CD4+ T cells was not linked to the frequency of the memory marker CD45RO. Thus, the quantity of PD-1 molecules on each CD4+ T cell is more likely to be a prognostic surrogate for T cell pathogenesis, potentially because of its potent ability to hamper central memory CD4+ T cell homeostasis (33). The correlation between increased baseline MFI of PD-1 and poor absolute CD4 recovery after ART initiation further indicate that PD-1 probably induce inhibitory signals, prohibiting the proliferation and adequate homeostasis of CD4+ T cells.

In this study, we used multiparametric bioinformatics to identify 139 distinct immunopathological T cell populations that primarily correlated with the CD4/CD8 ratio. Most of these populations were found to be fairly rare and only distinguishable in the HIV-infected subjects. Therefore, we also conducted threshold analyses, where a given population needed to occupy at least >0.01% (median) of all populations. This reduced the number of T cell populations to 125, but these data still showed similar results with even better and more correlations for most routine parameters including the CD4/CD8 ratio (data not shown). Based on the 139 T cell pathological populations, we showed that memory CD8+ T cells expressing CD38, HLA-DR, and PD-1 were highly elevated in the HIV-infected cohort and were associated with HIV disease progression. These findings are in agreement with previous studies that demonstrated that the CD8+ T cell upregulation of PD-1 occurs in the presence of HLA-DR and CD38 (13). Later, the triple combination of CD38, HLA-DR, and PD-1 was found to correlate strongly with the CD4 count and VL (34). Many of the other individual immunological markers (e.g., CD45RO) also correlated significantly with the various laboratory parameters, but...
only before Bonferroni corrections were applied. Diverse expression of the senescence markers (CD57+ and CD28+ cells) was observed within the populations that expressed CD38, HLA-DR, and PD-1. A lack of CD28 expression was prominent in many of the CD8+ T cell populations that were highly elevated within the HIV-infected subjects (data not shown), indicating that the absence of CD28 appears to be a better predictor of immunopathological events than CD57. Another marker that was poorly expressed on the pathological T cell populations was Tim-3. Tim-3 was included in this study, because its expression was previously observed to be elevated on total and HIV-specific CD4+ and CD8+ T cells (16). In this study, we confirmed that the frequency of Tim-3 expression was elevated on total CD4+ and CD8+ T cells, but Tim-3 was not expressed in any of the identified top-10 pathological T cell populations. The frequency of Tim-3+ T cells was also previously correlated with disease progression (CD4 count and VL) and immune activation (16), but neither of these results was confirmed in this analysis. Whether these discrepancies were due to experimental procedures, cohort differences, or other effects remains to be identified. However, an interesting linkage between increased frequencies of Tim-3 and poor absolute CD4 recovery after ART was observed. Like PD-1, these results suggest that inhibitory receptors in general might impede the homeostasis of CD4+ T cells to generate proper reconstitution after ART.

The CD4/CD8 ratio is well-known to gradually decline with age in the general population. During the 1990s, researchers clarified in longitudinal studies that the CD4/CD8 ratio is a surrogate marker of immunosenescence and is predictive of mortality (35, 36). Similar results have also been observed in other cohorts (37, 38). Later, a CD4/CD8 ratio <1 was also confirmed to be a major predictor of mortality in very elderly people (>100 y) (39). Whether an inverted CD4/CD8 ratio is due to bystander effects or other causes in the general population remains unclear, but the expansion of CMV-specific CD8+ T cells with age might be one possible explanation for this principle (40, 41). As CMV burden increases with age, many researchers have suggested that the virus drives or at least exacerbates T cell senescence that eventually leads to an inverted CD4/CD8 ratio. Recent evidence implicates that CMV might impact the evolution and prognosis of transplantation, cancer, immunodeficiency, and other diseases through so-called early immunosenescence. Most probably, however, CMV in combination with other variables together lead to an age-associated alteration of immunity that deteriorates the CD4/CD8 ratio (42).

A limited number of studies have been conducted to verify the significance of the CD4/CD8 ratio in HIV infection. Taylor et al. (43) demonstrated in 1989 that the CD4/CD8 ratio is a slightly better predictor of the time to AIDS development than the CD4 count. Today, it is widely known that in many cases, despite fully recovered CD4 counts after long-term ART, the CD4/CD8 ratio rarely reaches normal levels. The reason for this is unknown, but recent data from HIV-infected children, adolescents, and adults who were receiving ART have shown strong associations between the CD4/CD8 ratio and T cell activation, senescence, and activation/exhaustion (23, 24). All of our diverse analyses confirm that the CD4/CD8 ratio is a better surrogate of combined T cell pathogenesis than the CD4 count and VL. Hypothetically, our results could be of interest at ART initiation, because individuals with increased pathological signs of the immune system (including T cell activation) have an increased risk for immunological failure (44, 45) and, therefore, of non-AIDS-related morbidities (46). We have in this study largely confirmed these previous observations, and find that the state of combined T cell pathology is predictive of whether subjects initiating ART will have a good CD4 recovery. In addition, low CD4/CD8 ratios can exist in the presence of moderate CD4 count, and such individuals might therefore be at an increased risk for immunological failure. This observation was defined in our study, because the CD4 count at baseline was not predictive of CD4 recovery 2 y after ART introduction. Future studies should assess the linkage between lower CD4/CD8 ratios, immunopathology, and the prognoses of immune recovery and non-AIDS morbidities after ART initiation in larger cohorts.

Limitations of this study include the lack of comparisons between routine laboratory parameters and other inflammatory variables such as IL-6, TNF, CRP and microbial translocation markers, which have been shown to be significant correlates of immunopathogenesis (19). The CMV burden would also be an interesting variable to quantify, due to its linkage to T cell senescence (19). Additionally, knowledge about the duration of infection was not available for most individuals, where it would have been interesting to longitudinally follow individuals from early HIV infection and further investigate whether the CD4/CD8 ratio is an improved surrogate of combined immunopathogenesis and disease outcomes. Although we acknowledge the importance of monitoring fluctuations of laboratory parameters over time, we wanted to conduct an observational study in untreated patients to identify a proper correlating factor between combined pathological signs of the T cell repertoire and routine laboratory markers. The knowledge of a single measurement remains of clinical importance, independently of whether the laboratory parameters will be assessed over time (43).

In an era during which we cannot eradicate the virus itself, studies of the immunopathological events that occur during HIV infection remain important to increase the quality of life of HIV-infected individuals. Using an interdisciplinary approach, wherein multiparametric immunology and advanced statistical bioinformatics were combined, we conclude that the CD4/CD8 ratio is a suitable surrogate marker of combined, ongoing T cell pathological events in natural HIV infection. These findings are of particular interest to future therapy or cure studies, in which simple measurements are required to monitor ongoing pathological events of the T cell repertoire in HIV-infected subjects.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

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2108 IMMUNOPATHOLOGICAL CORRELATES IN HIV INFECTION


