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The CSF immune response in HIV-1-associated cryptococcal meningitis: macrophage activation, correlates of disease severity and effect of antiretroviral therapy

James E Scriven[1,2,3,4], Lisa M Graham[1], Charlotte Schutz[1,4], Thomas J Scriba[5], Katalin A Wilkinson[1,7], Robert J Wilkinson[1,4,6,7], David R Boulware[8], Britta C Urban[2], Graeme Meintjes[1,4,6], David G Lalloo[2,3]

1. Clinical Infectious Diseases Research Initiative, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa
2. Liverpool School of Tropical Medicine, L3 5QA, UK
3. Wellcome Trust Liverpool Glasgow Centre for Global Health Research, L69 3GF, UK
4. Department of Medicine, University of Cape Town and Groote Schuur Hospital, Observatory 7925, South Africa
5. South African TB Vaccine Initiative, Institute of Infectious Disease and Molecular Medicine and Division of Immunology, Department of Pathology, University of Cape Town 7925, South Africa
6. Department of Medicine, Imperial College, London, W2 1PG, UK
7. The Francis Crick Institute Mill Hill Laboratory, London, NW7 1AA, UK
8. Department of Medicine, University of Minnesota, MN 55455, USA

Correspondence: James Scriven: jscriven@liv.ac.uk, Wellcome Trust Centre for Global Health Research, 70 Pembroke Place, Liverpool, L69 3GF, UK

ADDITIONAL INFORMATION

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Conflicts of Interest

No authors have any conflicts of interest to declare

Presentations

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Abstract

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 IL-6, G-CSF and IL-5 concentrations. High intracranial pressure (≥30cmH2O) 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.

Keywords: Cryptococcus; immune response; alternatively activated macrophages; flow cytometry; fungal burden; raised intracranial pressure

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] The vast majority of 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 meningoencephalitis frequently complicated by high intracranial pressure.[4] The majority of 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 CSF levels of pro-inflammatory cytokines (interferon-γ, interleukin-6 and interleukin-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 following the initiation of anti-retroviral 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 following successful anti-fungal 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 following the recruitment of IFN-γ producing CD4 T cells, stimulating macrophages to become classically (M1) activated.[15] However, macrophages may also become alternatively activated (M2) due to 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 to 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. Following enrolment, 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 intracranial pressure. Anti-fungal therapy comprised amphotericin B deoxycholate 1mg/kg and fluconazole 800mg daily for 14 days, then fluconazole 400mg daily for 10 weeks, and 200mg daily thereafter. Participants were followed for 6 months. ART was started at 4 weeks if participants were not taking ART at enrolment.

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 (CFU/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); anti-CD16-APCH7 and anti-CD3-PacBlue (BD Biosciences, San Jose, CA). During optimization experiments, additional cells were permeabilized with 1ml 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 analysed 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 following the exclusion of neutrophils and T cells (CD14+MM) [**Figure 1: D**]. 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+) [**Figure 1: D, E1, E2**]. Expression of CD206, CD163, CD16 and HLA-DR were measured on both CD14+ and CD14- monocyte-macrophages using median fluorescence intensity (MFI) and cell percentage expressing the marker [**Figure 1: E3-6**]. HLA-DR expression was measured on all T cell subsets. Some participants were noted to have CD8 T cells with significantly increased size (forward scatter); these were termed “large T cells” **[Figure 1: C2, C5]**. NK cells were defined as CD16+ cells following exclusion of neutrophils and monocyte-macrophages [**Figure 1: F**]. Cryptococci were defined as CD45- cells as demonstrated elsewhere;[20] *Cryptococcus* size was measured using forward scatter (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: Interleukin (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), tumour necrosis factor (TNF)-α, interferon(IFN)-α, IFN-γ, vascular endothelial growth factor (VEGF), chemokine ligand 2 (CCL2), CCL3, CCL4 and C-X-C chemokine ligand 9 (BioRad, Hercules, CA; Invitrogen). The concentrations of two soluble markers of macrophage activation (sCD163 and sCD14) were measured using commercial ELISA (R&D, Minneapolis, MN).

Data analysis

Baseline characteristics were summarized and analysed using descriptive statistics as appropriate. Data from flow cytometry and biomarker analysis were combined (resulting in a dataset of 89 variables [Supplementary Data 1]) and analysed 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] Prior to 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 neighbours technique.[22] Heatmap analysis with non-hierarchical clustering was performed as described elsewhere.[23] Variables were filtered using statistical tests prior to 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 CFU/mL CSF), high ICP (CSF opening pressure >30cmH20), 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 (FDR) using the Benjamini-Hochberg procedure for multiple-testing correction[24]). Analysis was performed using Stata version 12. (Stata Corp, College Station, Texas,) and Qlucore Omics Explorer version 3.0 (Qlucore AB, Lund, Sweden).

RESULTS

Participants

Sixty participants were enrolled, CSF flow cytometry was performed on 57 (three had insufficient CSF available for analysis). The median age was 36 years (interquartile range (IQR) 30-43) and median CD4 count was 34 cells/μL (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 enrolment (26%); six of these had clear evidence of virological failure (detectable viral load after ≥6 months ART), and one later reported non-adherence; eight 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 prior to 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%]) [**Supplementary Figure 1: A**]. 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]) **[Supplementary Figure 1: B]**.

Immune factors significantly associated with high fungal culture burden

We first explored the correlation between CSF immune and baseline fungal burden. 12 variables that were significantly correlated with CSF fungal burden (Pearson's correlation, $p<0.05$ and $q<0.1$) were entered into a principal component analysis (PCA). To avoid the confounding effect of anti-fungal therapy, analysis was restricted to 36 persons who had not received amphotericin B at enrolment. Flow cytometry *Cryptococcus* counts were also removed due to the strong correlation with quantitative fungal culture previously reported (R=.93, *P*<.0001)[20]). Participants with higher fungal burdens clearly clustered together on a PCA plot with particularly low scores for Principal component 1 (PC1) [**Figure 2: A**]. 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] [**Figure 2: B**]. CSF and blood CD4 counts were closely correlated (Pearson's R=0.66 P<.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=.002, Q=.09 and R=-0.56, P=.001, Q=.05 respectively). There was no significant correlation between fungal burden and CD206 expression (MFI) on CSF macrophages (P=.89).

Immune factors significantly associated with high intracranial pressure

We next aimed to determine whether the raised intracranial pressure (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 enrolment or at any time during their hospitalization (ICP≥30cm H_2O, n=35), to 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 [**Figure 3: A, C**]. This difference was primarily due to 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” [**Figure 3: B**].

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 to participants who survived (n=34) (geometric mean 52 pg/mL (95%CI 19-139) vs. 131 pg/mL (95%CI 97-176) 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 3.3-7.2) vs. 8.7% (95%CI 7.4-10.4), p=0.002 and 3.1% (95%CI 2.2-4.4) vs. 4.8% (95%CI 4.0-5.9), p=0.018, respectively) [**Supplementary Figure 2**]. These findings lost statistical significance (q≥0.1) when adjusted for multiple comparisons. IFN-γ was significantly correlated with the numbers of CD4-CD8- T cells (Pearson’s R=.31 P=.022), CD8 T cells (R=.26, P=.047), and NK cells (R=.35

P=.001) but not CD4 T cells (R=.23, P=.092). There was no association between macrophage CD206 expression and mortality (P=.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 1st line ART or switched to 2nd line ART in the 12 weeks prior to 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 non-hierarchal cluster analysis according to their CSF immune response [**Figure 4: A, C**]. In this 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 [**Figure 4: B**]. The increase in CD4 T cells at the site of disease was noticeably greater than that observed in the blood [**Supplementary Figure 3**].

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's R=-0.59, P<.001) and in an analysis restricted to participants who were not taking ART (Pearson's R=-0.57, P<.001) [**Supplementary Figure 4**].

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. This 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 pro-inflammatory cytokines (IL-6, IFNγ and TNFα) 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 (GXM) shed by the large numbers of *C. neoformans* within the central nervous system.[34-36]

Raised intracranial pressure 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 intracranial pressure in cryptococcal meningitis occurs predominantly due to 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 towards 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 pro-inflammatory classically activated phenotype predominating in untreated HIV-1 infection, shifting towards an alternatively activated state (with increased 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 towards 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 pro-inflammatory agents warrant further study.

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FIGURE LEGENDS

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, E2) CD14 + and CD14- monocyte-macrophages have similar physical characteristics (FSC-SSC) and similar expression of CD68; (E3, E4, E5, 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.

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 colour (scale at left of plot displays $\log_{10}$ CFU/mL CSF). Participants with a high fungal burden (red, ~10^6CFU/mL) cluster together at the bottom of the plot while participants with low fungal burden (green ~10^1CFU/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 towards the first three principal components; variables located in close proximity contribute similarly.

Colour indicates direction of correlation with fungal load (red – positive correlation, green – negative correlation). Absolute cell counts are expressed in cells /ml CSF while relative counts are expressed as a percentage of all CSF leukocytes (%CD45 cells). Abbreviations: CD45 (leukocytes), DNT (double negative T cells, i.e. CD4-CD8-), NK (Natural Killer cells), WCC (white cell count/µL by microscopy), Lymph (lymphocytes/µL by microscopy), MFI (median fluorescence intensity).

Figure 3. Differences in CSF immune response between participants who developed high intracranial pressure 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 intracranial pressure (ICP). Axes indicate the first three principal components. Participants who developed high ICP during admission (≥30cm H_2O – blue) cluster together and broadly separate from those who do not develop high ICP (<30cm H_2O – yellow) according to CSF characteristics. **(b)** PCA plot illustrating the 12 variables that significantly differed between the two 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/ml 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 non-hierarchal 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. Abbreviations: ICP (intra-cranial pressure), Crypto (Cryptococcus), FSC (forward scatter, 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 Figure

1), DNT (double negative T cells, CD4-CD8-), WCC (white cell count), Lymph (lymphocyte count).

Figure 4. Principal component analysis (PCA) and non-hierarchical 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 towards 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 non-hierarchical 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 colour of square (red – increased; green – decreased). Abbreviations: ART (anti-retroviral therapy); %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).

Table 1. Comparison of clinical and laboratory features at enrolment between participants taking effective ART and no ART (n=53).

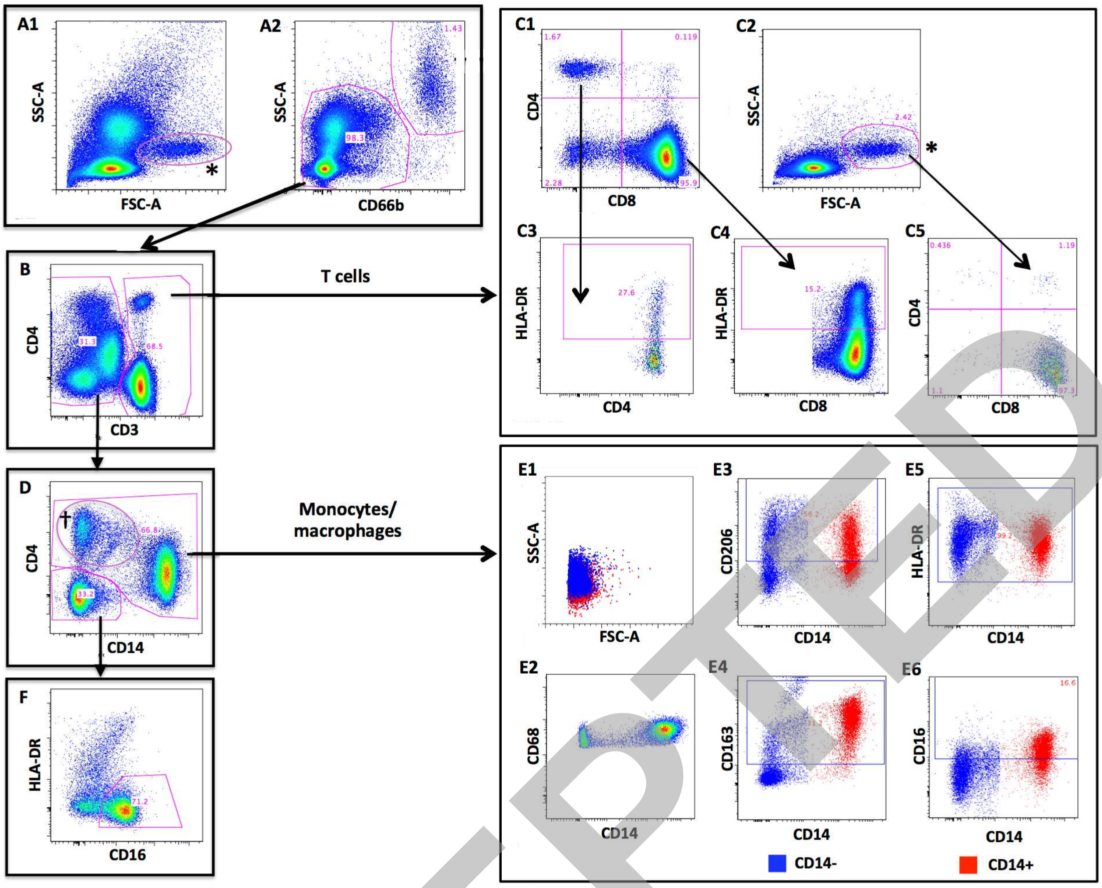
Baseline Parameters	**Recent ART (n=10)**	**No ART (n=43)**	**P-value**
Age, years	32 (27-40)	37 (29-43)	0.369
Male	3 (30%)	25 (58%)	0.162
Blood CD4 count /µL	60 (45-85)	29 (12-67)	0.024
HIV-1 viral load $\log_{10}$ copies/mL	2.4 (1.3-3.3)	5.3 (5.1-5.6)	<0.001
HIV-1 viral load <40 copies/mL	3 (30%)	0 (0%)	0.005
Altered consciousness	1 (10%)	9 (21%)	0.665
CSF opening pressure at Day 0 cmH_2O	25 (12-31)	25 (16-40)	0.465
Max CSF opening pressure[a], cmH_2O	27 (24-33)	38 (22-50)	0.255
OP>30 cmH_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

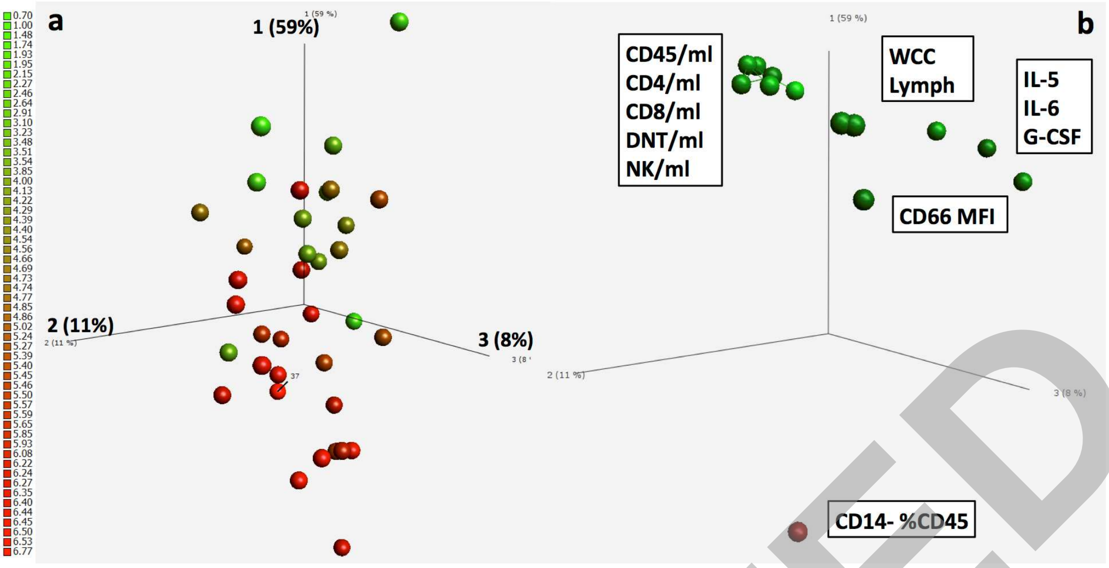
Data are numbers with percentages or median with interquartile range (IQR). P-values derived from Wilcoxon rank-sum or Fisher's exact test as appropriate.

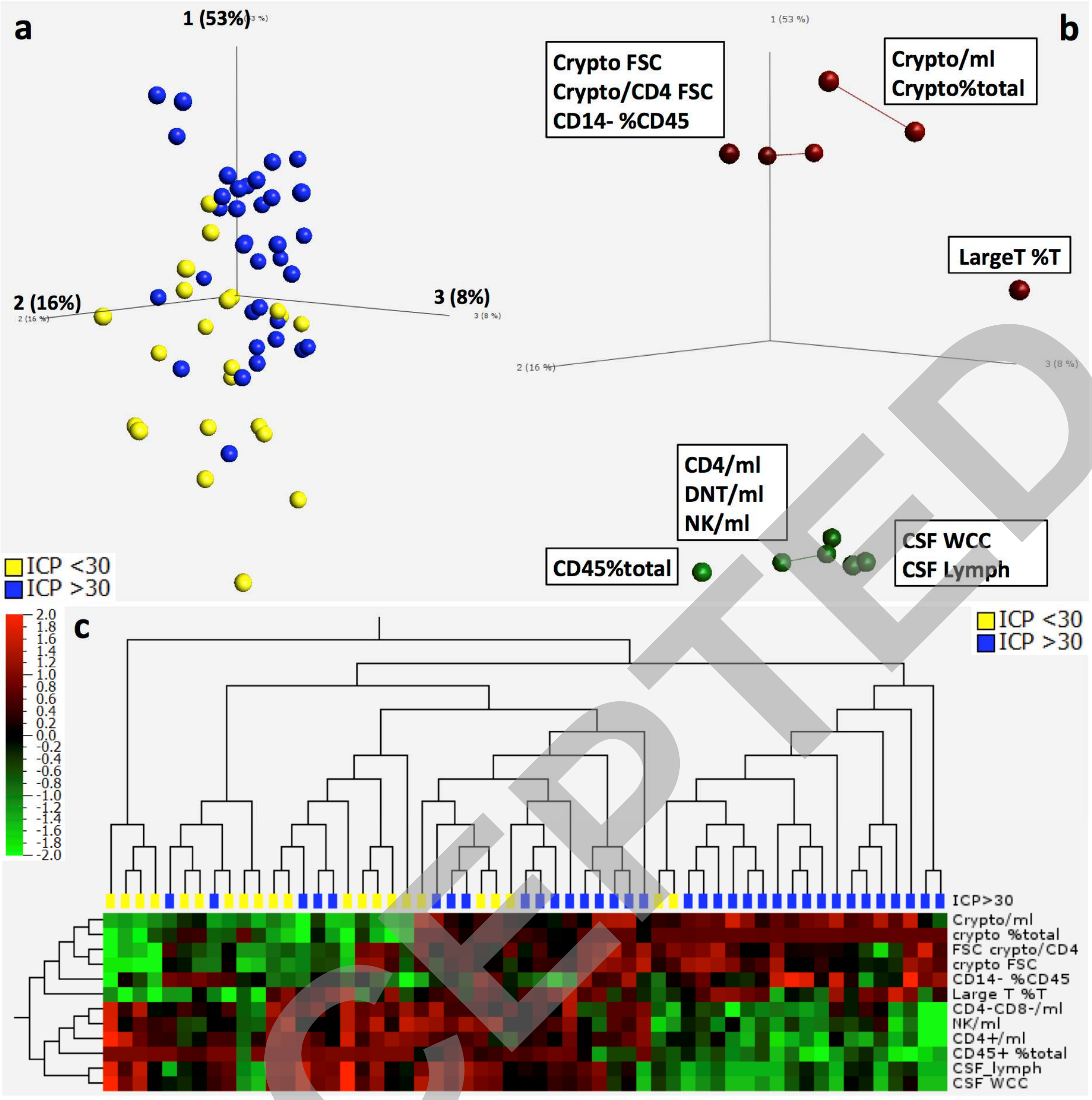
[a] Recent ART defined as starting 1st line ART or switching to second line ART in the 12 weeks prior to presentation.

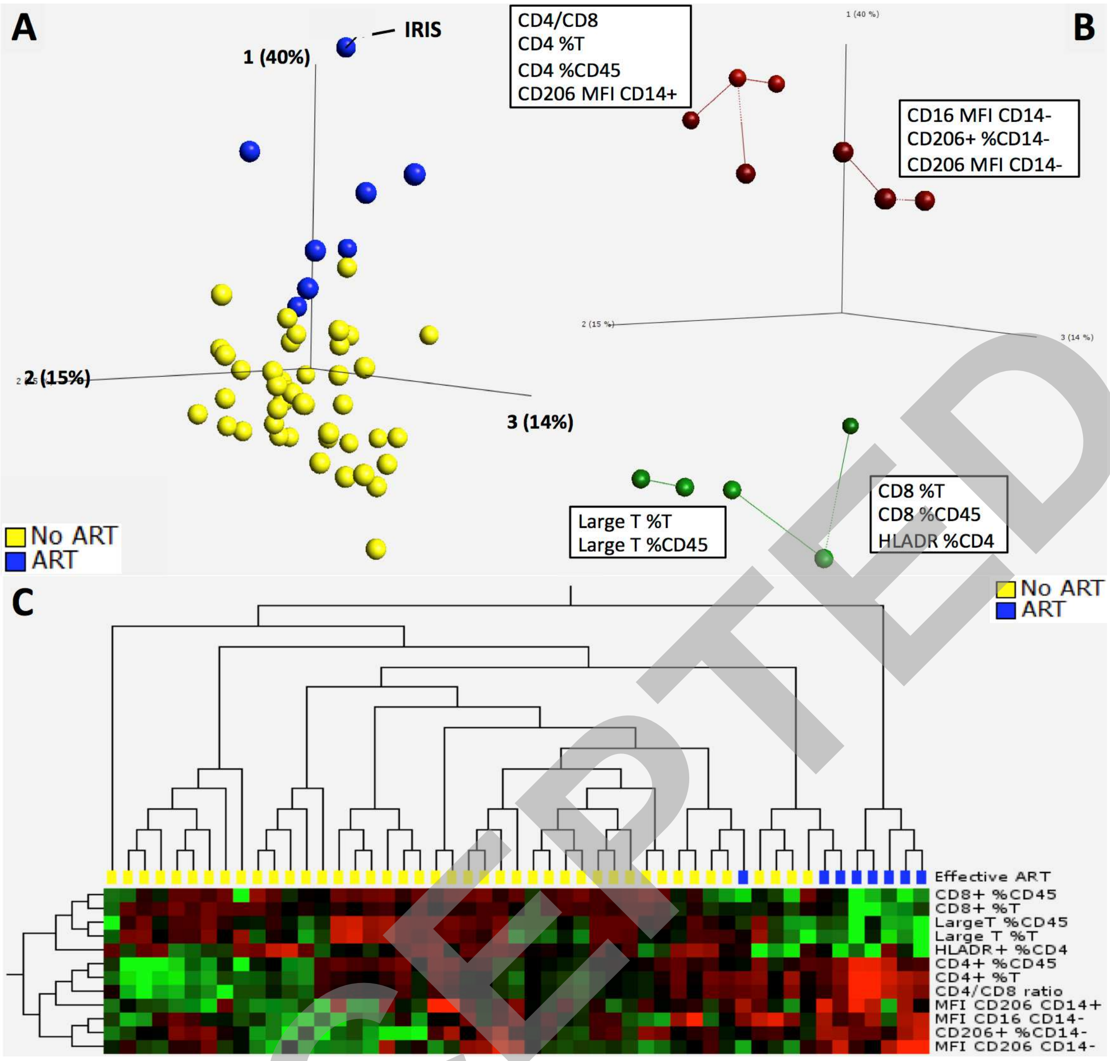
[b] Maximum CSF opening pressure during first 14 days of admission

Abbreviations: ART (anti-retroviral therapy); CSF (cerebrospinal fluid); CFU (colony forming units)


A1
SSC-A
FSC-A
*
A2
SSC-A
CD66b
1.43
98.3
B
CD4
CD3
31.3
68.5
T cells
C1
CD4
CD8
1.67
0.119
2.28
95.9
C2
SSC-A
FSC-A
2.42
*
C3
HLA-DR
CD4
27.6
C4
HLA-DR
CD8
15.2
C5
CD4
CD8
0.436
1.19
1.1
97.3
D
CD4
CD14
†
66.8
33.2
Monocytes/
macrophages
E1
SSC-A
FSC-A
E3
CD206
CD14
56.2
E5
HLA-DR
CD14
99.2
E2
CD68
CD14
E4
CD163
CD14
E6
CD16
CD14
16.6
F
HLA-DR
CD16
71.2
🟦 CD14-
🟥 CD14+


0.70
1.00
1.48
1.74
1.93
1.95
2.15
2.27
2.46
2.64
2.91
3.10
3.23
3.48
3.51
3.54
3.85
4.00
4.13
4.22
4.29
4.39
4.40
4.54
4.56
4.66
4.69
4.73
4.74
4.77
4.85
4.86
5.02
5.24
5.27
5.39
5.40
5.45
5.46
5.50
5.57
5.59
5.65
5.85
5.93
6.08
6.22
6.24
6.27
6.35
6.40
6.44
6.45
6.50
6.53
6.77
a
1 (59%)
1 (59 %)
2 (11%)
2 (11 %)
3 (8%)
3 (8 %)
37
b
1 (59 %)
2 (11 %)
3 (8 %)
CD45/ml
CD4/ml
CD8/ml
DNT/ml
NK/ml
WCC
Lymph
IL-5
IL-6
G-CSF
CD66 MFI
CD14- %CD45


a
1 (53%)
2 (16%)
3 (8%)
ICP <30
ICP >30
b
1 (53 %)
2 (16 %)
3 (8 %)
Crypto FSC
Crypto/CD4 FSC
CD14- %CD45
Crypto/ml
Crypto%total
LargeT %T
CD4/ml
DNT/ml
NK/ml
CD45%total
CSF WCC
CSF Lymph
c
ICP <30
ICP >30
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
ICP>30
Crypto/ml
crypto %total
FSC crypto/CD4
crypto FSC
CD14- %CD45
Large T %T
CD4-CD8-/ml
NK/ml
CD4+/ml
CD45+ %total
CSF_lymph
CSF WCC


A
1 (40%)
IRIS
2 (15%)
3 (14%)
No ART
ART
B
1 (40 %)
2 (15 %)
3 (14 %)
CD4/CD8
CD4 %T
CD4 %CD45
CD206 MFI CD14+
CD16 MFI CD14-
CD206+ %CD14-
CD206 MFI CD14-
Large T %T
Large T %CD45
CD8 %T
CD8 %CD45
HLADR %CD4
C
No ART
ART
Effective ART
CD8+ %CD45
CD8+ %T
LargeT %CD45
Large T %T
HLADR+ %CD4
CD4+ %CD45
CD4+ %T
CD4/CD8 ratio
MFI CD206 CD14+
MFI CD16 CD14-
CD206+ %CD14-
MFI CD206 CD14-