Interleukin-6, Tumor Necrosis Factor-α Expression and Monocyte Subset Phenotype are Associated with Cryptococcal Immune Reconstitution Inflammatory Syndrome

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Abstract Number: 815

ABSTRACT

Introduction. Immune Reconstitution Inflammatory Syndrome (IRIS) occurs in up to 30% of HIV-infected patients with Cryptococcal Meningitis (CM), resulting from exaggerated immune responses upon initiation of antiretroviral therapy (ART). The contribution of monocytes to the immunopathogenesis of cryptococcal IRIS is not well understood.

Methods. We isolated peripheral blood mononuclear cells from 10 subjects in Kampala, Uganda at CM diagnosis, 11 subjects at time of CM-IRIS and 6 subjects with CM without IRIS matched for ART duration (Controls). Upon *in vitro* stimulation with IFN-γ, a proxy for CD4$^+$ T cell help, monocytes expressing IL-6 and TNF-α were quantified by intracellular flow cytometry. Monocytes defined as CD4^{lo}CD11c$^+$CD14^{++}CD16$^{+/-}$ were either classical (CD14^{++}CD16$^-$); intermediate (CD14^{++}CD16$^+$) or non-classical (CD14$^+$CD16^{++}).

Results. At CM diagnosis, non-classical monocytes were less frequent in persons who later developed IRIS vs CM controls, median 0% vs 4% (*P*<0.001), as were activated (PD-L1$^+$CD25$^+$) non-classical monocytes in CM-IRIS vs controls; median 0% vs 11% (*P*<0.001). No differences in frequencies of classical and intermediate monocytes occurred between CM-IRIS and controls at baseline.
TNF-α expression by activated classical monocytes was more frequent in CM-IRIS vs controls, whereas TNF-α- and IL-6-producing non-classical monocytes were higher among controls. During CM-IRIS, IL-6$^+$ classical and intermediate were increased compared with baseline pre-ART among 10 subjects with paired samples. Moreover, subjects with CM-IRIS showed a higher frequency of activated monocytes and increased frequencies of IL-6$^+$, TNF-α$^+$ classical and IL-6$^+$ intermediate monocytes vs. controls. These results were seen in unstimulated monocytes and augmented with IFN-γ stimulation.

Conclusions. At CM diagnosis, persons who later developed CM-IRIS had increased activation and TNF-α responses compared with those who did not develop CM-IRIS. At CM-IRIS, monocyte activation was higher than matched controls; classical and intermediate monocytes were the predominant source of cytokines. Distinct monocyte populations, activation profiles and cytokine responses at CM diagnosis may be involved in the development and pathogenesis of CM IRIS.

INTRODUCTION

- *Cryptococcus neoformans* is the most common cause of adult meningitis in sub-Saharan Africa (SSA) and accounts for 20 to 25% of acquired immunodeficiency syndrome (AIDS)-related mortality[1].
- The primary immune response to *Cryptococcus* involves fungal recognition by innate immune cells and subsequent expansion of *Cryptococcus*-specific CD4$^+$ T cells to control the infection.
- Up to 30% of patients with cryptococcal meningitis (CM) develop paradoxical deterioration in their clinical status after initiating antiretroviral therapy (ART).
- This phenomenon is thought to be due to aberrant inflammatory responses to residual cryptococcal antigens during recovery of the host's immune system, and is termed paradoxical immune reconstitution inflammatory syndrome (IRIS)[1].
- The contribution of monocytes to the immunopathogenesis of cryptococcal IRIS is not well understood.
- We evaluated the role and function of peripheral blood monocytes from HIV+ subjects with meningitis treated with ART in CM-IRIS by characterizing their phenotype, activation, and cytokine responses upon *in vitro* stimulation with IFN-γ, a proxy for CD4$^+$ T cell driven activation.

METHODS

- **Subjects:** Participants ≥18 years of age with HIV infection (ART naïve) and clinical evidence of meningitis were prospectively enrolled in the Cryptococcal Optimal Antiretroviral Timing (COAT) Trial (Clinicaltrials.gov: NCT01075152), or in the Neurological Outcomes on ART (NOAT) study, a prospective observational cohort of HIV-infected patients with clinical meningitis. Peripheral blood mononuclear cells (PBMCs) were obtained at initial screening, at CM-IRIS and from control subjects without CM-IRIS matched for ART duration.

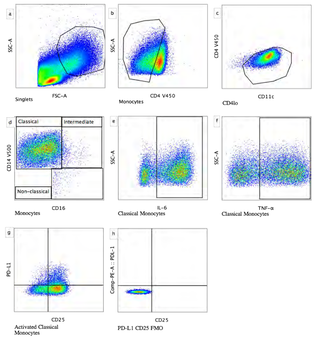
- **CM-IRIS**: A diagnosis of definite/probable/possible CM-IRIS was made according to a published consensus case definition[2].

- **Stimulation**: Lipopolysaccharide (LPS) (10ng/mL) was added as the positive control, Phosphate Buffered saline (PBS) as the negative control and Recombinant Interferon-γ (IFN-γ; 150U/mL) as the test well for 2 hours in 5% CO_2 at 37°C, followed by Brefeldin A (100µL/mL) for another 4 hours. Cells were stored in the dark overnight at 4°C and stained for lineage, activation and intracellular cytokines using 8-color flow cytometry **(Table 1)**.

Table 1. Monocyte Subsets, Activation and Intracellular Cytokines

	Lineage Markers	Activation markers	Intracellular cytokines
Classical monocytes	CD14^{++}CD16$^-$	PD-L1	TNF-
Intermediate monocytes	CD14^{++}CD16$^+$	CD25	IL-6
Non-Classical monocytes	CD14$^+$CD16^{++}		

Figure 1. Flow cytometry gating strategy for Monocytes.



RESULTS

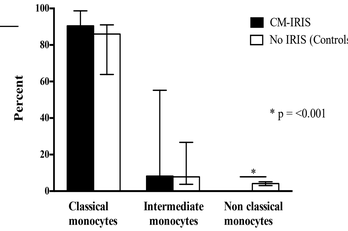
BASELINE PARAMETERS

- Age, baseline CD4$^+$ and CD8$^+$ T cell counts, cerebrospinal fluid(CSF) cryptococcal antigen (CRAG) titer, HIV viral load, CSF protein and white blood cells were similar among subjects with CM-IRIS vs controls **(Table 2).**
- At CM diagnosis, there was a trend to higher quantitative cryptococcal colony counts among subjects who developed CM-IRIS, median, 5.33 (IQR; 4.96, 5.46) vs 3.97 (IQR; 2.45, 5.26) $\log_{10}$ colony forming units/mL among control subjects who did not develop IRIS.
- Classical monocytes were the predominant monocyte subset among subjects who developed CM IRIS, 90% (IQR: 42, 99) and controls, 86% (IQR: 64, 91). However, the frequency of non-classical monocytes was significantly higher among control subjects without IRIS vs those who developed CM-IRIS (4.1% vs 0%, p = <0.001) **(Figure 2).**
- Activated (PD-L1$^+$CD25$^+$) non-classical monocytes were also less frequent in CM-IRIS vs controls; median 0% (IQR, 0, 0) vs 11% (IQR, 6, 16), *P*<0.001.

Table 2. Characteristics of subjects with CM-IRIS vs subjects without CM-IRIS

	Controls (N= 6)	CM-IRIS (N= 11)	p-value
Male (%)	17	73	0.05
Age (years)	35 (±9)	35 (±8)	0.937
Baseline CD4 T cells/µL	8 (5, 166)	6.5 (4, 28)	0.828
CD4 T cells ≥3 mo ART/µL	156 (55, 309)	68 (33, 79)	0.256
Baseline CD8 T cells/µL	163 (97, 784)	256 (140, 591)	0.515
CD8 T cells ≥3 mo ART/µL	1005 (615, 1086)	831 (565, 997)	0.463
Baseline HIV viral load ($\log_{10}$copies/mL)	4.93(±0.34)	5.24 (±0.5)	0.280
Baseline $\log_{10}$Cryptococcal colony forming units/mL	3.97 (2.45, 5.26)	5.33 (4.96, 5.46)	0.080
Baseline CSF CRAG titer	4512 (528, 12192)	7200 (4048, 16384)	0.455
CSF protein (mg/dL)	60 (47, 68)	53 (20, 70)	0.471
Baseline CSF WBC (cells/mL)	19.5 (<5, 45)	<5 (<5, <5)	0.169

Figure 2. Baseline Monocyte Subsets in subjects developing CM IRIS vs Controls



CM IRIS vs CONTROLS

- The proportion of activated TNF-α$^+$ non-classical monocytes was higher among controls, 3% (0, 7) compared to subjects with CM-IRIS, 0%, (p = 0.022).

- At CM-IRIS, the frequencies of IL-6$^+$monocytes, classical and intermediate monocyte subsets expressing IL-6 and TNF-α were higher among subjects with CM-IRIS compared to controls without IRIS in response to IFN-γ. Similar results were demonstrated when we compared the unstimulated samples, showing higher IL-6 and TNF-α responses among subjects with CM-IRIS.

- At CM-IRIS, immune responses were characterized by a predominance of dual function IL-6- and TNF-α-producing monocytes (median, IQR) 39% (20, 61) compared to controls, 8% (3, 10) with mono-functional TFN-α-producing cells, 22% (7, 52) the next most frequent phenotype **(Figure 3)**.

- The frequency of IL-6$^+$ and TN-α$^+$ monocytes was significantly higher among subjects experiencing CM IRIS vs controls. This difference in IL-6 expression was in both IFN-γ-stimulated and unstimulated cells **(Figure 4)**.

Figure 3. Functional Monocyte Cytokine responses following IFN-γ Stimulation.

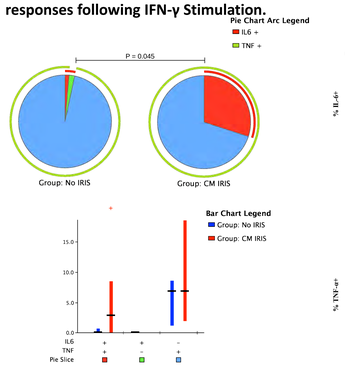
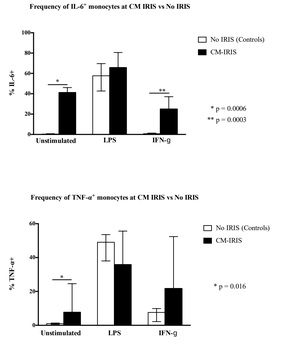


Figure 4. Monocyte IL-6 and TNF-α expression at CM IRIS



CONCLUSIONS

- Aberration in monocyte gene expression functionally related to infection and inflammation has been shown in TB-IRIS, it is not clear if this occurs in cryptococcal IRIS. However, we demonstrated differences in the monocyte expression of IL-6 and TNF-α among subjects with and without CM IRIS.

- At baseline, the differential monocyte expression of IL-6 and TNF-α among subjects with future CM IRIS suggests possible exaggerated innate immune responses to T cell signaling in the presence of cryptococcal infection, as a risk factor for CM IRIS.

- Absence of the non-classical monocyte phenotype in patients with CM was associated with future CM IRIS.

- Monocytes from patients with CM-IRIS showed differential responses to stimulation compared with non-IRIS controls: increased IL-6 and TNF-α cytokine in response to exogenous IFN-γ but similar in response to LPS. Results are driven largely by the predominant classical subset.

- Similar cytokine response in unstimulated and IFN-γ stimulated cells may result from prior in vivo IFN-γ signaling during CM-IRIS or monocyte exhaustion.

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2. Haddow LJ, et al. (2010) Cryptococcal immune reconstitution inflammatory syndrome in HIV-1-infected individuals: proposed clinical case definitions. Lancet Infect Dis 10: 791-802.

ACKNOWLEDGMENT

- The Welcome Trust (Training Health Researchers into Vocational Excellence (THRiVE) in East Africa, grant number 087540)
- National Institutes of Health [R01AI078934, U01AI089244, NS065713]