

1 Article

2 **HIV-associated Cryptococcal Immune Reconstitution Inflammatory Syndrome is**
3 **associated with Aberrant T cell function and Increased Cytokine Responses.**

4 **David B. Meya,^{1,2,3} Samuel Okurut,¹ Godfrey Zziwa,⁴ Stephen Cose,^{5,6} David R.**
5 **Boulware,² Edward N. Janoff ⁷**

6 ¹ Infectious Diseases Institute, Makerere University, Kampala, P.O. Box 22418, Uganda

7 ² Dept of Medicine, Center for Infectious Diseases and Microbiology Translational
8 Research, University of Minnesota, Minneapolis, MN 55455, USA; bohja001@umn.edu
9 (P.R.B.); boulw001@umn.edu (D.R.B.)

10 ³ School of Medicine, College of Health Sciences, Makerere University, Kampala, P.O. Box
11 7072, Uganda; david.meya@gmail.com (DBM)

12 ⁴ Research Department, Makerere University Walter Reed Project, Plot 42, Nakasero Road,
13 Kampala, P.O. Box 1624, Uganda; okurut@gmail.com (S.O.); gzziwa@muworp.org (G.Z.)

14 ⁵ London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK;
15 stephen.cose@mrcuganda.org

16 ⁶ MRC/UVRI and LSHTM Uganda Research Unit, Entebbe, Plot 51-59 Nakiwogo Road,
17 Uganda

18 ⁷ Mucosal and Vaccine Research Program Colorado (MAVRC), ⁹ University of Colorado
19 Denver, Aurora, CO 80045, USA; Edward.Janoff@ucdenver.edu (E.N.J);

20

21 * Correspondence should be addressed to: David Meya david.meya@gmail.com; Tel.
22 +256312207224, Infectious Diseases Institute, Makerere University, Kampala, P.O. Box
23 22418, Uganda.

24 Received: date; Accepted: date; Published: date

25 **Abstract:** Cryptococcal meningitis remains a significant opportunistic infection among
26 HIV-infected patients, contributing 15%-20% of HIV-related mortality. A complication of
27 initiating Antiretroviral therapy (ART) following opportunistic infection is Immune
28 Reconstitution Inflammatory Syndrome (IRIS). IRIS afflicts 10-30% of HIV-infected patients
29 with cryptococcal meningitis (CM), but its immunopathogenesis is poorly understood. We
30 compared circulating T cell memory subsets and cytokine responses among 17 HIV-infected
31 Ugandans with CM: 11 with and 6 without CM-IRIS. At meningitis diagnosis, stimulation
32 with cryptococcal capsule component, glucuronoxylomannan (GXM) elicited consistently

33 lower frequencies of CD4⁺ and CD8⁺ T cell memory subsets expressing intracellular
34 cytokines (IL-2, IFN- γ and IL-17) among subjects who subsequently developed CM-IRIS.
35 After ART initiation, T cells evolved to show a decreased CD8⁺ central memory phenotype.
36 At the onset of CM-IRIS, stimulation more frequently generated polyfunctional IL-2⁺/IL-17⁺
37 CD4⁺ T cells in patients with CM-IRIS. Moreover, CD8⁺ central and effector memory T cells
38 from CM-IRIS subjects also demonstrated more robust IL-2 responses to antigenic
39 stimulation vs. controls. Thus, ART during CM elicits distinct differences in T cell cytokine
40 production in response to cryptococcal antigens both prior to and during the development
41 of IRIS, suggesting an immunologic foundation for the development of this morbid
42 complication of CM infection.

43 **Keywords:** Cryptococcal meningitis; Cryptococcus; HIV; CD4 T cells, CD8 T cells; adaptive immune
44 response; IRIS,

45 1. Introduction

46 Cryptococcal meningitis causes 15-20% of AIDS-related mortality worldwide [1]. In sub-
47 Saharan African countries with high HIV prevalence (>5%), *Cryptococcus* is the most
48 common cause of meningitis in adults, accounting for 26% of cases in Malawi, 45% in
49 Zimbabwe, 30% in South Africa [2-5], and 60% in Uganda [3, 5-7]. In 2014, an estimated
50 162,500 cases of CM occurred in sub-Saharan Africa resulting in more than 90,000 deaths [1].
51 Despite improved immune function in antiretroviral therapy (ART)-treated HIV-infected
52 patients in low and middle-income countries, a significant proportion of patients with a new
53 HIV diagnosis still present with advanced disease (CD4⁺ T cells <200/ μ L) and are at risk for
54 opportunistic infections (OI) such as cryptococcal meningitis [8].

55 ART suppresses HIV replication and CD4⁺ T cell loss by apoptosis allowing immune
56 reconstitution to occur. However, in addition to its benefits, immune reconstitution with
57 ART can also be detrimental. A proportion of patients treated with ART experience a
58 constellation of symptoms and signs in which sub-clinical or pre-existing infections trigger
59 an exaggerated inflammatory response that leads to clinical deterioration, presenting as
60 Immune Reconstitution Inflammatory Syndrome (IRIS) [9]. IRIS can present as unmasking
61 or paradoxical phenomena. In unmasking IRIS, subclinical infections become overtly

62 symptomatic with a first episode of the OI after ART initiation. Conversely, in paradoxical
63 IRIS, there is usually evidence of initial microbiological and clinical response to treatment
64 of the OI pre-ART, which evolves into recrudescence of symptoms following ART without
65 microbiological evidence of the associated OI following ART initiation.

66 Depending on the site and activity, IRIS can present with a range of symptoms from
67 minor to severe inflammation resulting in organ failure, hospitalization, or death [10-13].
68 Whether the current shift to a 'Test & Treat' ART strategy with limited screening and
69 treatment of OIs prior to ART will increase the incidence of unmasking IRIS remains to be
70 seen. Reversal of CD4⁺ T cell lymphopenia and increased T cell activation have been
71 associated with the development of IRIS [14]. Studies *in vitro* have demonstrated that
72 attenuated *Cryptococcus*-specific IFN- γ responses prior to starting ART are associated with
73 cryptococcal meningitis-IRIS when patients who developed CM-IRIS were compared to
74 HIV-infected controls who did not develop IRIS [15]. However, the ontogeny of antigen-
75 specific T cell responses prior to and during cryptococcal IRIS are not well defined.

76 We have previously demonstrated that lower levels of inflammation, demonstrated
77 by decreased numbers of CSF leukocytes and levels of CSF protein, IFN- γ , IL-6, IL-8, and
78 TNF- α were predictors for developing cryptococcal IRIS [16]. It is imperative that the
79 mechanisms underlying cryptococcal IRIS are understood in order to prevent or optimize
80 interventions against the deleterious effects of cryptococcal IRIS. We hypothesized that prior
81 to ART initiation, T cell phenotype and function would distinguish patients who
82 subsequently did and did not later develop paradoxical cryptococcal IRIS. We therefore
83 investigated the quantitative and functional reconstitution of CD4⁺, CD8⁺ T cells,
84 characterizing the association of T cell responses with the development or absence of
85 cryptococcal IRIS in HIV-infected patients receiving ART after cryptococcal meningitis
86 treatment in order to understand the contribution of these components to the
87 immunopathogenesis of cryptococcal IRIS.

88 2. Materials and Methods

89 2.1 Study Subjects and Procedures

90 As previously described [17], we sequentially screened participants presenting with
91 suspected meningitis at Mulago National Referral Hospital in Kampala, Uganda. We
92 enrolled adults with a first episode of CM diagnosed by cerebrospinal fluid (CSF)
93 cryptococcal antigen or positive *Cryptococcus neoformans* culture [18]. Participants received
94 amphotericin B (0.7-1 mg/kg/day) for 2 weeks with oral fluconazole (800 mg/day) which
95 was continued for ~5 weeks, then later decreased to 400 mg/day for 8 weeks and 200
96 mg/day thereafter [19]. ART (zidovudine, lamivudine and efavirenz) was started within 6
97 weeks of CM diagnosis [20].

98 We collected blood from subjects longitudinally with isolation of peripheral blood
99 mononuclear cells (PBMCs) by density centrifugation gradient (Ficoll 1077, Sigma) followed
100 by cryopreservation in RPMI-1640 with fetal bovine serum (20%), dimethyl sulphoxide
101 (10%), and penicillin/streptomycin, 1%) in liquid nitrogen.

102 A diagnosis of definite/probable/possible CM-IRIS was made according to the
103 published consensus case definition [9], with external adjudication by a three-physician
104 panel whose members were not part of the clinical team. Grading was classified as definite,
105 probable, or possible IRIS based on the available clinical and CSF information. Institutional
106 review board approvals were obtained from the School of Medicine Ethics review
107 committee at Makerere University (REF 2009-022) and the University of Minnesota
108 (0810M49622), and written informed consent was obtained.

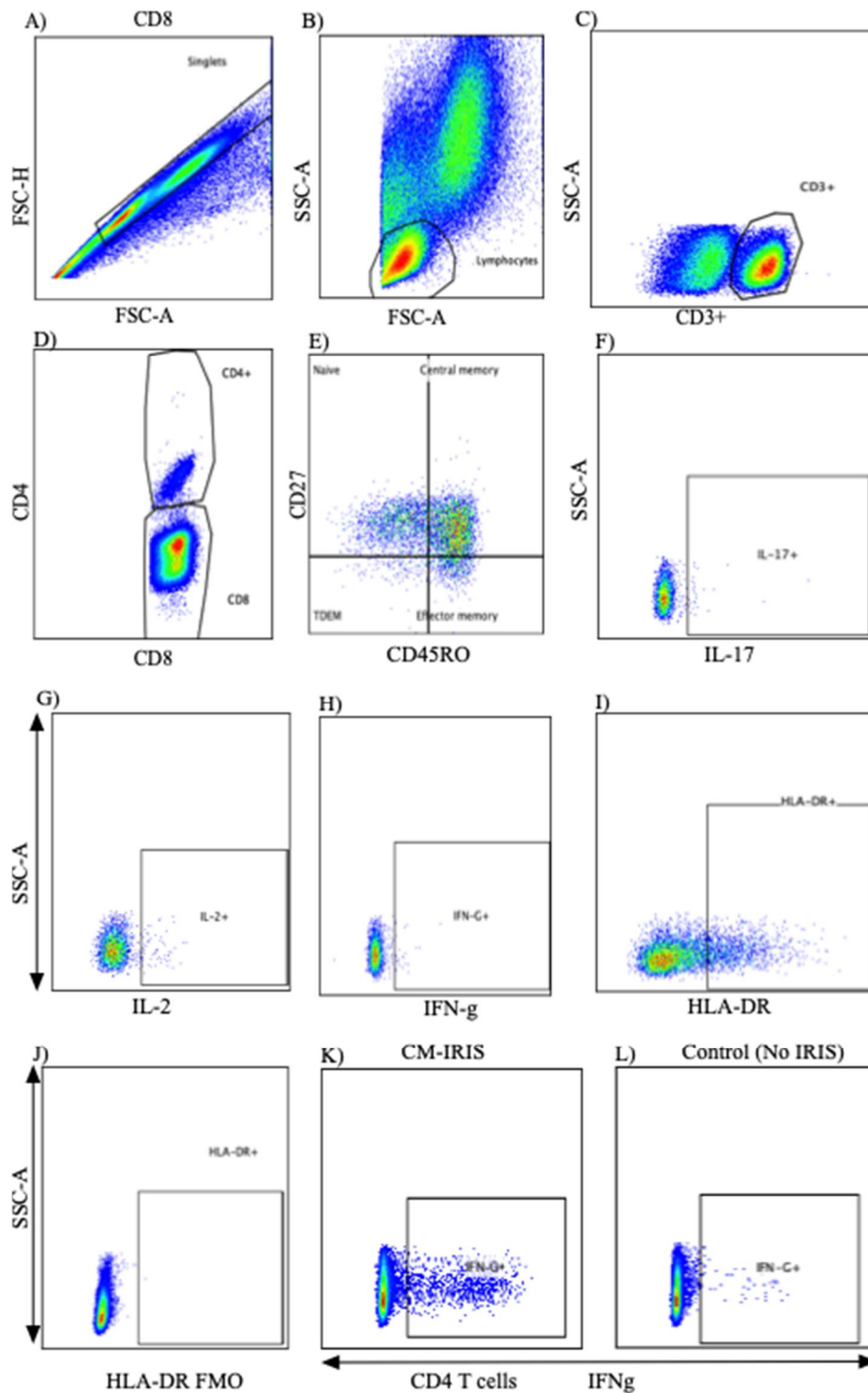
109

110 2.2 PBMC Stimulation and Surface Flow Cytometric Staining

111 Cryopreserved PBMCs at initial CM diagnosis, during CM-IRIS and from control
112 subjects without CM-IRIS matched for ART duration were thawed and stimulated *in vitro*.
113 PBMCs were rapidly thawed and diluted in complete media (RPMI-1640 with 10% FBS, 2%
114 HEPES, 2% L-Glutamine, 1% Pen/Strep) and 1×10^6 cells were added to each of three wells.
115 Glucuronoxylomannan (GXM) was added to the test well with co-stimulatory anti-CD28
116 and anti-49d monoclonal antibodies to enhance detection of cytokine-secreting T cells.
117 Staphylococcal enterotoxin B (SEB; 10ng/mL) (Invivogen, France), a non-specific polyclonal
118 activator, was used as the positive control and phosphate buffered saline (PBS) (100 μ l/well)

119 (Sigma-Aldrich, USA) as the negative control. After 2 hours of incubation in 5% carbon
120 dioxide at 37°C, Brefeldin A (100µL/mL) (BD, Golgi Stop, catalogue # 554724) was added
121 to each well and cells were incubated for another 4 hours then refrigerated in the dark
122 overnight at 4°C.

123 We assessed *in vitro* cytokine responses to cryptococcal GXM in circulating T cells at CM
124 diagnosis, at the time of CM-IRIS, or a matched ART time point for CM controls without
125 IRIS. We stained cells with commercial monoclonal antibodies reactive with
126 CD45RO^{PerCPy5.5} (clone UCHL1, BD Biosciences), CD27^{APC-H7} (clone MT271, BD Biosciences),
127 CD3^{V500} (clone UCHT1, BD Biosciences) and CD4^{V450} (clone RPA-T4, BD Horizon).
128 CD3⁺CD4⁻ cells were considered as CD8⁺ T cells. We assessed T cell activation using the
129 proportion of T cells expressing HLA-DR^{PECy7} (clone LN3, BD Biosciences). We prepared
130 fluorescence minus one (FMO) controls on blood samples to set gates for CD27 and
131 CD45RO. Following selection of singlet cells based on FSC height vs. FSC area, differential
132 gating of lymphocytes was based on size and granularity (Figure 1). T cell phenotype,
133 activation state, and percentage of T cell subsets were determined by 8-color flow cytometry
134 using a FACSCanto II (BD Biosciences).

135 **Figure 1. Gating Strategy for Flow cytometry analysis**

136

137 **Figure 1. Flow Cytometry Gating Strategy for T cells.** Multiparameter flow cytometry was used to
 138 identify the frequency, phenotype and post-stimulation cytokine expression of CD4⁺ and CD8⁺ T cells
 139 within total peripheral blood mononuclear cells of patients with and without CM-IRIS at baseline
 140 and at CM-IRIS event. Representative staining shows the analytic gating strategy : A) FSC-H/FSC-A
 141 showing the singlet gate; B) FSC/SSC for lymphocytes selected from singlet gate C) T cells expressing

142 CD3 were selected; **D**) CD3⁺ cells expressing CD4 and CD8 were then identified; **E**) gating by
143 differential expression of CD27 and CD45RO identified naïve and memory T cells subsets with naïve
144 T cells as (CD27⁺CD45RO⁻); central memory as (CD27⁺CD45RO⁺), Effector memory as (CD27⁻
145 CD45RO⁺), and Terminally differentiated effector memory as (CD27⁻CD45RO⁻); **F**) CD4⁺ expression
146 of IL-17 (unstimulated); **G**) representative example of IL-2 expression by CD4⁺ T cells (unstimulated);
147 **H**) representative example of IFN- γ expression by CD4⁺ T cells (unstimulated); **I**) representative
148 example of HLA-DR expression by CD4⁺ T cells and; **J**) HLA-DR Fluorescence minus one gating; **K**)
149 representative example of IFN- γ expression by CD4⁺ T cells in a subject with CM-IRIS and; **L**) IFN- γ
150 expression by CD4⁺ T cells in a control subject without CM IRIS.

151

152

153 *2.3 Intracellular Cytokine Staining*

154 We determined T cell cytokine responses by intracellular cytokine staining. Following 6
155 hours of stimulation at 37°C and overnight incubation at 4°C, cells were washed, fixed and
156 permeabilized with successive washes in FACS Permeabilizing Solution (BD Biosciences)
157 and stained with intracellular monoclonal antibodies reactive with IFN- γ ^{PE} (clone 4S.B3,
158 Biolegend), and IL-17^{Alexa647} (clone SPCL 1362, BD Biosciences) and IL-2^{FITC} (clone 5433.111,
159 BD Biosciences). One million events were acquired the following day using an 8-color FACS
160 Canto II (BD Biosciences), and data were analyzed using FlowJo version 10.0.5 (TreeStar,
161 USA).

162

163 *2.4 Statistical Analysis*

164 Data were analyzed using GraphPad Prism, version 6.0b (GraphPad software Inc.,
165 California) and Spice, version 5.35 (NIAID, NIH, Bethesda, MD). We compared paired
166 samples at meningitis diagnosis and at CM-IRIS event using the non-parametric Wilcoxon
167 signed-rank test. We compared cell phenotype and activation variables between CM-IRIS
168 and time-matched controls using the non-parametric Mann-Whitney rank sum test.
169 Statistical significance was defined as a P-value ≤ 0.05 . We compared cytokine expression
170 profiles by permutation analysis to determine combinations of cytokine expression

171 following background subtraction, using a 10,000-iteration Monte Carlo simulation model
172 described in detail elsewhere [21].

173 3. Results

174 Among 11 HIV-1-infected adults who developed CM-IRIS cases, cryopreserved PBMCs
175 were available from 10 at CM diagnosis and 11 at time of CM-IRIS event. Among 6 HIV-
176 1-infected control subjects with CM but no IRIS, PBMC were available from 5 at the time of
177 CM diagnosis and all at a subsequent visit time-matched to the timing of CM-IRIS in the
178 other group (67 vs. 78 days).

179 Age, baseline CD4 and CD8 T cell counts, cryptococcal antigen (CrAg) titer, HIV
180 viral load, CSF protein and white blood cells were similar among subjects with CM-IRIS vs
181 controls (**Table 1**). At meningitis diagnosis, subjects who subsequently developed CM-IRIS
182 showed a trend to higher quantitative CSF *Cryptococcus* colony forming units (CFU) on
183 culture, median 213,796 (IQR: 91,201- 288,403) CFU/mL compared to control subjects who
184 did not develop IRIS median 9,332 (IQR: 281 -181,970) CFU/mL.

185

186 **Table 1. Characteristics of Subjects Who Developed CM-IRIS vs Subjects without CM-**
187 **IRIS**

	Controls (n=6)	CM-IRIS (n=11)	
Men, N (%)	1 (17%)	8 (73%)	0.05
Age, years	35 (28, 40)	35 (29, 42)	0.937
CD4 ⁺ T cells/ μ L - Diagnosis	8 (5, 166)	6.5 (4, 28)	0.828
- >3 mon	156 (55, 309)	68 (33, 79)	0.256
ART			
CD8 ⁺ T cells/ μ L - Diagnosis	163 (97, 784)	256 (140, 591)	0.515
- >3 mon	1005 (615, 1086)	831 (565, 997)	0.463
ART			
Plasma HIV RNA (log ₁₀ copies/mL)	5.1 (4.6, 5.2)	5.3 (4.8, 5.6)	0.260

CSF Cryptococcus (\log_{10} CFU/mL)	3.97 (2.45, 5.26)	5.33 (4.96, 5.46)	0.078
CSF CRAG titer, 1:x	4512 (528, 12192)	7200 (4048, 16384)	0.455
CSF protein (mg/dL)	60 (47, 68)	53 (20, 70)	0.471
CSF WBC/ μ L	20 (<5, 45)	<5 (<5, <5)	0.169
Duration from ART initiation (days)	67 (48, 92)	78 (43, 202)	0.737

188 Values listed as median (IQR) or mean (\pm SD). Values are at time of cryptococcal meningitis diagnosis
 189 unless otherwise stated. Abbreviations: ART- Antiretroviral Therapy; CRAG- Cryptococcal Antigen;
 190 CSF- Cerebrospinal Fluid; CFU - colony forming units; HIV- Human immunodeficiency virus.

191

192 3.1 T cell Phenotype and activation at CM diagnosis vs Controls

193 At CM diagnosis, circulating CD4⁺ T cell numbers were similarly very low in both
 194 groups. The frequencies of total CD8⁺ T cells were comparable. Central memory CD4⁺ T cells
 195 (CD27⁺CD45RO⁺) and naive CD8⁺ T cells (CD27⁺CD45RO⁻) were the predominant T cell
 196 subsets (Figure 2) without significant differences in T cell subsets between groups. Similarly,
 197 baseline activation of CD4⁺ T cells expressing HLA-DR was very high at baseline but
 198 comparable among subjects with future CM-IRIS vs controls, 81% (IQR: 66, 90) vs 72% (IQR:
 199 46, 80), (P= 0.196), respectively. Further, CD8 T cell activation, also high, did not differ, with
 200 frequencies of 91% IQR: 85, 95) vs 94% (IQR: 59, 98), (p= 0.853) in the two groups,
 201 respectively.

202

203

204

205

206

207

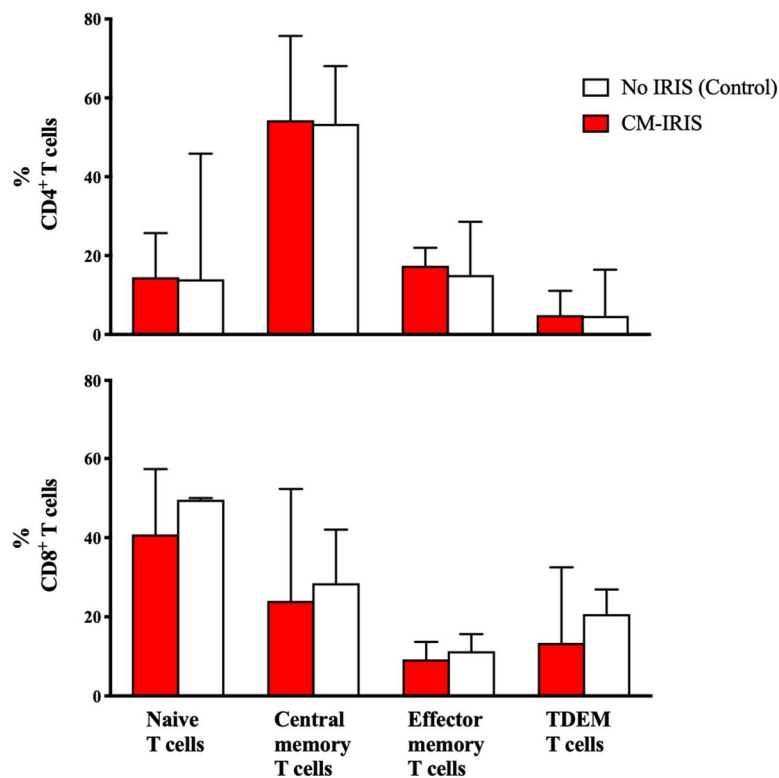
208

209

210

211 **Figure 2.** Baseline Frequency of T cells and Memory Phenotype at Cryptococcal Meningitis
 212 Diagnosis among subjects who later developed CM-IRIS vs CM-Controls without future
 213 IRIS.

214



215

216 **Figure 2.** Frequencies of CD4⁺ and CD8⁺ memory T cell subsets at time of initial cryptococcal
 217 meningitis diagnosis among ART-naïve subjects who later developed CM-IRIS vs controls without
 218 IRIS. CD4⁺ Central memory and CD8⁺ naïve T cells predominated without significant differences
 219 between groups. Bars represent median values and error bars show interquartile ranges.
 220 Abbreviations: TDEM- terminally differentiated effector memory. White bars represent CM
 221 diagnosis, red bars represent CM-IRIS.

222

223 3.2 T cell cytokine responses at baseline

224 At baseline, the frequencies of CD4⁺ and CD8⁺ memory subset T cells expressing
 225 intracellular cytokines (IL-2, IFN- γ and IL-17) after GXM stimulation were consistently
 226 lower among subjects who later developed CM-IRIS (Table 2) for each subset and each
 227 intracellular cytokine. Unstimulated and SEB stimulation did not demonstrate significant
 228 differences in responses.

229

230

231 Table 2. Cytokine responses by T cell phenotype among subjects with CM-IRIS vs

232 Controls following GXM stimulation at CM diagnosis.

	<i>Controls</i> (n=5)	<i>CM-IRIS</i> (n=10)	<i>P-value</i>
<u>CD4⁺ T cells</u>			
Central memory (T_{CM})			
IFN- γ ⁺	6 (3, 11)	0.8 (0, 3)	0.005
IL-2 ⁺	5 (2, 22)	1 (0.1, 3)	0.012
IL-17 ⁺	2 (1, 6)	0.5 (0, 2)	0.054
Effector memory (T_{EM})			
IFN- γ ⁺	8 (4, 16)	0.5 (0, 3)	0.027
IL-2 ⁺	3 (2, 13)	0 (0, 0.1)	0.004
IL-17 ⁺	2 (1.9, 3)	0	<0.001
Terminally differentiated effector memory (T_{TDEM})			
IFN- γ ⁺	3 (1, 16)	0 (0, 0.4)	0.005
IL-2 ⁺	74 (12, 86)	0	<0.001
IL-17 ⁺	0.1 (0, 1.1)	0 (0, 2)	0.624
<u>CD8⁺ T cells</u>			
Central memory (T_{CM})			
IFN- γ ⁺	2.4 (2.2, 3.1)	0.4 (0.1, 0.5)	<0.001
IL-2 ⁺	1.1 (0.5, 2.1)	0.07 (0.03, 0.3)	0.005
IL-17 ⁺	1.2 (0.9, 1.4)	0.08 (0.02, 0.2)	0.003
Effector memory (T_{EM})			
IFN- γ ⁺	6.2 (3.1, 9.9)	0.7 (0.2, 1.5)	0.005
IL-2 ⁺	1.2 (0.5, 1.7)	0.01 (0, 0.1)	<0.001
IL-17 ⁺	1.2 (1.0, 1.3)	0.06 (0, 0.2)	<0.001
Terminally differentiated effector memory (T_{TDEM})			
IFN- γ ⁺	1.3 (0.6, 3.5)	0.3 (0.2, 0.9)	0.037

IL-2 ⁺	1.1 (0.3, 1.2)	0.03 (0, 0.6)	0.068
IL-17 ⁺	0.09 (0.08, 0.25)	0.01 (0, 0.17)	0.119

233 P-values obtained by Mann Whitney U test. Data are presented as median (Interquartile Range)

234

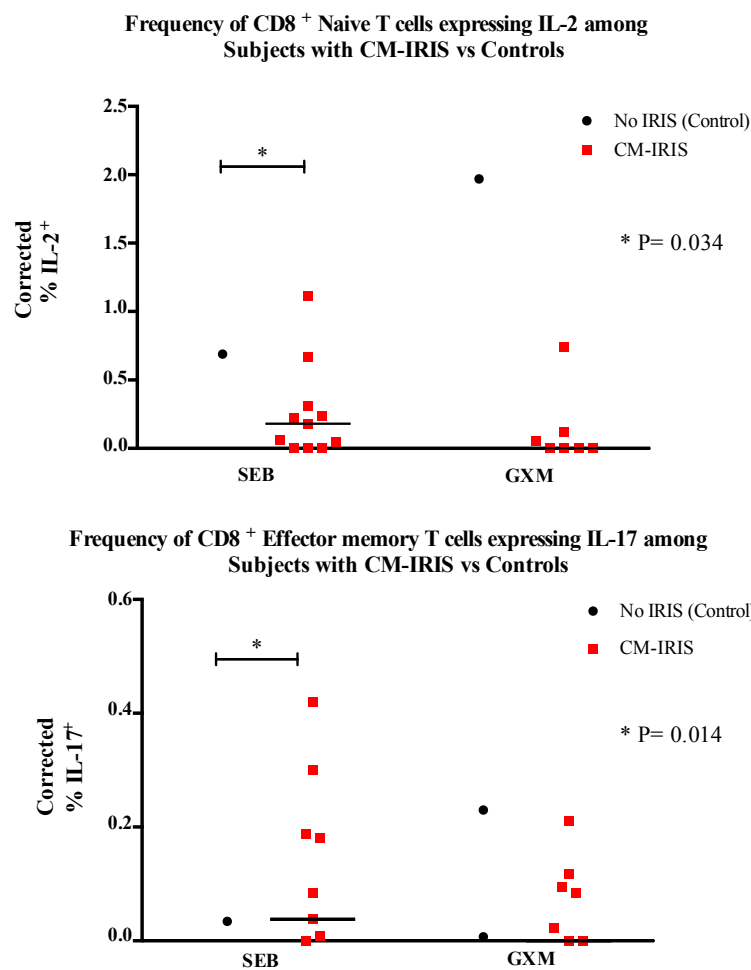
235

236 3.3 Cytokine responses at CM-IRIS vs Controls

237 Upon correcting for the cytokine expression in unstimulated samples, we found that
 238 mitogen-induced IL-2 responses by total CD8⁺ (P =0.034) and naïve CD8⁺ T cells (P=0.020)
 239 were significantly elevated among patients with CM-IRIS compared with controls, (Figure
 240 3) while there was a trend for higher CD8⁺ TDEM T cell IL-2 responses among subjects with
 241 CM-IRIS compared with controls. These results suggest that T cells are primed to increase
 242 IL-2 expression during CM-IRIS. This could result either in expanding the number and
 243 function of GXM-specific T cell clones or could be an effect of IL-2 inhibiting T cell
 244 proliferation during CM-IRIS.

245

246 Figure 3. Frequency of IL-2 and IL-17-producing CD8⁺ cells⁺ at CM-IRIS vs matched
247 Controls.



248

249 **Figure 3.** Mitogen-induced IL-2 responses by naïve CD8⁺ T cells and mitogen-induced IL-17
250 responses by effector memory CD8 T cells were elevated among participants with CM IRIS compared
251 to controls without CM-IRIS. Responses were calculated by subtracting the value for unstimulated
252 samples from the value for mitogen- or GXM-stimulated samples. Negative corrected values were
253 reported as zero. The horizontal line represents median. Differences were determined using
254 Wilcoxon rank sum test for paired samples. P-value from Mann Whitney U test. Abbreviations: IL-
255 Interleukin, SEB- Staphylococcal Enterotoxin B, GXM- Glucuronoxylomannan.

256

257 **3.4 Phenotype and Cytokine responses among subjects with Paired Samples at CM diagnosis vs CM-**
258 **IRIS**

259 We compared phenotype and cytokine expression at CM diagnosis and during CM-IRIS
260 from 10 subjects with paired samples. CD4⁺ T cell frequency was significantly higher at CM-

261 IRIS, 8% (IQR, 4 - 13%) compared to CM diagnosis, 3% (IQR, 1 - 3%) (P= 0.014). At CM-IRIS,
262 the frequency of CD4⁺ T cells expressing HLA-DR was significantly decreased compared to
263 CM diagnosis, 66% (IQR, 60%-79%) vs 81% (IQR, 66%-90%), (P = 0.014) respectively. CD4⁺
264 T cells with a central memory phenotype expressing IL-17 were less frequent at CM-IRIS
265 compared with CM diagnosis following GXM stimulation (Supplementary Figure 1). In
266 contrast, without GXM stimulation, CD4⁺ T cells with effector phenotype expressing IL-2
267 were more frequent at CM-IRIS compared to CM diagnosis, 0.92% (IQR, 0%-2.4%) vs 0%, (P
268 = 0.016) respectively (Figure 3). No differences were observed in the frequency of CD4⁺ T_{CM}
269 cells expressing IFN- γ on stimulation with GXM.

270

271 *3.5 CD4⁺ and CD8⁺ T cell Polyfunctional Cytokine Responses at CM-IRIS*

272 Upon stimulation with GXM, subjects at the time of IRIS CM-IRIS more frequently
273 expressed dual-functional IL-2⁺IL-17⁺IFN- γ ⁻ CD4⁺T cells vs Controls (0.34% vs 0.02%; p=
274 0.010) (Figure 4). When we compared CD8⁺ T cell responses at CM-IRIS vs controls, mono-
275 functional IL-2⁺IL-17⁻IFN- γ ⁻ CD8⁺ T cells were more frequent at CM-IRIS, 0.6% (IQR: 0.2, 1.0)
276 compared to controls, 0.05% (IQR: 0.0, 0.1), P= 0.01 (Supplementary Figure 2). These data
277 confirm the increased immune CD4⁺ T cell responses with co-expression of IL-2/IL-17 and
278 CD8⁺ T cells expressing IL-2 during CM-IRIS thereby contributing to the
279 immunopathogenesis of this syndrome.

280

281

282

283

284

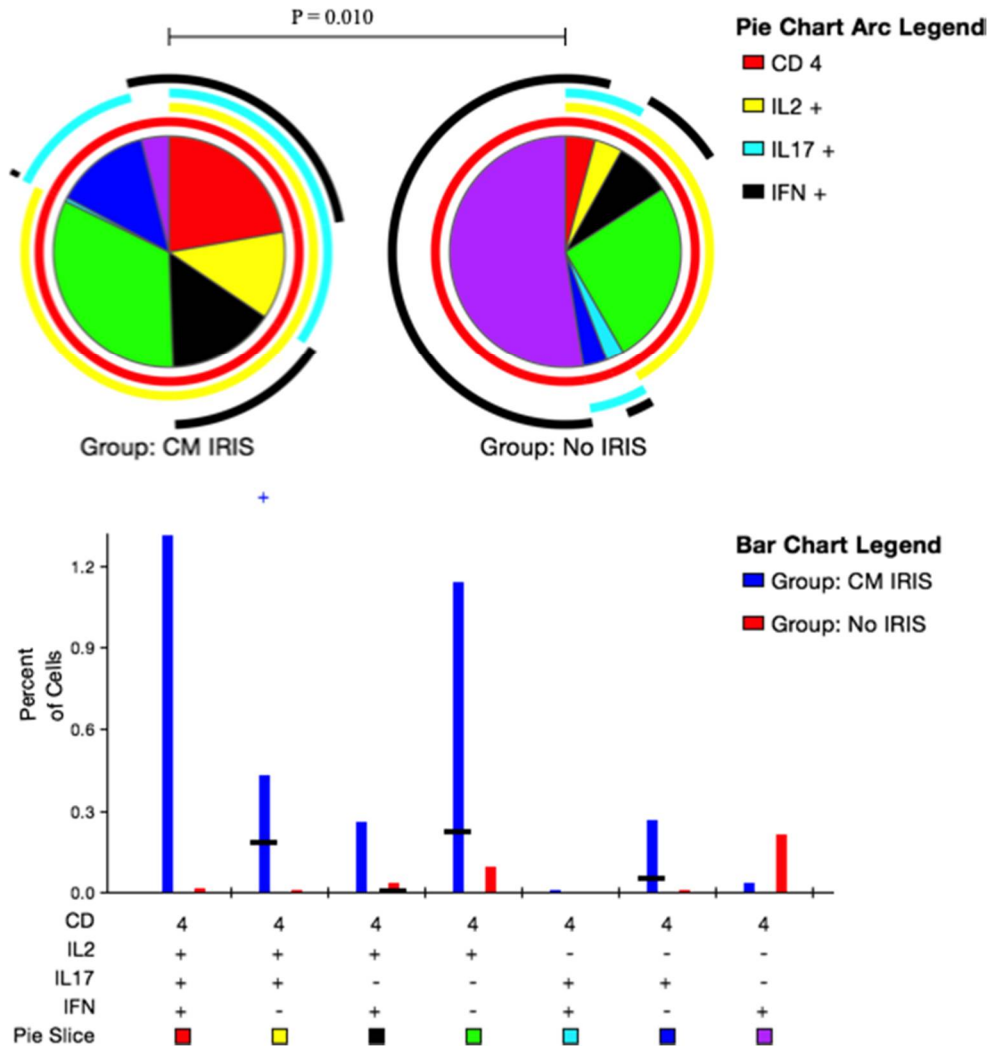
285

286

287

288 **Figure 4.** CD4⁺ T cell Cytokine responses at CM-IRIS vs Controls in response to GXM
 289 Stimulation

290



291

292 **Figure 4.** Peripheral blood mononuclear cells from subjects with cryptococcal meningitis were
 293 stimulated with Glucuronoxylomannan (GXM). Intracellular Interleukin-2 (IL-2), IL-17 and
 294 Interferon- γ (IFN- γ) expression by CD4⁺ T cells was quantified using flow cytometry. The bar chart
 295 shows each of the 3 possible combination responses on the x-axis. The percentage of the total cytokine
 296 response is shown on the y-axis, with the filled bar representing the interquartile range and a black
 297 line at the median. Statistically significant differences ($P < .05$) by rank-sum testing are indicated by
 298 the plus sign. Responses from ART matched control subjects who did not develop CM-IRIS are shown
 299 in blue, responses from subjects with CM-IRIS are in red on the bar graph. The pie charts show the
 300 fractions according to the pie-slice colors shown at the bottom of the bar chart, with color-coded arcs
 301 indicating the contributions of IL-2 (yellow), IFN- γ (black) and IL-17 (cyan) to the 3-, 2- and 1-function

302 responses. Statistical comparisons of the overall responses by permutation testing are shown in the
303 pie category test result chart where the red represents IL-2⁺IL-17⁺IFN- γ ⁺ CD4⁺ T cells; yellow
304 represents IL-2⁺IL-17⁺IFN- γ ⁻ CD4⁺T cells; black represents IL-2⁺IL-17⁻IFN- γ ⁺ CD4⁺ T cells; green
305 represents IL-2⁺IL-17⁻IFN- γ ⁻ CD4⁺ T cells; cyan represents IL-2⁻IL-17⁺IFN- γ ⁺ CD4⁺ T cells; blue
306 represents IL-2⁻IL-17⁺IFN- γ ⁻ CD4⁺ T cells and purple represents IL-2⁻IL-17⁻IFN- γ ⁺ CD4⁺ T cells.
307 Patients with CM-IRIS had a significantly elevated proportion of duo functional IL-2⁺IL-17⁺IFN- γ ⁻
308 CD4⁺T cells compared with matched Controls following GXM stimulation.

309 In summary, patients who developed CM-IRIS exhibited aberrant CD4⁺ T cell
310 responses during the primary CM episode as demonstrated by their poor mitogenic and
311 GXM-specific IL-2 responses. Notably, patients who developed CM-IRIS had diminished
312 IFN- γ ⁺ CD4⁺ T cells responsive to GXM at baseline and this response was markedly different
313 at CM-IRIS. CM-IRIS was associated with robust dual-functional CD4⁺IL-2⁺IL-17⁺, CD8⁺IL-
314 2⁺ and CD8⁺IL-17⁺ T cell responses. The selective expansion of these GXM-specific T cells is
315 consistent with other studies of T cell responses during IRIS [14] which confirms
316 exaggerated Th1 and Th17 responses during IRIS.

317 4. Discussion

318 Cryptococcal IRIS remains a clinical challenge in populations where advanced HIV
319 disease persists and yet the precise immunopathogenic mechanisms remain unclear. The
320 phenotype of CD4⁺ T cell effector memory responses to *Cryptococcus* is associated with
321 disease severity and outcome in HIV-associated cryptococcal meningitis [22]. We found that
322 at baseline, those who subsequently developed CM-IRIS showed a characteristically lower
323 immune response with decreased CD4⁺IFN- γ ⁺ T cells and poor CD8⁺IL-17⁺ mitogenic
324 responses at CM-IRIS. Indeed, during CM-IRIS, this pattern shifted to exhibit robust
325 CD8⁺IL-2⁺ and CD4⁺IL-2⁺IL-17⁺IFN- γ ⁻ T cell responses to GXM, which also distinguished
326 them from the non-IRIS patients. We found no association between T cell memory
327 phenotype and the incidence of CM-IRIS.

328 Antigen-specific immune responses during CM-IRIS have only been rarely studied.
329 Most studies have looked at the expression of cytokines in blood or CSF but not the source

330 of these cytokines. An instructive feature of this study was the detection of the cytokines
331 expressed by T cells at the single cell level before and during cryptococcal IRIS.

332 To understand why some patients presenting with cryptococcal meningitis
333 subsequently develop CM-IRIS after initiating ART, we evaluated the immune response to
334 cryptococcal antigen. The high-molecular-mass capsular polysaccharide,
335 glucuronoxylomannan, is found in high titers in patients with disseminated cryptococcosis
336 [23, 24]. The host response to GXM involves a granulomatous inflammatory response, intact
337 cell-mediated immunity and a Th1 pattern of cytokine release. GXM has anti-phagocytic
338 properties and inhibits leukocyte migration and proliferation [25, 26] and could explain the
339 significantly diminished baseline immune response among patients who subsequently
340 developed CM-IRIS. This diminished immune response could predispose to subsequent
341 IRIS events resulting from failure to completely clear the antigen burden, which sets the
342 stage for a possible over exaggerated response as hypothesized by Barber and colleagues
343 when CD4 T cell recovery delivers the missing IFN- γ stimulus to partially activated
344 macrophages that subsequently become fully activated *en masse* with a resulting 'cytokine
345 storm' [27].

346 Consistent with this suppressive activity by GXM, participants who developed CM-
347 IRIS had a trend to higher fungal burden at CM diagnosis when compared to control
348 subjects, suggesting that the presence of persistent antigen when immune reconstitution is
349 initiated may underlie, in part, the development of IRIS. This finding is consistent with data
350 showing that patients with disseminated cryptococcal fungaemia have a six fold higher risk
351 of subsequently developing IRIS due to the high fungal burden and poor clearance.
352 Similarly, patients with a cryptococcal antigen titer >1:1024 are reported to show an
353 increased risk of IRIS [28]. These findings are also consistent with data showing that in the
354 CSF of patients infected with *C. neoformans*, the pro-inflammatory cytokines IL-6, TNF- α and
355 IFN- γ were inversely correlated with cryptococcal fungal burden [29].

356 Murine models suggest that late cryptococcal clearance is impaired in the absence of
357 IL-17 and in a cohort of HIV-infected Ugandans, IL-17 was significantly lower in those who
358 developed CM-IRIS compared with controls prior to ART initiation [30, 31]. It is therefore

359 plausible that more robust immune responses during the primary cryptococcal infection
360 induced cryptococcal clearance and mitigated the risk of CM-IRIS among controls who had
361 no IRIS.

362 It is also possible that patients who developed CM-IRIS had aberrant or dysregulated
363 CD4⁺ T cell function during the primary cryptococcal infection resulting in persistent
364 cryptococcal antigen, known to be a risk factor for CM-IRIS [28, 32]. These findings are
365 consistent with a study suggesting that robust CD4⁺ T cell responses during IRIS represent
366 a dysregulated response against residual antigen [14]. This aberrant function appears to
367 result in poor immune responses during the primary cryptococcal infection and
368 subsequently in dysregulated robust T cell responses characteristic of IRIS. This T cell
369 dysfunction could also explain the poor inflammatory response observed in the CSF of
370 patients who developed CM-IRIS in a Ugandan cohort [16].

371 Interferon- γ plays an important role in the host defense against intracellular
372 pathogens including *Cryptococcus neoformans* at the site of infection and has been studied as
373 adjunctive therapy against cryptococcal meningitis [23, 33]. At CM diagnosis, we found
374 decreased frequencies of CD4⁺ IFN- γ ⁺ T cells in patients who developed CM-IRIS compared
375 with controls similar to the lower IFN- γ responses induced by cryptococcal mannoprotein
376 in a cohort of patients with CM in Durban [15]. Of note, the cytokine responses in the Durban
377 cohort were measured in whole blood cultures and not by intracellular flow cytometry at
378 single cell level.

379 Differential gene expression between patients who develop CM-IRIS and those who
380 do not has been demonstrated previously in a Ugandan cohort of HIV-infected patients. The
381 most common molecular and cellular functions of the up-regulated genes were cell
382 proliferation, cell apoptosis, and immune response (antigen presentation, innate responses,
383 and inflammatory responses) [34]. More recent data from a Durban cohort suggests that
384 CM-IRIS occurring within 12 weeks of ART initiation was predicted by the low expression
385 of interferon-inducible genes, whereas late CM-IRIS events, occurring after 12 weeks of ART
386 were characterized by abnormal upregulation of transcripts expressed in T, B, and natural
387 killer cells such as IFNG, IL27 and LRB1 [35]. In addition to immunoactive cytokines, Yoon

388 *et al* have also demonstrated significantly low plasma levels of IgM, Lam-binding IgM, Lam-
389 binding IgG, and GXM-IgG among patients who developed CM-IRIS [36]. Together, these
390 data suggest the involvement of genetic, innate, and adaptive (T and B cell) mechanisms in
391 the development of CM-IRIS.

392 Interleukin-2 has a dual role in the regulation of the immune system. On one hand, IL-
393 2 is involved in the activation of the immune system by promoting the proliferation of
394 lymphocytes, macrophages, and natural killer cells, as well as aiding in the differentiation
395 of CD4 T cells [37]. On the other hand, IL-2 works to regulate the immune system through
396 regulatory T cells, thus inhibiting T cell proliferation. In the current study, GXM-induced
397 CD4⁺ T cells expressing IL-2 were significantly elevated among participants at the time of
398 CM-IRIS compared with Controls. This immune response appeared to be driven by the
399 naïve and terminally differentiated memory CD4⁺ T cells.

400 Similarly, we found significantly elevated CD4⁺ effector memory T cells expressing IL-
401 17 among those with CM-IRIS compared with Controls matched for ART duration. The
402 differential expression of these cytokines in response to GXM suggests an exaggerated
403 GXM-specific response among patients with CM-IRIS. This response could induce other
404 pro-inflammatory cytokines that contribute to the immunopathology occurring at CM-IRIS.
405 Indeed, a distinctive feature of CM-IRIS was the quality of the immune response at CM-IRIS
406 compared to Controls where we found significant elevation of dual-function CD4⁺IL-2⁺IL-
407 17⁺ and mono functional CD8⁺ IL-2⁺ T cells compared with controls without IRIS. This
408 finding is consistent with data showing elevated IL-2 and IL-17 among subjects with CM-
409 IRIS compared to time matched controls in a cohort of HIV-infected Ugandans [31]. IL-2 and
410 IL-17 have been implicated in promoting protective immune responses against *Cryptococcus*
411 [30, 38] in murine models. Thus, the differential expression of GXM-induced IL-2 and IL-17
412 among participants with CM-IRIS suggests that these two cytokines are involved in the
413 immunopathogenesis of CM-IRIS. During CM-IRIS, IL-2 could play the role of inducing
414 immune activation and driving the inflammatory process that is measurable in CSF. The
415 Th17 differentiation factor, IL-6 is associated with IRIS [39-41] and inhibits T regulatory cell
416 proliferation in favor of Th17 cell induction in the presence of TGF- β [42, 43]. IL-17 is known

417 to induce tissue inflammation and could also induce the inflammation observed during CM-
418 IRIS [42].

419 The small sample size and low T cell counts preclude firm conclusions on differences
420 between T cell phenotypes, markers of T cell activation and some of the cytokine responses
421 among subjects with and without CM-IRIS. The ideal compartment to study would have
422 been the CSF, however, due to the limited number of cells available in CSF and the challenge
423 of obtaining CSF from ART-time matched controls, the *in vitro* stimulation studies were
424 performed with peripheral blood and are only reflective of the immune response in the
425 periphery rather than the local immune response in the central nervous system, where CM-
426 IRIS symptoms occur.

427 We only evaluated T cell responses against GXM. Approximately 90% of the
428 cryptococcal cell wall is comprised of GXM [44]. Thus, the T cell responses in this study may
429 not be reflective of what occurs *in vivo* where responses against other cryptococcal capsular
430 antigens including galactoxylomannan and mannoproteins may be dissimilar. It is also
431 possible that the cellular and cytokine responses we observed with CM-IRIS may represent
432 a more natural or healthy response to this pathogen as the subjects' immune function was
433 restored following ART. We did not evaluate T regulatory cells, which would have given a
434 broader picture of the adaptive immune response and the balance between pro-
435 inflammatory and anti-inflammatory pathways, which are now thought to contribute to
436 fungal IRIS [45]. Larger prospective studies are needed to concurrently examine T cell
437 responses against various cryptococcal antigens in both CSF and peripheral blood prior to
438 ART initiation, after patients initiate ART and at CM-IRIS events.

439 In conclusion, cryptococcal IRIS after ART initiation occurs as a consequence of both
440 host and pathogen factors. Aberrant T cell function during the primary cryptococcal
441 infection observed in our study could have contributed to the pathogenesis of CM-IRIS. The
442 elevated expression of GXM-specific IL-2 during CM-IRIS may indicate GXM-specific T cell
443 proliferation during the IRIS event. These responses occurring in the enclosed CNS may be
444 detrimental, resulting in the subsequent immunopathology associated with cryptococcal
445 IRIS.

446

447

448

449 Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

450 **Supplementary Figure S1:** Frequency of CD4⁺IL-17⁺Central memory and CD4⁺IL-2⁺

451 Effector memory cells from paired samples at CM Diagnosis vs CM-IRIS.

452 **Supplementary Figure S2:** CD8⁺ T cell Cytokine responses at CM-IRIS vs Controls in
453 response to GXM Stimulation.

454

455 **Author Contributions:** David B. Meya, Edward N. Janoff, Carol Yukari Manabe, and
456 Stephen Cose conceptualized the study and contributed to framing of the research questions
457 and manuscript editing. David R. Boulware and Edward N. Janoff contributed to critical
458 review of the manuscript and manuscript editing. David B. Meya, Samuel Okurut, and
459 Godfrey Zziwa contributed to designing the experiments, carried out the immunoassays,
460 analyses, and manuscript editing. All authors read and approved the final manuscript.

461

462 **Funding:** This work was funded by the Wellcome Trust (Training Health Researchers into
463 Vocational Excellence (THRiVE) in East Africa, grant number 087540-DBM) and the
464 DELTAS Africa Initiative grant # DEL-15-011 to THRiVE-2. The DELTAS Africa Initiative is
465 an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for
466 Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership
467 for Africa's Development Planning and Coordinating Agency (NEPAD) with funding from
468 the Wellcome Trust grant # 107742/Z/15/Z and the UK government. The views expressed
469 in this publication are those of the authors (s) and not necessarily those of AAS, NEPAD,
470 Wellcome Trust or the UK government. The clinical cohorts were supported in part by the
471 National Institutes of Health (R01AI078934, U01AI089244, R01AI108479, K24AI096925,
472 T32AI055433 DBM, DRB) and (R01AI1084791 to ENJ); the GlaxoSmithKline Collaborative
473 Investigator Research Award (CIRA) and the Veterans Affairs Research Service
474 (I01CX001464; ENJ). The funding agencies had no role in study design, data collection and
475 analysis, decision to publish, or preparation of the manuscript.

476 **Acknowledgments:** We thank Abdu Musubire, Nabeta Henry, Joshua Rhein, Jane Francis
477 Ndyetukira, Cynthia Ahimbisibwe, Florence Kugonza, Alisat Sadiq, Radha Rajasingham,
478 Catherine Nanteza, Richard Kwizera, and Darlisha Williams for patient care. We thank
479 Tihana Bicanic, Lewis Haddow, and Jason Baker for generously serving on the external IRIS
480 adjudication committee. We also thank the institutional support from the Infectious
481 Diseases Institute, specifically Alex Coutinho and Keith McAdam. We also thank Britta
482 Flach and Alison Taylor from Makerere University Walter Reed Project for using the
483 Laboratory at Walter Reed in Kampala. We thank Brent Palmer and Harsh Pratap
484 (University of Colorado Denver, Denver Veterans Affairs Medical Center) for their help
485 with flow cytometry data analysis and interpretation.

486

487 **Conflicts of Interest:** The authors declare no conflict of interest.
488

489 **References**

- 490 1. Rajasingham R, Smith RM, Park BJ, et al. Global burden of disease of HIV-associated cryptococcal meningitis:
491 an updated analysis. *The Lancet infectious diseases* **2017**; 17:873-81.
- 492 2. Gordon SB, Walsh AL, Chaponda M, et al. Bacterial meningitis in Malawian adults: pneumococcal disease is
493 common, severe, and seasonal. *Clinical infectious diseases : an official publication of the Infectious Diseases
494 Society of America* **2000**; 31:53-7.
- 495 3. Hakim JG, Gangaidzo IT, Heyderman RS, et al. Impact of HIV infection on meningitis in Harare, Zimbabwe:
496 a prospective study of 406 predominantly adult patients. *Aids* **2000**; 14:1401-7.
- 497 4. Jarvis JN, Meintjes G, Williams A, Brown Y, Crede T, Harrison TS. Adult meningitis in a setting of high HIV
498 and TB prevalence: findings from 4961 suspected cases. *BMC infectious diseases* **2010**; 10:67.
- 499 5. Durski KN, Kuntz KM, Yasukawa K, Virnig BA, Meya DB, Boulware DR. Cost-effective diagnostic checklists
500 for meningitis in resource-limited settings. *Journal of acquired immune deficiency syndromes* **2013**; 63:e101-8.
- 501 6. Cohen DB, Zijlstra EE, Mukaka M, et al. Diagnosis of cryptococcal and tuberculous meningitis in a resource-
502 limited African setting. *Tropical medicine & international health : TM & IH* **2010**; 15:910-7.
- 503 7. Jarvis JN, Meintjes G, Williams A, Brown Y, Crede T, Harrison TS. Adult meningitis in a setting of high HIV
504 and TB prevalence: findings from 4961 suspected cases. *BMC infectious diseases* **2010**; 10:67.
- 505 8. IeDea, Collaborations ARTC, Avila D, et al. Immunodeficiency at the start of combination antiretroviral
506 therapy in low-, middle-, and high-income countries. *Journal of acquired immune deficiency syndromes* **2014**;
507 65:e8-16.
- 508 9. Haddow LJ, Colebunders R, Meintjes G, et al. Cryptococcal immune reconstitution inflammatory syndrome
509 in HIV-1-infected individuals: proposed clinical case definitions. *The Lancet infectious diseases* **2010**; 10:791-802.
- 510 10. DeSimone JA, Pomerantz RJ, Babinchak TJ. Inflammatory reactions in HIV-1-infected persons after initiation
511 of highly active antiretroviral therapy. *Annals of internal medicine* **2000**; 133:447-54.
- 512 11. Lederman MM, Valdez H. Immune restoration with antiretroviral therapies: implications for clinical
513 management. *Jama* **2000**; 284:223-8.
- 514 12. Post MJ, Thurnher MM, Clifford DB, et al. CNS-immune reconstitution inflammatory syndrome in the setting
515 of HIV infection, part 2: discussion of neuro-immune reconstitution inflammatory syndrome with and without
516 other pathogens. *AJNR American journal of neuroradiology* **2013**; 34:1308-18.
- 517 13. Jenny-Avital ER, Abadi M. Immune reconstitution cryptococcosis after initiation of successful highly active
518 antiretroviral therapy. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of
519 America* **2002**; 35:e128-33.
- 520 14. Mahnke YD, Greenwald JH, DerSimonian R, et al. Selective expansion of polyfunctional pathogen-specific
521 CD4(+) T cells in HIV-1-infected patients with immune reconstitution inflammatory syndrome. *Blood* **2012**;
522 119:3105-12.
- 523 15. Chang CC, Lim A, Omarjee S, et al. Cryptococcosis-IRIS is associated with lower cryptococcus-specific IFN-
524 gamma responses before antiretroviral therapy but not higher T-cell responses during therapy. *The Journal of
525 infectious diseases* **2013**; 208:898-906.
- 526 16. Boulware DR, Bonham SC, Meya DB, et al. Paucity of initial cerebrospinal fluid inflammation in cryptococcal
527 meningitis is associated with subsequent immune reconstitution inflammatory syndrome. *The Journal of
528 infectious diseases* **2010**; 202:962-70.

- 529 17. Meya DB, Okurut S, Zziwa G, et al. Monocyte Phenotype and IFN-gamma-Inducible Cytokine Responses
530 Are Associated with Cryptococcal Immune Reconstitution Inflammatory Syndrome. *J Fungi (Basel)* **2017**; 3.
- 531 18. Boulware DR, Rolfes MA, Rajasingham R, et al. Multisite validation of cryptococcal antigen lateral flow assay
532 and quantification by laser thermal contrast. *Emerg Infect Dis* **2014**; 20:45-53.
- 533 19. Rajasingham R, Rhein J, Klammer K, et al. Epidemiology of meningitis in an HIV-infected Ugandan cohort.
534 *The American journal of tropical medicine and hygiene* **2015**; 92:274-9.
- 535 20. Boulware DR, Meya DB, Muzoora C, et al. Timing of antiretroviral therapy after diagnosis of cryptococcal
536 meningitis. *The New England journal of medicine* **2014**; 370:2487-98.
- 537 21. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate
538 datasets. *Cytometry Part A : the journal of the International Society for Analytical Cytology* **2011**; 79:167-74.
- 539 22. Jarvis JN, Casazza JP, Stone HH, et al. The phenotype of the Cryptococcus-specific CD4+ memory T-cell
540 response is associated with disease severity and outcome in HIV-associated cryptococcal meningitis. *The Journal*
541 *of infectious diseases* **2013**; 207:1817-28.
- 542 23. Rodrigues ML, Alviano CS, Travassos LR. Pathogenicity of *Cryptococcus neoformans*: virulence factors and
543 immunological mechanisms. *Microbes and infection / Institut Pasteur* **1999**; 1:293-301.
- 544 24. Buchanan KL, Murphy JW. What makes *Cryptococcus neoformans* a pathogen? *Emerging infectious diseases*
545 **1998**; 4:71-83.
- 546 25. Feldmesser M, Casadevall A. Mechanism of action of antibody to capsular polysaccharide in *Cryptococcus*
547 *neoformans* infection. *Frontiers in bioscience : a journal and virtual library* **1998**; 3:d136-51.
- 548 26. Yauch LE, Lam JS, Levitz SM. Direct inhibition of T-cell responses by the *Cryptococcus* capsular
549 polysaccharide glucuronoxylomannan. *PLoS pathogens* **2006**; 2:e120.
- 550 27. Barber DL, Andrade BB, Sereti I, Sher A. Immune reconstitution inflammatory syndrome: the trouble with
551 immunity when you had none. *Nature reviews Microbiology* **2012**; 10:150-6.
- 552 28. Lortholary O, Fontanet A, Memain N, et al. Incidence and risk factors of immune reconstitution inflammatory
553 syndrome complicating HIV-associated cryptococcosis in France. *Aids* **2005**; 19:1043-9.
- 554 29. Siddiqui AA, Brouwer AE, Wuthiekanun V, et al. IFN-gamma at the site of infection determines rate of
555 clearance of infection in cryptococcal meningitis. *Journal of immunology* **2005**; 174:1746-50.
- 556 30. Murdock BJ, Huffnagle GB, Olszewski MA, Osterholzer JJ. Interleukin-17A enhances host defense against
557 cryptococcal lung infection through effects mediated by leukocyte recruitment, activation, and gamma
558 interferon production. *Infection and immunity* **2014**; 82:937-48.
- 559 31. Boulware DR, Meya DB, Bergemann TL, et al. Clinical features and serum biomarkers in HIV immune
560 reconstitution inflammatory syndrome after cryptococcal meningitis: a prospective cohort study. *PLoS medicine*
561 **2010**; 7:e1000384.
- 562 32. Chang CC, Dorasamy AA, Gosnell BI, et al. Clinical and mycological predictors of cryptococcosis-associated
563 immune reconstitution inflammatory syndrome. *Aids* **2013**; 27:2089-99.
- 564 33. Jarvis JN, Meintjes G, Rebe K, et al. Adjunctive interferon-gamma immunotherapy for the treatment of HIV-
565 associated cryptococcal meningitis: a randomized controlled trial. *Aids* **2012**; 26:1105-13.
- 566 34. Boulware DR MD, Bergemann TL, Wiesner D, Rhein J, Williams D, Vlasova I, Kambugu A, Janoff EN,
567 Bohjanen PR. Gene Expression Biomarkers in Blood Predict Cryptococcal Immune Reconstitution Inflammatory
568 Syndrome (IRIS). 5th IAS Conference on HIV Pathogenesis, Treatment & Prevention **2009**; Abstract no.
569 TUPEB163.

- 570 35. Vlasova-St Louis I, Chang CC, Shahid S, French MA, Bohjanen PR. Transcriptomic Predictors of Paradoxical
571 Cryptococcosis-Associated Immune Reconstitution Inflammatory Syndrome. *Open Forum Infect Dis* **2018**;
572 5:ofy157.
- 573 36. Yoon HA, Nakouzi A, Chang CC, et al. Association between plasma antibody responses and risk for
574 Cryptococcus-associated immune reconstitution inflammatory syndrome. *The Journal of infectious diseases*
575 **2018**.
- 576 37. Hoyer KK, Dooms H, Barron L, Abbas AK. Interleukin-2 in the development and control of inflammatory
577 disease. *Immunol Rev* **2008**; 226:19-28.
- 578 38. Zhou Q, Gault RA, Kozel TR, Murphy WJ. Immunomodulation with CD40 stimulation and interleukin-2
579 protects mice from disseminated cryptococcosis. *Infection and immunity* **2006**; 74:2161-8.
- 580 39. Barber DL, Andrade BB, McBerry C, Sereti I, Sher A. Role of IL-6 in Mycobacterium avium--associated
581 immune reconstitution inflammatory syndrome. *Journal of immunology* **2014**; 192:676-82.
- 582 40. Goovaerts O, Jennes W, Massinga-Loembe M, et al. LPS-binding protein and IL-6 mark paradoxical
583 tuberculosis immune reconstitution inflammatory syndrome in HIV patients. *PloS one* **2013**; 8:e81856.
- 584 41. Narendran G, Andrade BB, Porter BO, et al. Paradoxical tuberculosis immune reconstitution inflammatory
585 syndrome (TB-IRIS) in HIV patients with culture confirmed pulmonary tuberculosis in India and the potential
586 role of IL-6 in prediction. *PloS one* **2013**; 8:e63541.
- 587 42. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature* **2008**;
588 453:1051-7.
- 589 43. Tajima M, Wakita D, Noguchi D, et al. IL-6-dependent spontaneous proliferation is required for the induction
590 of colitogenic IL-17-producing CD8+ T cells. *The Journal of experimental medicine* **2008**; 205:1019-27.
- 591 44. Zaragoza O, Rodrigues ML, De Jesus M, Frases S, Dadachova E, Casadevall A. The capsule of the fungal
592 pathogen *Cryptococcus neoformans*. *Advances in applied microbiology* **2009**; 68:133-216.
- 593 45. Delliere S, Guery R, Candon S, et al. Understanding Pathogenesis and Care Challenges of Immune
594 Reconstitution Inflammatory Syndrome in Fungal Infections. *J Fungi (Basel)* **2018**; 4.
595