The CSF Immune Response in HIV-1–Associated Cryptococcal Meningitis: Macrophage Activation, Correlates of Disease Severity, and Effect of Antiretroviral Therapy

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Background: Immune modulation may improve outcome in HIV-associated cryptococcal meningitis. Animal studies suggest alternatively activated macrophages are detrimental but human studies are limited. We performed a detailed assessment of the cerebrospinal fluid (CSF) immune response and examined immune correlates of disease severity and poor outcome, and the effects of antiretroviral therapy (ART).

Methodology: We enrolled persons ≥18 years with first episode of HIV-associated cryptococcal meningitis. CSF immune response was assessed using flow cytometry and multiplex cytokine analysis. Principal component analysis was used to examine relationships between immune response, fungal burden, intracranial pressure and mortality, and the effects of recent ART initiation (<12 weeks).

Findings: CSF was available from 57 persons (median CD4 34/μL). CD206 (alternatively activated macrophage marker) was expressed on significantly increased macrophage expression of CD206. High fungal burden was not associated with CD206 expression but with a paucity of CD4+, CD8+, and CD4+CD8− T cells and lower interleukin-6, G-CSF, and interleukin-5 concentrations. High intracranial pressure (>30 cm H2O) was associated with fewer T cells, a higher fungal burden, and larger Cryptococcus organisms. Mortality was associated with reduced interferon-gamma concentrations and CD4−CD8− T cells but lost statistical significance when adjusted for multiple comparisons. Recent ART was associated with increased CSF CD4/CD8 ratio and a significantly increased macrophage expression of CD206.

Conclusions: Paucity of CSF T cell infiltrate rather than alternative macrophage activation was associated with severe disease in HIV-associated cryptococcosis. ART had a pronounced effect on the immune response at the site of disease.

Key Words: Cryptococcus, immune response, alternatively activated macrophages, flow cytometry, fungal burden, raised intracranial pressure, mortality

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INTRODUCTION

Host immunity is central to the pathogenesis of cryptococcosis. Cryptococcus neoformans is found widely in the environment and serological studies suggest exposure is common. Most infections are asymptomatic with the infecting organism contained within pulmonary granulomas. However, when cell-mediated immunity is impaired, C. neoformans can disseminate throughout the body resulting in...
mengoencephalitis frequently complicated by high intracranial pressure (ICP). Most cases worldwide are associated with HIV-1 infection and cryptococcosis remains a leading cause of death in sub-Saharan Africa.5-7

Impaired immunity also influences disease presentation and response to treatment. Previous studies have shown that low cerebrospinal fluid (CSF) levels of proinflammatory cytokines [interferon-γ, interleukin (IL)-6, and IL-8] are associated with a higher fungal burden, slower clearance of infection and increased mortality.6,9 However, cryptococcosis may also be complicated by an over exuberant inflammatory response after the initiation of antiretroviral therapy (ART). This is known as immune reconstitution inflammatory syndrome (IRIS) and either develops during the first manifestation of cryptococcosis (unmasking IRIS) or as a recurrence of meningitis symptoms after successful antifungal treatment (paradoxical IRIS).10 There are increasing reports from sub-Saharan Africa of patients developing cryptococcal meningitis after recently starting ART,11,12 whether these cases represent unmasking IRIS or a state of immune deficiency not yet reversed by ART, has not been fully elucidated.

Central to host immunity is the interaction between macrophages and Cryptococcus. The yeast is easily phagocy-tosed by macrophages but can resist intracellular killing through permeabilization of the phagosome membrane.13 This enables Cryptococcus to avoid immune surveillance and replicate within the cell and may facilitate migration to the central nervous system.14 Infection is controlled after the recruitment of interferon (IFN)-γ producing CD4 T cells, stimulating macrophages to become classically (M1) activated.15 However, macrophages may also become alternatively activated (M2) because of the stimulation by IL-4 or IL-13, a state better suited to tissue repair.16 In animal models of cryptococcosis, alternatively activated macrophages (identified by expression of CD206) along with a Th2 T-cell response were detrimental, resulting in uncontrolled fungal infection and death. By contrast, classically activated macrophages and a Th1 response were beneficial.17 The role of macrophage activation in determining outcome in human disease has not been studied.

This study aimed to better understand the host-immune response at the site of disease in HIV-1-associated cryptococcal meningitis. We performed a detailed examination of the CSF immune response using flow cytometry and biomarker analysis and concentrated particularly on the cellular immune response and the activation state of monocyte/macrophages. We examined how this immune phenotype related to markers of disease severity and clinical outcome. To better understand the pathophysiology of ART-associated cryptococcal meningitis, we also examined the effects of recent ART initiation (≤12 weeks) on the CSF immune response. We hypothesized that macrophages in the CSF of persons with cryptococcal meningitis would express CD206, a marker of alternative activation, and that the degree of CD206 expression would be correlated with outcome, such that individuals with the highest expression of CD206 would have the highest fungal burden and be more likely to die. We also hypothesized that persons recently started on ART would have a more inflammatory CSF with lower macrophage CD206 expression compared with persons not taking ART.

METHODS

Participant Recruitment and Clinical Care
A prospective cohort study was conducted in Cape Town, South Africa between April 2012 and July 2013. Ethical approval was obtained from the University of Cape Town Human Research Ethical Committee (reference 408/2010, 371/2013) and Liverpool School of Tropical Medicine Research Ethics Committee (reference 11.92). All participants provided written informed consent; family members provided surrogate consent for patients with impaired consciousness. Consecutive persons ≥18 years with a first episode of HIV-1-associated cryptococcal meningitis (positive CSF culture or cryptococcal antigen test) were enrolled within 48 hours of presentation. After enrollment, clinical details were recorded and lumbar puncture (LP) performed for management of CSF opening pressure and CSF sampling. Additional LPs were performed at attending physicians’ discretion to manage raised ICP. Antifungal therapy comprised amphotericin B deoxycholate 1 mg/kg and fluconazole 800 mg daily for 14 days, then fluconazole 400 mg daily for 10 weeks, and 200 mg daily thereafter. Participants were followed for 6 months. ART was started at 4 weeks if participants were not taking ART at enrollment.

CSF Processing and Analysis of Immune Response
CSF was transferred to the laboratory on ice and processed in real-time. Fungal burden was measured using quantitative culture as previously described and recorded as colony forming units per milliliter of CSF (colony-forming units/mL).18 The remaining CSF was centrifuged, the supernatant frozen at −80°C for batch biomarker analysis, and the cell pellet stained immediately for flow cytometry analysis.

Flow Cytometry Staining of CSF Cells
CSF cells were incubated at 4°C for 30 minutes with an amine viability dye (AQUA; Invitrogen, Carlsbad, CA); anti-CD45-PECy5.5, anti-CD4-PECy7, anti-CD66b-PE, anti-CD206-AF488, anti-HLADR-AF700, anti-CD163-APC (Biolegend, San Diego, CA); anti-CD8-Qdot655, anti-CD14-Qdot605 (Invitrogen); and anti-CD16-APCH7 and anti-CD3-PacBlue (BD Biosciences, San Jose, CA). During optimization experiments, additional cells were permeabilized with 1 mL of PermWash (BD Biosciences) and stained with anti-CD68-PE (Biolegend) to better characterize macrophages. FACS lysing solution (BD Biosciences) was used to remove any erythrocytes and the sample fixed using 2% paraformaldehyde in flow buffer. Cells were protected from light at all times and analyzed within 24 hours on a BD LSR Fortessa Flow Cytometer using FACS-Diva software (BD Biosciences). Note was made of the total CSF volume and the sample was acquired in its entirety with forward scatter (FSC) threshold set at 5000 to exclude debris. Species appropriate positive and negative compensation beads were used along with ArC Amine Reactive Compensation Bead Kit to ensure accurate compensation (BD Biosciences; Invitrogen). Fluorescence minus
one experiments were used during optimization steps to ensure accurate gating as previously described.\textsuperscript{19} Flow cytometry data were analyzed using FlowJo version 9.5.3 (Tree Star software, OR); gating strategy is detailed in Figure 1. Flow cytometry allowed accurate identification and quantification of neutrophils, T cells (CD8\textsuperscript{+}, CD4\textsuperscript{+}, CD4\textsuperscript{+}CD8\textsuperscript{+}, and CD4\textsuperscript{+}CD8\textsuperscript{−}), and monocyte-macrophages. Monocyte-macrophages were initially identified as CD14\textsuperscript{+} cells after the exclusion of neutrophils and T cells (CD14\textsuperscript{+}MM) (Fig. 1D). A second population of CD14\textsuperscript{−} monocyte-macrophages (CD14\textsuperscript{−}MM) was also identified with similar physical characteristics and CD68 expression to CD14\textsuperscript{+}MM (CD3\textsuperscript{−}CD4\textsuperscript{−}CD14\textsuperscript{−}HLADR\textsuperscript{+}) (Figs. 1D, E1, E2). Expression of CD206, CD163, CD16, and HLA-DR were measured on both CD14\textsuperscript{+} and CD14\textsuperscript{−} monocyte-macrophages using median fluorescence intensity and cell percentage expressing the marker (Figs. 1E3–E6). HLA-DR expression was measured on all T-cell subsets. Some participants were noted to have CD8 T cells with significantly increased size (FSC); these were termed “large T cells” (Figs. 1C2, C5). Natural killer (NK) cells were defined as CD16\textsuperscript{+} cells after exclusion of neutrophils and monocyte-macrophages (Fig. 1F). Cryptococci were defined as CD45\textsuperscript{−} cells as demonstrated elsewhere\textsuperscript{20}; Cryptococcus size was measured using

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\caption{CSF flow cytometry gating. A1, FSC-SSC plot of CSF cells after exclusion of singlets, aggregates, Cryptococcus yeasts, and dead cells. Cells with high FSC noted (circled and marked *); (A2) neutrophils defined as CD66\textsuperscript{+} and high SSC; (B) CD3 used to identify T cells; (C1) T-cell subsets analyzed using CD4 and CD8; (C2) FSC-SSC view of T cells, “Large” T cells circled and marked *; (C3) HLA-DR expression on CD4\textsuperscript{+} T cells; (C4) HLA-DR expression on CD8\textsuperscript{+} T cells; (C5) analysis of “large T cells”—majority comprise CD8\textsuperscript{+} T cells; (D) further gating on non-T cells using CD14 and CD4 identifies monocyte-macrophages. Population of CD14\textsuperscript{−} monocyte-macrophages are circled and marked †; (E1 and E2) CD14\textsuperscript{+} and CD14\textsuperscript{−} monocyte-macrophages have similar physical characteristics (FSC-SSC) and similar expression of CD68; (E3–E6) expression of CD206, CD163, HLA-DR, and CD16 (respectively) on CD14\textsuperscript{+} and CD14\textsuperscript{−} MM; (F) CD3\textsuperscript{−}CD4\textsuperscript{−}CD14\textsuperscript{−}CD16\textsuperscript{+} cells identified—likely NK cells.
\end{figure}
FSC, as an absolute measurement and in relation to CD4+ T cells (FSC crypto/CD4).

Biomarker Analysis

Commercial multiplex assays were used to measure the concentrations of 23 cytokines/chemokines: IL-1RA, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, granulocyte and granulocyte-macrophage colony stimulating factors (G-CSF and GM-CSF), tumor necrosis factor-α, IFN-α, IFN-γ, vascular endothelial growth factor, chemokine ligand 2 (CCL2), CCL3, CCL4, and C-C chemokine ligand 9 (Bio-Rad, Hercules, CA; Invitrogen). The concentrations of 2 soluble markers of macrophage activation (sCD163 and sCD14) were measured using commercial ELISA (R&D, Minneapolis, MN).

Data Analysis

Baseline characteristics were summarized and analyzed using descriptive statistics as appropriate. Data from flow cytometry and biomarker analysis were combined [resulting in a dataset of 89 variables (Supplemental Digital Content 1, http://links.lww.com/QAI/A1000)] and analyzed using principal component analysis (PCA), a mathematical technique used to simplify complex datasets by examining them in terms of a series of principal components rather than individual variables.21 Before PCA, variables were log-transformed and scaled such that the geometric mean equaled zero and variance equaled one. Missing values were imputed by K-nearest neighbors technique.22 Heatmap analysis with nonhierarchical clustering was performed as described elsewhere.23 Variables were filtered using statistical tests before incorporation into PCA and cluster analysis such that, only variables with a statistically significant association with the dependent variable were used. Four main dependent variables were examined: fungal burden (log10 colony-forming units/mL CSF), high ICP (CSF opening pressure >30 cm H2O), mortality (death within 12 weeks), and recent ART initiation (<12 weeks). Statistical significance was defined as a P-value of <0.05 and q-value of less than 0.1 (q < 0.1 is equivalent to a 10% false discovery rate using the Benjamini–Hochberg procedure for multiple-testing correction).24 Analysis was performed using Stata version 12. (Stata Corp., College Station, TX.) and Quilocore Omics Explorer version 3.0 (Quilocore AB, Lund, Sweden).

RESULTS

Participants

Sixty participants were enrolled, CSF flow cytometry was performed on 57 (3 had insufficient CSF available for analysis). The median age was 36 years [interquartile range (IQR) 30–43] and median CD4 count was 34 cells per microliter (IQR 13–76). The cumulative case fatality rate was 23% at 2 weeks (13/57) and 38% at 12 weeks (21/56); one participant was lost to follow-up after hospital discharge. Fifteen participants were taking ART at enrollment (26%); 6 of these had clear evidence of virological failure (detectable viral load after ≥6 months ART), and one later reported nonadherence; 8 participants were defined as “Recent ART” having either initiated ART (n = 6), or switched to second line ART after virological failure (n = 2) in the 12 weeks before presentation (median 6 weeks); one had clinical features consistent with unmasking IRIS.10

CSF Flow Cytometry

A median of 7 mL (IQR 4.5–8) of CSF was available per participant for flow cytometry resulting in a median of 108,000 cells (IQR 30,877–294,500) per sample; cell viability remained high (median 100%, range 92%–100%). CD8+ T cells were the most abundant cell type [median 49.7% (IQR 30.2%–63.7%)], followed by neutrophils [11.9% (IQR 2.3%–29.4%)], monocyte-macrophages [6.74% (IQR 3.1%–14.1%)], and CD4+ T cells [6.2% (IQR 3.7%–9.6%)].

Immune Factors Significantly Associated With High Fungal Culture Burden

We first explored the correlation between CSF immune and baseline fungal burden. Twelve variables that were significantly correlated with CSF fungal burden (Pearson correlation, P < 0.05 and q < 0.1) were entered into a PCA. To avoid the confounding effect of antifungal therapy, analysis was restricted to 36 persons who had not received amphotericin B at enrollment. Flow cytometry Cryptococcus counts were also removed because of the strong correlation with quantitative fungal culture previously reported (R = 0.93, P < 0.0001). Participants with higher fungal burdens clearly clustered together on a PCA plot with particularly low scores for principal component 1 (PC1) (Fig. 2A). Analysis of the variables contributing to PC1 showed that the CSF of persons with high fungal burden was characterized by significantly lower numbers of CSF T cells (CD4, CD8, and CD4+CD8−) and NK cells, lower CSF concentrations of IL-5, IL-6 and G-CSF, and lower expression of the neutrophil activation marker CD66b (Fig. 2B). CSF and blood CD4 counts were closely correlated (Pearson R = 0.66 P < 0.001). Adjusting for blood CD4 count reduced the number of variables that were significantly negatively correlated with fungal burden to only CSF CD4−CD8− T cell numbers and IL-5 concentration (R = −0.51, P = 0.002, Q = 0.09 and R = −0.56, P = 0.001, Q = 0.05, respectively). There was no significant correlation between fungal burden and CD206 expression (median fluorescence intensity) on CSF macrophages (P = 0.89).
Immune Factors Significantly Associated With High ICP

We next aimed to determine whether the raised ICP observed in cryptococcal meningitis might be associated with a particular CSF immune response. To do this we compared participants who had evidence of high ICP at study enrollment or at any time during their hospitalization (ICP $\geq 30$ cm H$_2$O, $n = 35$), with those who did not develop high ICP ($n = 22$). Participants who experienced high ICP clearly grouped together on PCA and cluster analysis according to their CSF characteristics (Figs. 3A, C). This difference was primarily because of significantly higher Cryptococcus counts in the CSF of subjects who developed high ICP along with increased size of the Cryptococcus measured by flow cytometry. In addition, participants who developed high ICP had significantly lower CSF counts of CD4$^+$ T cells, NK cells and CD4$^+$CD8$^-$ T cells, and higher proportion of “large T cells” (Fig. 3B).

Associations Between CSF Immune Response and Mortality

We then examined immune correlates of mortality. Participants who died by week 12 ($n = 22$) had lower baseline CSF IFN-γ concentrations compared with participants who survived ($n = 34$) (geometric mean 52 pg/mL [95% confidence interval (CI): 19 to 139] vs. 131 pg/mL [95% CI: 97 to 176], respectively, $P = 0.032$), and a decreased frequency of CD4$^+$CD8$^-$ T cells as a proportion of CSF T cells and as a proportion of CSF CD45 cells (geometric means 4.9% [95% CI: 3.3 to 7.2] vs. 8.7% [95% CI: 7.4 to 10.4], $P = 0.002$ and 3.1% [95% CI: 2.2 to 4.4] vs. 4.8% [95% CI: 4.0 to 5.9], $P = 0.018$, respectively) (Supplemental Digital Content, Fig. 2, http://links.lww.com/QAI/A1000). These findings lost statistical significance ($q \leq 0.1$) when adjusted for multiple comparisons. IFN-γ was significantly correlated with the numbers of CD4$^+$CD8$^-$ T cells (Pearson $R = 0.31$, $P = 0.022$), CD8$^+$ T cells (R = 0.26, $P = 0.047$), and NK cells (R = 0.35, $P = 0.001$) but not CD4$^+$ T cells (R = 0.23, $P = 0.092$). There was no association between macrophage CD206 expression and mortality ($P = 0.26$).

Effect of ART on CSF Immune Response During Cryptococcal Meningitis

Finally, to characterize the CSF immune phenotype of ART-associated cryptococcal meningitis and understand the effects of recent ART initiation on the immune response at the site of disease, we compared participants not taking ART ($n = 43$) against those taking “Recent ART” (started first line ART or switched to second line ART in the 12 weeks before presentation, $n = 8$). “Recent ART” was associated with a significantly lower plasma HIV-1 viral load and significantly higher blood CD4 counts but no significant difference in CSF fungal burden, opening pressure, white cell count, or mortality (Table 1). Participants who had recently started/switched ART clustered together on PCA and nonhierarchical cluster analysis according to their CSF immune response (Figs. 4A, C). In this

FIGURE 2. Relationship between CSF immune response and fungal burden. A, PCA plot detailing distribution of participants according to CSF immune response after filtering for variables significantly correlated with CSF fungal burden ($P < 0.05$, $q < 0.1$). Axes represent the first three principal components; % displays the degree of total sample variability accounted for by component. Fungal burden is indicated by color (scale at left of plot displays log$_{10}$ CFU/mL CSF). Participants with a high fungal burden (red, $\sim 10^6$ CFU/mL) cluster together at the bottom of the plot while participants with low fungal burden (green $\sim 10^1$ CFU/mL) group together at the top. B, PCA plot of variables significantly correlated with fungal burden that contributed to the PCA. Position in PCA plot indicates the weighting toward the first 3 principal components; variables located in close proximity contribute similarly. Color indicates direction of correlation with fungal load (red—positive correlation, green—negative correlation). Absolute cell counts are expressed in cells per milliliter CSF while relative counts are expressed as a percentage of all CSF leukocytes (%CD45 cells). CD45, leukocytes; DNT, double negative T cells, ie, CD4$^-$CD8$^-$; WCC, white cell count/μL by microscopy; Lymph, lymphocytes/μL by microscopy; MFI, median fluorescence intensity.
Recent ART initiation was associated with significantly higher proportions of CSF CD4⁺ T cells and lower proportions of CSF CD8⁺ T cells, along with significantly increased expression of CD206 on CD14⁺ monocyte-macrophages and increased expression of CD206 and CD16 on CD14⁻ monocyte-macrophages suggesting increased alternative activation of macrophages (Fig. 4B). The increase in CD4 T cells at the site of disease was noticeably greater than that observed in the blood (Supplemental Digital Content, Fig. 3, http://links.lww.com/QAI/A1000).

We hypothesized that the effects of ART on macrophage activation were mediated via alterations in the HIV-1 viral load. This was supported by the observation of a significant inverse correlation between HIV-1 viral load in the blood and CD206 expression on CSF CD14⁺ monocyte-macrophages both in the whole cohort (Pearson $R = -0.59$, $P < 0.001$) and in an analysis restricted to participants who were not taking ART (Pearson $R = -0.57$, $P < 0.001$) (Supplemental Digital Content, Fig. 4, http://links.lww.com/QAI/A1000).
CSF Immune Response in Cryptococcosis

A number of cell populations were characterized by low numbers of T lymphocytes (CD4, CD8, and CD4–CD8–) and NK cells, along with decreased CD4 T cell predominance observed in healthy persons, but is consistent with other studies of HIV-1 disease progression. In contrast to animal studies, there was no association between alternative activation of CSF macrophages and fungal burden.8 Instead, high CSF fungal burden was clearly associated with higher CSF CD4+ T-cell counts in the CSF and blood are closely correlated suggests that the major factor determining fungal burden may simply be HIV-1–associated CD4 cell depletion. However, an alternative explanation for these findings is that infiltration of immune cells into the CSF may be inhibited by the immunomodulatory actions of the cell wall polysaccharide glucuronoxylomannan shed by the large numbers of C. neoformans within the central nervous system.34–36

Raised ICP within the first 14 days was significantly associated with a higher baseline fungal burden, significantly larger cryptococci in the CSF (increased FSC on flow cytometry), and decreased CSF CD4+ and CD4–CD8– T cell infiltrates. Although the role of large CD8 T cells needs to be further explored, our study did not convincingly suggest that high ICP occurs as a result of a pathological inflammatory response. These findings are similar to others demonstrating an association between raised CSF opening pressure and greater CSF fungal quantitative culture and increased Cryptococcus capsule size (measured ex vivo using microscopy).37,38 Our findings are therefore consistent with the concept that raised ICP in cryptococcal meningitis occurs predominantly because of obstruction of CSF drainage by huge numbers of encapsulated yeast rather than pathological inflammation.39

Fatal outcome was associated with reduced CSF CD4–CD8– T cells and IFN-γ concentration. Although these associations lost significance when adjusted for multiple comparisons, the findings are compatible with previous studies showing significantly slower fungal clearance and reduced survival in persons with lower CSF IFN-γ concentrations.8 The significant correlation between CSF IFN-γ and CD4–CD8– T cells (but not CD4 T cells) suggest CD4–CD8– T cells could be an additional source of IFN-γ. Given their presence was also associated with lower fungal burden, further study is warranted to determine their nature and function.

Finally, to better understand the pathology of ART-associated cryptococcal meningitis (including unmasking IRIS), we performed an exploratory analysis examining the effects of recent ART initiation on the CSF immune response. Recent ART initiation did not appear to influence the overall numbers of cells in the CSF but was associated with a noticeable increase in the CSF CD4/CD8 ratio, far more prominent than the changes observed in the blood. This is consistent with other studies in asymptomatic persons with HIV-1 infection and patients with paradoxical cryptococcal IRIS.28,29 Recent ART was also associated with significantly reduced activation of CD4 T cells (lower HLA-DR expression), fewer large T cells, and contrary to our hypothesis, a switch toward an alternatively activated macrophage phenotype (significantly higher expression of CD206 on both CD14+ and CD14– monocyte-macrophages25). The strong negative correlation between plasma HIV-1 viral load and CD206 expression on CSF CD14+ MM even in participants not taking ART caused us to hypothesize that ART-associated alterations in macrophage polarity may occur as a direct effect of HIV-1, with a proinflammatory classically activated phenotype predominating in untreated HIV-1 infection, shifting toward an alternatively activated state (with increased

### DISCUSSION

This study provides a comprehensive examination of the CSF cellular immune response in HIV-1–associated cryptococcal meningitis, with particular reference to CSF macrophage polarization. CD8 T cells were the predominant cell type followed by neutrophils and CD4 T cells. These contrasts with the CD4 T cell predominance observed in healthy persons, but is consistent with other studies of HIV-1–infected persons.27–29 A number of cell populations were identified in the CSF that are not commonly seen in blood and warrant further study. These included “large” CD8 T cells (which may represent activated CD8 T cells30), CD4–CD8– T cells (possibly a mixture of γδ T cells and invariant natural killer T cells as observed in other neurological conditions31,32), and CD14– monocyte-macrophages. CD206 expression was commonly observed on both CD14+ and CD14– monocyte-macrophages in keeping with previous work suggesting macrophages adopt an alternatively activated phenotype as HIV-1 disease progresses.33

In contrast to animal studies, there was no association between alternative activation of CSF macrophages and fungal burden.17 Instead, high CSF fungal burden was clearly associated with a paucicellular CSF immune response characterized by low numbers of T lymphocytes (CD4, CD8, and CD4–CD8–) and NK cells, along with decreased CSF concentrations of IL-5, IL-6, and G-CSF. This is consistent with Thai studies that also observed significantly lower concentrations of proinflammatory cytokines (IL-6, IFNγ, and tumor necrosis factor-α) in subjects with higher CSF fungal burden.8 Our finding that CD4+ T-cell counts in the CSF and blood are closely correlated suggests that the major factor determining fungal burden may simply be HIV-1–associated CD4 cell depletion. However, an alternative explanation for these findings is that infiltration of immune cells into the CSF may be inhibited by the immunomodulatory actions of the cell wall polysaccharide glucuronoxylomannan shed by the large numbers of C. neoformans within the central nervous system.34–36

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### TABLE 1. Comparison of Clinical and Laboratory Features at Enrollment Between Participants Taking Effective ART and No ART (n = 53)

| Baseline Parameters | Recent ART* (n = 10) | No ART (n = 43) | P  |
|---------------------|---------------------|----------------|----|
| Age, yrs            | 32 (27–40)          | 37 (29–43)     | 0.369 |
| Male                | 3 (30%)             | 25 (58%)       | 0.162 |
| Blood CD4 count/µL  | 60 (45–85)          | 29 (12–67)     | 0.024 |
| HIV-1 viral load log10 copies/µL | 2.4 (1.3–3.3)   | 5.3 (5.1–5.6) | <0.001 |
| HIV-1 viral load <40 copies/µL | 3 (30%)         | 0 (0%)         | 0.005 |
| Altered consciousness | 1 (10%)          | 9 (21%)        | 0.665 |
| CSF opening pressure at day 0 cm H2O | 25 (12–31)     | 25 (16–40)    | 0.465 |
| Max CSF opening pressure,† cm H2O | 27 (24–33)      | 38 (22–50)    | 0.255 |
| OP >30 cm H2O       | 3 (38%)             | 26 (60%)       | 0.268 |
| CSF white cells, µL | 8 (0–45)            | 21 (3–115)     | 0.227 |
| CSF protein, g/L    | 0.73 (0.57–1.3)     | 0.97 (0.56–1.7) | 0.502 |
| CSF glucose, mmol/L | 1.9 (1.5–2.7)       | 2.5 (1.7–3)    | 0.175 |
| Fungal burden, log10 CFU/mL | 4.1 (3.1–6.1) | 4.7 (3.5–5.5) | 0.838 |
| CSF IFN-γ             |                     |                |    |
| Death by day 14      | 2 (20%)             | 11 (26%)       | 0.601 |

Data are numbers with percentages or median with IQR. P-values derived from Wilcoxon rank-sum or Fisher exact test as appropriate.

*Recent ART defined as starting first line ART or switching to second line ART in the 12 weeks before presentation.

†Maximum CSF opening pressure during first 14 days of admission.
CD206 expression) when ART is started. This theory is supported by both in vitro and ex vivo studies that have shown HIV-1 replication to be associated with significant decreases in CD206 expression.\textsuperscript{40,41} Larger studies are now required to determine the clinical implications of recent ART initiation in cryptococcal meningitis.

There are a number of limitations to this work. This was an exploratory study of a relatively small, heterogeneous, cohort and the findings will need to be confirmed in larger studies. Comparisons with healthy controls and HIV-1-infected persons with no CNS pathology would have been helpful but ethical considerations limit access to CSF without a clinical indication for LP. Real-time flow cytometry removed the potential adverse effects of freezing on cell activation, but did preclude any ability to repeat assays. We only used one marker of alternative activation (CD206) in our antibody panel and the absence of CD56 means that findings regarding NK cells counts must be verified in other cohorts. Finally, we were unable to assess the contribution of resident microglial cells.

Despite these caveats, this exploratory study provides novel findings regarding the human immune response in cryptococcal meningitis at the site of disease. We have provided a detailed characterization of the CSF infiltrate,

FIGURE 4. PCA and nonhierarchical cluster analysis examining effect of recent ART initiation on CSF immune response. A, PCA plot showing distribution of subjects according to CSF immune response. Subjects who started taking ART in the previous 12 weeks (blue dots) group together and separate from subjects not taking ART (yellow dots). The participant with unmasking IRIS is marked. B, PCA plot displaying 12 variables that contributed to the PCA. Plot position reflects variable weightings toward the three principal components: red dot (variable significantly increased among participants taking ART); green dot (variable significantly decreased among participants taking ART). Variables with similar contributions are positioned in close proximity; those correlated $\geq 80\%$ are connected with lines. Statistical significance defined as $P < 0.05$ and $q < 0.1$. C, Heat map demonstrating nonhierarchical cluster analysis according to CSF immune response. Subjects who started ART in the previous 12 weeks (blue squares) group together due to similar expression of the 12 variables (rows) detailed in (B). Expression of variable in relation to geometric mean is indicated by color of square (red—increased; green—decreased). %T, relative frequency as a percentage of all CSF T cells; % CD45, relative frequency as a percentage of all CSF leukocytes; MFI, median fluorescence intensity; CD14\textsuperscript{+}, CD14\textsuperscript{2} monocyte-macrophages; CD14\textsuperscript{2}, CD14\textsuperscript{+} monocyte-macrophages; CD206\textsuperscript{+}, CD206\textsuperscript{2} monocyte-macrophages expressing CD206; HLA-DR %CD4, proportion of CD4 T cells expressing HLA-DR.
identified cell types not commonly found in the blood and assessed the activation state of CSF macrophages ex vivo. Although recent ART was associated with a shift toward an alternatively activated macrophage phenotype, contrary to animal studies this did not appear to be associated with severe disease or poor outcome. Instead, a T cell infiltrate appears central to the protective response. We conclude that efforts to augment this immune response with proinflammatory agents warrant further study.

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