OPEN

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

James E. Scriven, MBChB, PhD,†‡§ Lisa M. Graham, PhD,* Charlotte Schutz, MBChB, MPH,*§ Thomas J. Scriba, PhD,|| Katalin A. Wilkinson, PhD,*¶ Robert J. Wilkinson, FRCP, PhD,*§¶# David R. Boulware, MD, PhD,** Britta C. Urban, PhD,‡ Graeme Meintjes, MBChB, PhD,*§# and David G. Lalloo, FRCP, MD†‡*

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 54% $CD14^+$ and 35% $CD14^-$ monocyte-macrophages. 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 H_2O) 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

(*J Acquir Immune Defic Syndr* 2017;75:299–307)

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.[1,2] Most infections are asymptomatic with the infecting organism contained within pulmonary granulomas.[3] However, when cell-mediated immunity is impaired, *C. neoformans* can disseminate throughout the body resulting in

Received for publication September 26, 2016; accepted January 31, 2017.

From the *Clinical Infectious Diseases Research Initiative, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory, South Africa; †Liverpool School of Tropical Medicine, Liverpool, United Kingdom; ‡Wellcome Trust Liverpool Glasgow Centre for Global Health Research, Liverpool, United Kingdom; §Department of Medicine, University of Cape Town and Groote Schuur Hospital, Observatory, South Africa; ||South African TB Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa; ¶The Francis Crick Institute, Mill Hill Laboratory, The Ridgeway, London, United Kingdom; #Department of Medicine, Imperial College, London, United Kingdom; and **Department of Medicine, University of Minnesota, MN.

Supported by the Wellcome Trust through a training fellowship to J.E.S. (094013/B/10/Z). G.M. is supported by the Wellcome Trust (098316), the South African Medical Research Council and the South African Research Chairs Initiative of the Department of Science and Technology and National Research Foundation of South Africa (64787). C.S., D.R.B., and G.M. receive support from the National Institutes of Health (U01AI089244). R.J.W. receives funding from Medical research Council (U1175.02.002.00014.01), Wellcome Trust (104803), National Research Foundation of South Africa (96841), European Union (FP7-PEOPLE-2011-IRSES and FP7-HEALTH-F3-2012-305578). B.C.U. is supported by the Wellcome Trust (079082). The funders had no role in the study design, data collection, data analysis, data interpretation, or writing of this report. The opinions, findings and conclusions expressed in this manuscript reflect those of the authors alone.

Presented in part at the Conference for Retroviruses and Opportunistic Infections; February 25, 2015; Seattle, WA.

The authors have no conflicts of interest to disclose.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

Correspondence to: James E. Scriven, MBChB, PhD, Wellcome Trust Centre for Global Health Research, 70 Pembroke Place, Liverpool L69 3GF, United Kingdom (e-mail: jscriven@liv.ac.uk).

meningoencephalitis frequently complicated by high intracranial pressure (ICP).[4] 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.[8,9] 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 phagocytosed 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 batched 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.[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 quantitation of neutrophils, T cells ($CD8^+$, $CD4^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$), and monocyte-macrophages. Monocyte-macrophages were initially identified as $CD14^+$ cells after the exclusion of neutrophils and T cells ($CD14^+$MM) (Fig. 1D). A second population of $CD14^-$ monocyte-macrophages ($CD14^-$MM) was also identified with similar physical characteristics and CD68 expression to $CD14^+$MM ($CD3^-CD4^+CD14^-$HLADR+) (Figs. 1D, E1, E2). Expression of CD206, CD163, CD16, and HLA-DR were measured on both $CD14^+$ and $CD14^-$ 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 $CD16^+$ cells after exclusion of neutrophils and monocyte-macrophages (Fig. 1F). Cryptococci were defined as $CD45^-$ cells as demonstrated elsewhere[20]; *Cryptococcus* size was measured using

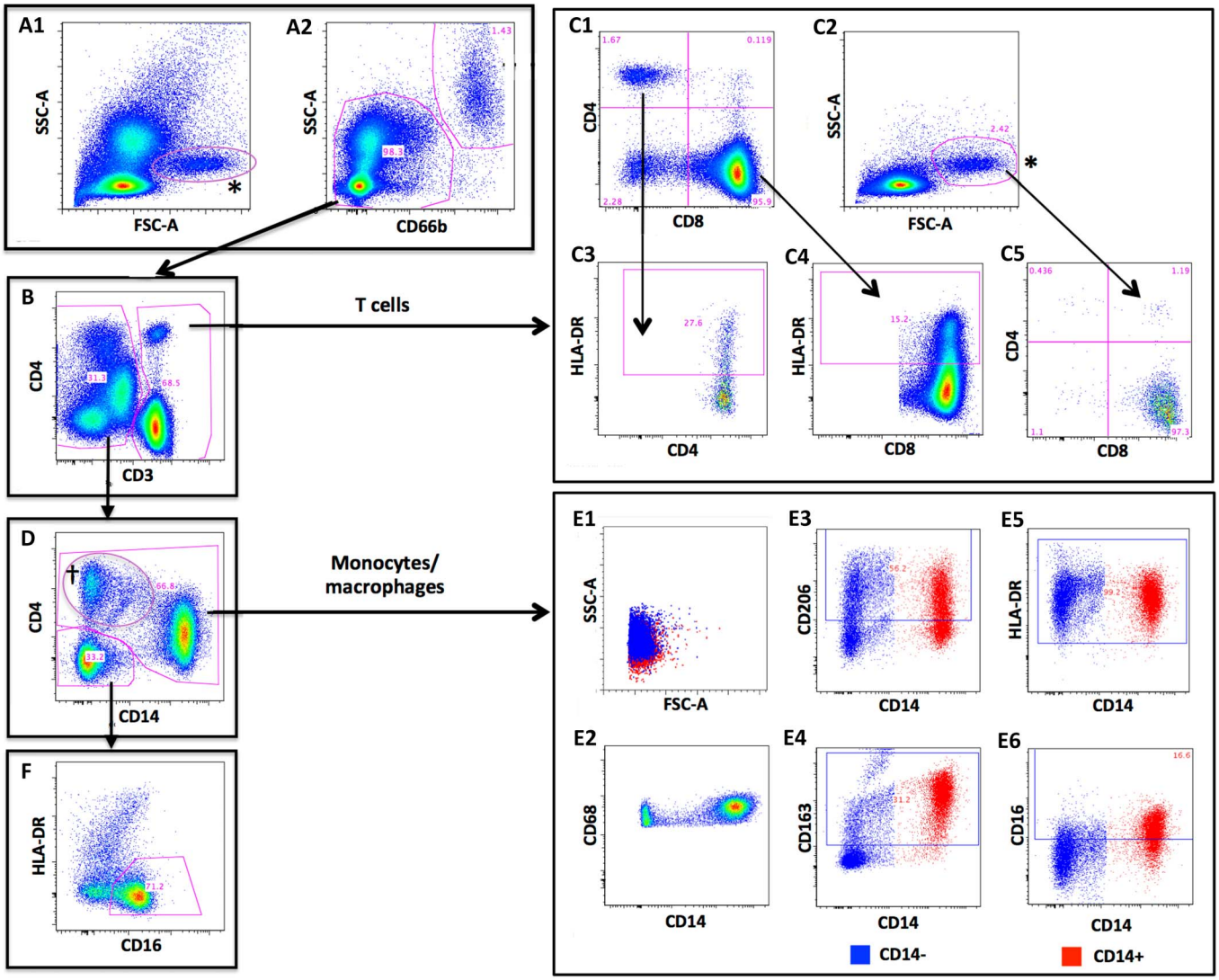

FIGURE 1. CSF flow cytometry gating. A1, FSC-SSC plot of CSF cells after exclusion of singlets, aggregates, Cryptococcus yeasts, and dead cells. Cells with high FSC noted (circled and marked *); (A2) neutrophils defined as $CD66^+$ and high SSC; (B) CD3 used to identify T cells; (C1) T-cell subsets analyzed using CD4 and CD8; (C2) FSC-SCC view of T cells, "Large" T cells circled and marked *; (C3) HLA-DR expression on $CD4^+$ T cells; (C4) HLA-DR expression on $CD8^+$ T cells; (C5) analysis of "large T cells"—majority comprise $CD8^+$ T cells; (D) further gating on non-T cells using CD14 and CD4 identifies monocyte-macrophages. Population of $CD14^-$ monocyte-macrophages are circled and marked †; (E1 and E2) $CD14^+$ and $CD14^-$ 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 $CD14^+$ and $CD14^-$ MM; (F) $CD3^-CD4^-CD14^-CD16^+$ cells identified—likely NK cells.

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-X-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 H20), 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 Qlucore Omics Explorer version 3.0 (Qlucore 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%)] (Supplemental Digital Content, Fig. 1A, http://links.lww.com/QAI/A1000). Large T cells comprised a median of 2.7% (IQR 0.93–4.55) of the total CD8 T-cell population. HLA-DR expression did not differ between large and normal CD8 T cells (not shown). Both $CD14^+$ and $CD14^-$ monocyte-macrophages expressed a range of activation markers including HLA-DR, CD206, CD16, and CD163. A median of 54% (IQR 37%–70%) $CD14^+$ monocyte-macrophages and 35% (IQR 20%–52%) $CD14^-$ monocyte-macrophages expressed the surface marker CD206 (consistent with alternative activation[25]) (Supplemental Digital Content, Fig. 1B, http://links.lww.com/QAI/A1000).

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 ($R = 0.93$, $P < 0.0001$).[20] 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[26] (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 ($P = 0.89$).

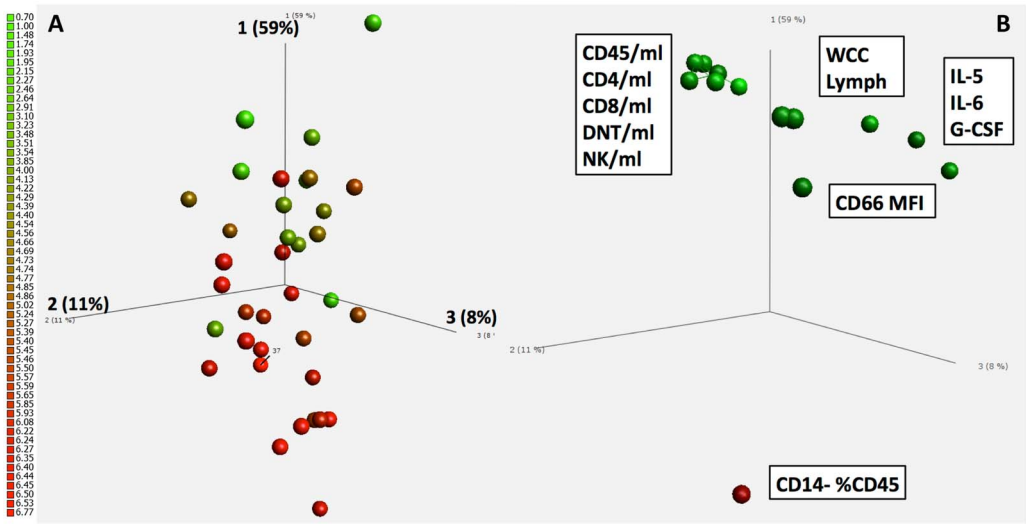

FIGURE 2. Relationship between CSF immune response and fungal burden. A, PCA plot detailing distribution of participants according to CSF immune response after filtering for variables significantly correlated with CSF fungal burden ($P < 0.05$, $q < 0.1$). Axes represent the first three principal components; % 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.

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 ≥ 30 cm H_2O, 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 ($q \geq 0.1$) 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 nonhierarchal cluster analysis according to their CSF immune response (Figs. 4A, C). In this

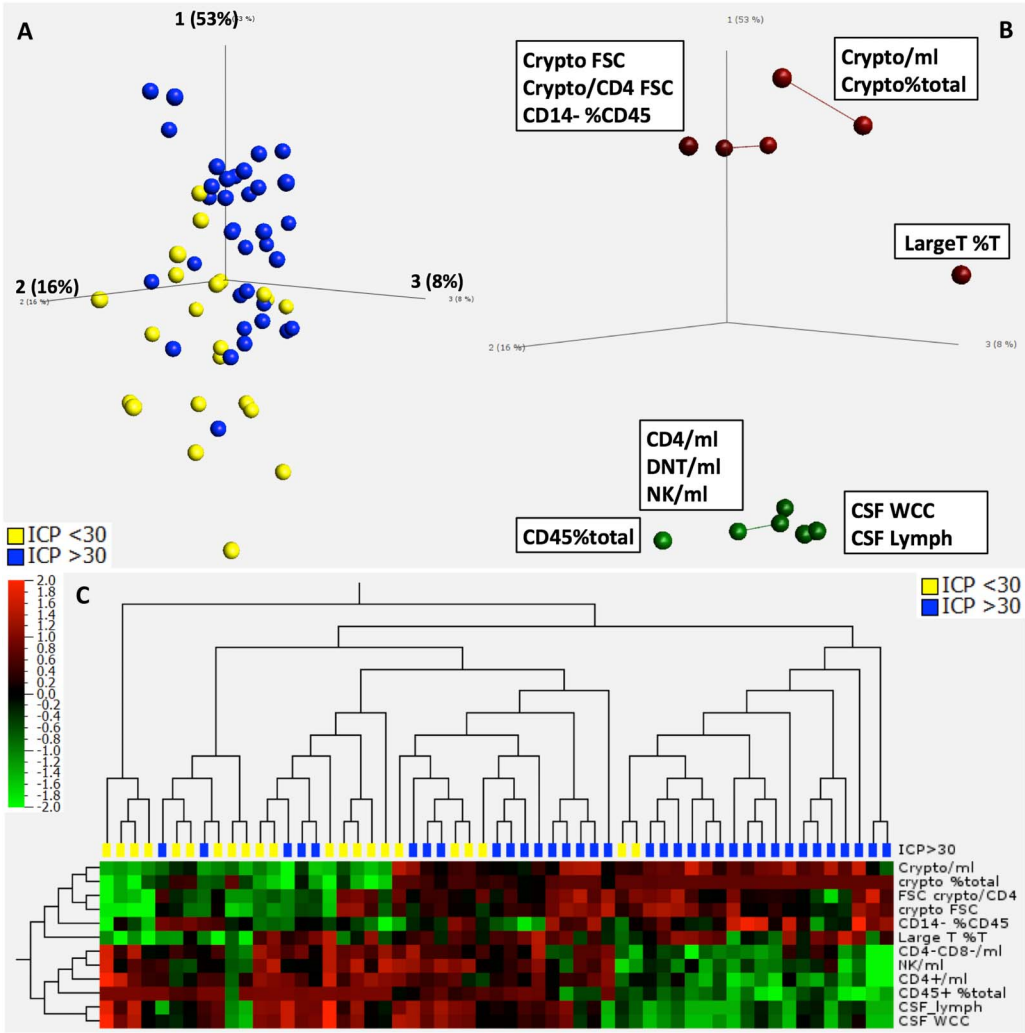

FIGURE 3. Differences in CSF immune response between participants who developed high intracranial pressure (ICP) during admission and those who did not. A, PCA plot showing distribution of participants according to CSF immune response after filtering for variables significantly associated with raised ICP. Axes indicate the first three principal components. Participants who developed high ICP during admission (≥30 cm H_2O—blue) cluster together and broadly separate from those who do not develop high ICP (<30 cm H_2O—yellow) according to CSF characteristics. B, PCA plot illustrating the 12 variables that significantly differed between the 2 groups and hence contributed to the PCA [red—significantly greater in subjects with high ICP, green—significantly lower in subjects with high ICP ($P < 0.05$ and $q < 0.1$)]. Absolute cell counts are expressed as cells per milliliter CSF; relative counts are expressed either as a percentage of CD45 cells (%CD45) or a percentage of all flow cytometry events (%total). C, Heat map illustrating nonhierarchal cluster analysis of participants according to the same 12 variables detailed in (B). Participants who develop high ICP during admission tend to cluster at the right end of the plot. Crypto, Cryptococcus; FAC, flow cytometry measurement of cell size; Crypto/CD4 FSC, relative size of Cryptococcus in relation to CD4 T cells; Large T, large T cells as detailed in Fig. 1; DNT, double negative T cells, $CD4^-CD8^-$; WCC, white cell count; Lymph, lymphocyte count.

analysis 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).

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 $\log_{10}$ copies/mL	2.4 (1.3–3.3)	5.3 (5.1–5.6)	<0.001
HIV-1 viral load <40 copies/mL	3 (30%)	0 (0%)	0.005
Altered consciousness	1 (10%)	9 (21%)	0.665
CSF opening pressure at day 0 cm H_2O	25 (12–31)	25 (16–40)	0.465
Max CSF opening pressure,† cm H_2O	27 (24–33)	38 (22–50)	0.255
OP >30 cm H_2O	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, $\log_{10}$ CFU/mL CSF	4.1 (3.1–6.1)	4.7 (3.5–5.5)	0.838
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.

CFU, colony-forming units.

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 cells[30]), $CD4^-CD8^-$ T cells (possibly a mixture of γδ T cells and invariant natural killer T cells as observed in other neurological conditions[31,32]), 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]

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-macrophages[25]). 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

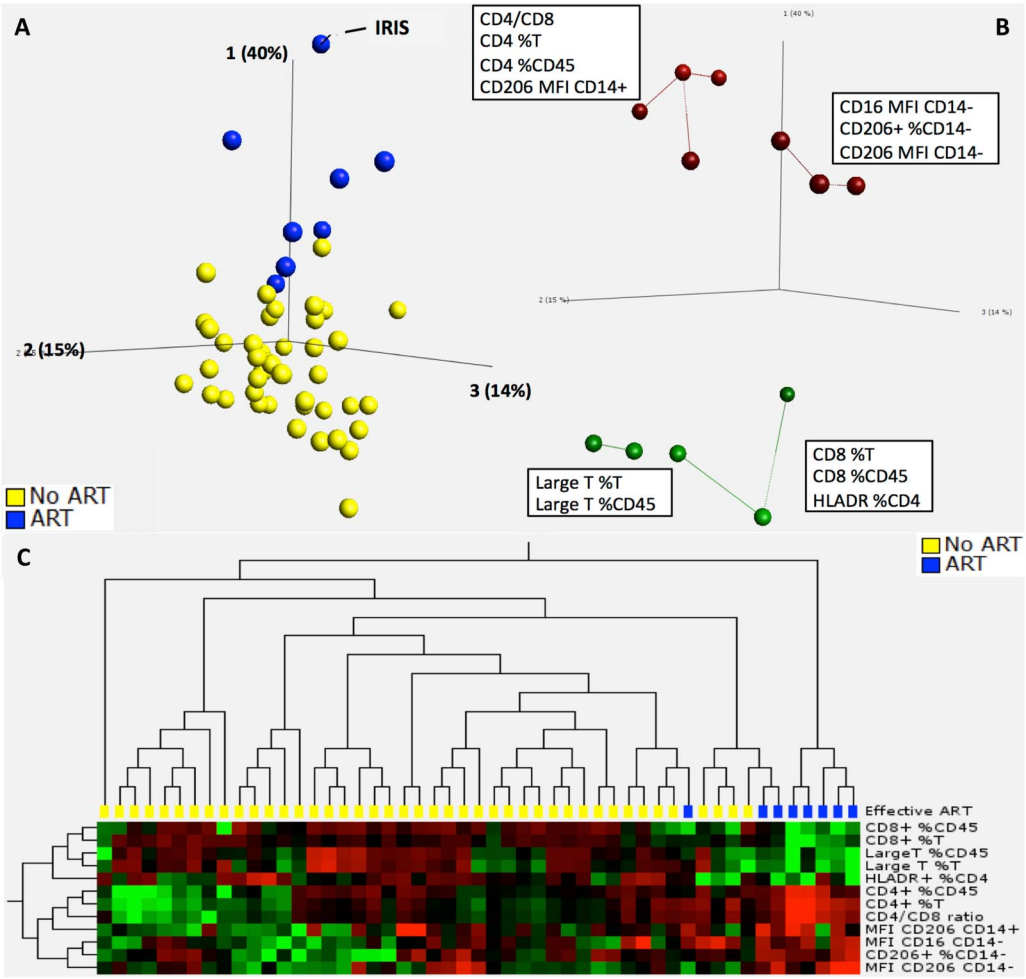

FIGURE 4. PCA and nonhierarchical cluster analysis examining effect of recent ART initiation on CSF immune response. A, PCA plot showing distribution of subjects according to CSF immune response. Subjects who started taking ART in the previous 12 weeks (blue dots) group together and separate from subjects 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 ≥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; $CD14^+$, $CD14^+$ monocyte-macrophages; $CD14^-$, $CD14^-$ monocyte-macrophages; $CD206^+$ $\%CD14^-$, proportion of $CD14^-$ monocyte-macrophages expressing CD206; HLADR %CD4, proportion of CD4 T cells expressing HLA-DR.

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.[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,

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.

ACKNOWLEDGMENTS

The authors are grateful for the assistance of Dr. Rosie Burton, Dr. Gavin van Wyk and Mr. Anthony Williams along with the clinical, laboratory and administrative staff of the Provincial Government of the Western Cape Department of Health for their support of the study. They also thank Prof. Brian Faragher for additional statistical input.

REFERENCES

1. Emmons CW. Saprophytic sources of Cryptococcus neoformans associated with the pigeon (Columba livia). *Am J Hyg.* 1955;62:227–232.
2. Goldman DL, Khine H, Abadi J, et al. Serologic evidence for Cryptococcus neoformans infection in early childhood. *Pediatrics*. 2001;107:E66.
3. Salyer WR, Salyer DC, Baker RD. Primary complex of cryptococcus and pulmonary lymph nodes. *J Infect Dis.* 1974;130:74–77.
4. Bicanic T, Harrison TS. Cryptococcal meningitis. *Br Med Bull.* 2004;72: 99–118.
5. Cohen DB, Zijlstra EE, Mukaka M, et al. Diagnosis of cryptococcal and tuberculous meningitis in a resource-limited African setting. *Trop Med Int Health.* 2010;15:910–917.
6. Jarvis JN, Meintjes G, Williams A, et al. Adult meningitis in a setting of high HIV and TB prevalence: findings from 4961 suspected cases. *BMC Infect Dis.* 2010;10:67.
7. Park BJ, Wannemuehler KA, Marston BJ, et al. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS.* 2009;23:525–530.
8. Siddiqui AA, Brouwer AE, Wuthiekanun V, et al. IFN-gamma at the site of infection determines rate of clearance of infection in cryptococcal meningitis. *J Immunol.* 2005;174:1746–1750.
9. Jarvis JN, Meintjes G, Bicanic T, et al. Cerebrospinal fluid cytokine profiles predict risk of early mortality and immune reconstitution inflammatory syndrome in HIV-associated cryptococcal meningitis. *PLoS Pathog.* 2015;11:e1004754.
10. Haddow LJ, Colebunders R, Meintjes G, et al. Cryptococcal immune reconstitution inflammatory syndrome in HIV-1-infected individuals: proposed clinical case definitions. *Lancet Infect Dis.* 2010;10:791–802.
11. Rhein J, Morawski BM, Hullsiek KH, et al. Efficacy of adjunctive sertraline for the treatment of HIV-associated cryptococcal meningitis: an open-label dose-ranging study. *Lancet Infect Dis.* 2016;16:809–818.
12. Scriven JE, Lalloo DG, Meintjes G. Changing epidemiology of HIV-associated cryptococcosis in sub-Saharan Africa. *Lancet Infect Dis.* 2016; 16:891–892.
13. Feldmesser M, Tucker S, Casadevall A. Intracellular parasitism of macrophages by Cryptococcus neoformans. *Trends Microbiol.* 2001;9:273–278.
14. Casadevall A. Cryptococci at the brain gate: break and enter or use a Trojan horse? *J Clin Invest.* 2010;120:1389–1392.
15. Kawakami K, Kohno S, Kadota J, et al. T cell-dependent activation of macrophages and enhancement of their phagocytic activity in the lungs of mice inoculated with heat-killed Cryptococcus neoformans: involvement of IFN-gamma and its protective effect against cryptococcal infection. *Microbiol Immunol.* 1995;39:135–143.
16. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol.* 2003;3:23–35.
17. Stenzel W, Müller U, Köhler G, et al. IL-4/IL-13-dependent alternative activation of macrophages but not microglial cells is associated with uncontrolled cerebral cryptococcosis. *Am J Pathol.* 2009;174:486–496.
18. Brouwer AE, Teparrukkul P, Pinpraphapom S, et al. Baseline correlation and comparative kinetics of cerebrospinal fluid colony-forming unit counts and antigen titers in cryptococcal meningitis. *J Infect Dis.* 2005;192:681–684.
19. Herzenberg LA, Tung J, Moore WA, et al. Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol.* 2006;7:681–685.
20. Scriven JE, Graham LM, Schutz C, et al. Flow cytometry to assess CSF fungal burden in cryptococcal meningitis. *J Clin Microbiol.* 2016;54: 802–804.
21. Ringnér M. What is principal component analysis? *Nat Biotechnol.* 2008; 26:303–304.
22. Troyanskaya O, Cantor M, Sherlock G, et al. Missing value estimation methods for DNA microarrays. *Bioinformatics*. 2001;17:520–525.
23. Bergkvist A, Rusnakova V, Sindelka R, et al. Gene expression profiling —clusters of possibilities. *Methods*. 2010;50:323–335.
24. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B.* 1995;57: 289–300.
25. Ambarus CA, Krausz S, van Eijk M, et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. *J Immunol Methods.* 2012;375:196–206.
26. Stocks SC, Kerr MA, Haslett C, et al. CD66-dependent neutrophil activation: a possible mechanism for vascular selectin-mediated regulation of neutrophil adhesion. *J Leukoc Biol.* 1995;58:40–48.
27. De Graaf MT, Smitt PAES, Luitwieler RL, et al. Central memory CD4+ T cells dominate the normal cerebrospinal fluid. *Cytometry B Clin Cytom.* 2011;80:43–50.
28. Ho EL, Ronquillo R, Altmeppen H, et al. Cellular composition of cerebrospinal fluid in HIV-1 infected and uninfected subjects. *PLoS One*. 2013;8:e66188.
29. Meya DB, Okurut S, Zziwa G, et al. Cellular immune activation in cerebrospinal fluid from Ugandans with cryptococcal meningitis and immune reconstitution inflammatory syndrome. *J Infect Dis.* 2014;211:1597–1606.
30. Rathmell JC, Elstrom RL, Cinalli RM, et al. Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. *Eur J Immunol.* 2003;33: 2223–2232.
31. Bertotto A, Spinozzi F, Gerli R, et al. Gamma delta T lymphocytes in mumps meningitis patients. *Acta Paediatr.* 1995;84:1268–1270.
32. Hamzaoui K, Kamoun M, Houman H, et al. Discrepancies of NKT cells expression in peripheral blood and in cerebrospinal fluid from Behçet's disease. *J Neuroimmunol.* 2006;175:160–168.
33. Herbein G, Varin A. The macrophage in HIV-1 infection: from activation to deactivation? *Retrovirology.* 2010;7:33.
34. Yauch LE, Lam JS, Levitz SM. Direct inhibition of T-cell responses by the Cryptococcus capsular polysaccharide glucuronoxylomannan. *PLoS Pathog.* 2006;2:e120.
35. Piccioni M, Monari C, Kenno S, et al. A purified capsular polysaccharide markedly inhibits inflammatory response during endotoxic shock. *Infect Immun.* 2013;81:90–98.
36. Retini C, Vecchiarelli A, Monari C, et al. Encapsulation of Cryptococcus neoformans with glucuronoxylomannan inhibits the antigen-presenting capacity of monocytes. *Infect Immun.* 1998;66:664–669.
37. Bicanic T, Brouwer AE, Meintjes G, et al. Relationship of cerebrospinal fluid pressure, fungal burden and outcome in patients with cryptococcal meningitis undergoing serial lumbar punctures. *AIDS*. 2009;23:701–706.
38. Robertson EJ, Najjuka G, Rolfes MA, et al. Cryptococcus neoformans ex vivo capsule size is associated with intracranial pressure and host immune response in HIV-associated cryptococcal meningitis. *J Infect Dis.* 2014;209:74–82.
39. Loyse A, Wainwright H, Jarvis JN, et al. Histopathology of the arachnoid granulations and brain in HIV-associated cryptococcal meningitis: correlation with cerebrospinal fluid pressure. *AIDS*. 2010; 24:405–410.
40. Porcheray F, Samah B, Léone C, et al. Macrophage activation and human immunodeficiency virus infection: HIV replication directs macrophages towards a pro-inflammatory phenotype while previous activation modulates macrophage susceptibility to infection and viral production. *Virology*. 2006;349:112–120.
41. Jambo KC, Banda DH, Kankwatira AM, et al. Small alveolar macrophages are infected preferentially by HIV and exhibit impaired phagocytic function. *Mucosal Immunol.* 2014;7:1116–1126.