Compartmentalization of innate immune responses in the central nervous system during cryptococcal meningitis/HIV coinfection

Vivek Naranbhai[a,b,c,*], Christina C. Chang[b,d,e,*], Raveshni Durgiah[b], Saleha Omarjee[b], Andrew Lim[f], Mahomed-Yunus S. Moosa[g], Julian H. Elliot[d,e], Thumbi Ndung'u[b,h,i], Sharon R. Lewin[d,e], Martyn A. French[f] and William H. Carr[b,j]

Objective: The role of innate immunity in the pathogenesis of cryptococcal meningitis is unclear. We hypothesized that natural killer (NK) cell and monocyte responses show central nervous system (CNS) compartment-specific profiles, and are altered by antifungal therapy and combination antiretroviral therapy (cART) during cryptococcal meningitis/HIV coinfection.

Design: Substudy of a prospective cohort study of adults with cryptococcal meningitis/HIV coinfection in Durban, South Africa.

Methods: We used multiparametric flow cytometry to study compartmentalization of subsets, CD69 (a marker of activation), CXCR3 and CX3CR1 expression, and cytokine secretion of NK cells and monocytes in freshly collected blood and cerebrospinal fluid (CSF) at diagnosis ($n = 23$), completion of antifungal therapy induction ($n = 19$), and after a further 4 weeks of cART ($n = 9$).

Results: Relative to blood, CSF was enriched with $CD56^{bright}$ (immunoregulatory) NK cells ($P = 0.0004$). At enrolment, CXCR3 expression was more frequent among blood $CD56^{bright}$ than either blood $CD56^{dim}$ ($P < 0.0001$) or CSF $CD56^{bright}$ ($P = 0.0002$) NK cells. Antifungal therapy diminished blood ($P < 0.05$), but not CSF $CXCR3^{pos}$ NK-cell proportions nor $CX3CR1^{pos}$ NK-cell proportions. $CD56^{bright}$ and $CD56^{dim}$ NK cells were more activated in CSF than blood ($P < 0.0001$). Antifungal therapy induction reduced $CD56^{dim}$ NK-cell activation in CSF ($P = 0.02$). Activation of blood $CD56^{bright}$ and $CD56^{dim}$ NK cells was diminished following cART commencement ($P < 0.0001$, $P = 0.03$). Immunoregulatory NK cells in CSF tended to secrete higher levels of CXCL10 ($P = 0.06$) and lower levels of tumor necrosis factor α ($P = 0.06$) than blood immunoregulatory NK cells. CSF was enriched with nonclassical monocytes ($P = 0.001$), but antifungal therapy restored proportions of classical monocytes ($P = 0.007$).

Conclusion: These results highlight CNS activation, trafficking, and function of NK cells and monocytes in cryptococcal meningitis/HIV and implicate immunoregulatory

[a]Centre for the AIDS Programme of Research in South Africa, [b]HIV Pathogenesis Programme, Nelson R Mandela School of Medicine, University of KwaZulu Natal, Durban, South Africa, [c]Nuffield Department of Medicine, University of Oxford, Oxford, UK, [d]Department of Infectious Diseases, Monash University and Alfred Hospital, [e]Centre for Biomedical Research, Burnet Institute, Melbourne, [f]School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Australia, [g]Department of Infectious Diseases, Nelson R Mandela School of Medicine, [h]KwaZulu-Natal Research Institute for Tuberculosis and HIV(K-RITH), University of KwaZulu Natal, Durban, South Africa, [i]Max Planck Institute for Infection Biology, Berlin, Germany, and [j]Medgar Evers College (City University of New York), Brooklyn, New York, USA.

Correspondence to Vivek Naranbhai, Nuffield Department of Medicine, University of Oxford, Oxford OX3 7BN, UK.

E-mail: vivekn@well.ox.ac.uk

[*]Vivek Naranbhai and Christina C. Chang contributed equally to the writing of the article.

Received: 12 November 2013; revised: 6 January 2014; accepted: 6 January 2014.

DOI:10.1097/QAD.0000000000000200

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

NK cells and proinflammatory monocytes as potential modulators of cryptococcal meningitis pathogenesis during HIV coinfection.

© 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins

AIDS 2014, **28**:657–666

Keywords: cerebrospinal fluid, cryptococcal meningitis, HIV-1, monocytes, natural killer cells

Introduction

Cryptococcal meningitis is a major cause of morbidity and mortality in patients with HIV and AIDS. Annually, approximately 957 900 cases of cryptococcal meningitis occur, resulting in 624 700 deaths by 3 months after infection, with sub-Saharan Africa bearing the largest burden of disease [1]. The underlying mechanisms causing death and disability include development of persistently high intracranial pressures, vasculopathies, and local brain inflammation with bystander neuronal damage. Both innate and adaptive immune responses contribute to the immunopathogenesis of cryptococcal meningitis, but the regulation and timing of their development remain poorly understood.

Natural killer (NK) cells are key effectors of innate immunity that are able to mediate pathogen elimination by direct killing or modulating innate and adaptive immune responses through secretion of cytokines. In humans, expression of CD56, but a lack of CD3, CD14, and CD19, defines their phenotype. Functionally, they can be further subdivided into subsets with primarily cytokine-secretion capabilities ($CD56^{bright}CD16^{neg}$) or cytolytic capabilities ($CD56^{dim}CD16^{pos}$) [2]. Typically, $CD56^{bright}$ NK cells are enriched at extravascular sites [2]. During HIV disease, a third subset, $CD56^{neg}$ $CD16^{pos}$, increases disproportionately in blood, but this subset is deficient in both cytokine production and cytolysis [3]. In-vivo mouse and in-vitro human studies suggest that NK cells are able to directly kill cryptococci by perforin-mediated cytotoxicity [4,5], or indirectly by the potentiation of macrophage antifungal activity [6]. NK cells are able to enter the central nervous system (CNS) during inflammatory disease such as multiple sclerosis (MS) [7]; indeed, they have been shown to play a major role in a variety of CNS infections [8]. Therefore, it is plausible that *in vivo*, NK cells may traffic to the site of cryptococcal infection and exert antifungal activity. Alternatively, NK cells may secrete immunoregulatory cytokines that affect recruitment and function of other innate and adaptive immune cells. The phenotype, function, and mechanisms of NK-cell infiltration/trafficking into the CNS are not well described in cryptococcal meningitis/HIV coinfection, and have only recently been examined in HIV monoinfection [9]. Thus, delineating the profiles of NK cells in the cerebrospinal fluid (CSF) during treated cryptococcal meningitis may allow identification of parameters that play a role in cryptococcal meningitis/HIV pathogenesis.

Monocytes/macrophages are a second innate immune leukocyte subset that play a role in the pathogenesis of some inflammatory CNS diseases, and with which NK cells have substantial cross-talk. NK cells are required for monocyte differentiation into dendritic cells in several inflammatory disorders [10]. Conversely, monocytes/macrophages are able to activate NK cells through their secretion of proinflammatory cytokines, interleukin-12 (IL-12) and IL-18 [11]. Monocytes can be divided into three functionally distinct subsets based on their relative expression of CD16 and CD14 (i.e. classical: $CD14^{++}CD16^{-}$; intermediate: $CD14^{++}$ $CD16^{+}$; and nonclassical: $CD14^{+}CD16^{++}$) [12]. Among these subsets, the nonclassical monocytes have the greatest capacity for secreting proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) [13], intermediate monocytes have superior reactive-oxygen species production, and classical monocytes appear to have superior phagocytic function [12]. The role of monocytes in cryptococcal meningitis pathogenesis is unresolved; some reports suggest that monocytes may act as a 'Trojan horse' allowing entry of intracellular cryptococci into the CNS [14], whereas others suggest that monocytes may mediate cryptococcal elimination [15]. Similar to other infections by intracellular pathogen disorders, in cryptococcal meningitis, monocytes are likely required for pathogen elimination, but also to harbor pathogens intracellularly and impose clinically relevant immunopathology with their activity in cryptococcal meningitis [16].

Here we aimed to identify changes in the innate immune response in blood and CSF in patients with cryptococcal meningitis and HIV in South Africa. We prospectively characterized blood and CSF NK-cell phenotypes, monocyte subsets and, to a lesser extent, NK-cell function in patients with HIV/cryptococcal meningitis coinfection at admission for care, after induction of antifungal therapy, and after a further 4 weeks following commencement of combination antiretroviral therapy (cART) in some patients.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Methods

This study was conducted as a substudy of the Cryptococcal Immune Restoration Disease (IRD) study, which has been described previously [17]. We prospectively enrolled consenting cART-naive, HIV-infected adults with a first episode of microbiologically confirmed cryptococcal meningitis at the King Edward VIII Hospital in Durban, South Africa. Briefly, whole blood and CSF were obtained at enrolment (median 2 days after diagnosis, range 0–8 days) from 23 participants. Amphotericin B was commenced immediately on diagnosis for a protocolled time of 14 days. About half of all patients had persistent cryptococcal growth after amphotericin B therapy [17]. Following this induction period of antifungal therapy, 19 patients were re-sampled for blood and CSF (median 14 days after diagnosis, range 10–15 days) and were commenced on cART as per contemporary guidelines [18] and continued on oral fluconazole. After 4 weeks of cART, a final whole blood specimen was obtained from a subset of nine patients based on their availability. Serial therapeutic lumbar punctures were conducted as required for therapeutic purposes while continuing antifungal therapy.

At enrolment, the mean age of participants in this substudy was 34.7 years (range 21–55 years), and 43% were women, similar to the overall cohort [17]. The median baseline $CD4^+$ T-cell count was 22 cells/μl (interquartile range, IQR 6.5–43), and the median plasma HIV viral load was $3.18 \log_{10}$ copies/ml (IQR 1.14–5.95). After 4 weeks of cART, among nine participants from whom blood samples were available, the median $CD4^+$ T-cell count was 74 cells/μl (IQR 49–153), and the median plasma HIV viral load was $2.56 \log_{10}$ copies/ml (IQR 2.31–2.91).

This study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee, the Monash University ethics committee, and the University of Western Australia ethics committee.

Flow cytometry analyses

The cellular profile of CSF can be taken as a measure of cells in an intermediate compartment between blood and CNS parenchyma. We used methods that have been previously used to study CSF T-cell profiles in healthy and HIV-infected patients to characterize NK cells and monocytes in the CNS [19–21]. Peripheral blood and CSF leukocytes were simultaneously stained with a panel of fluorophore-conjugated antibodies and subjected to multiparametric flow cytometry using conventional methods. Briefly, for whole blood staining, 150 μl undiluted whole blood collected in tubes containing ethylenediaminotetraacetic acid (EDTA) was incubated with the following antibodies for 20 min at 4°C: anti-CD3 APC, anti-CD8 Qdot 655, anti-CD14 Pacific Orange (PO), anti-CD16 Pacific Blue (PB), anti-CD45 AlexaFluor700 (AF700), anti-CD56 PC7 (Beckman Coulter, Pasadena, California, USA), anti-CD69 FITC, anti-CX3CR1 PE, and anti-CXCR3 PerCP-Cy5.5. All antibodies were from Becton Dickinson (Franklin Lakes, New Jersey, USA), unless otherwise indicated. Red blood cells were lysed with VersaLyse (Beckman Coulter) as per the manufacturer's directions, leacocytes were pelleted by centrifugation, and fixed with a paraformaldehyde-containing fixative (Reagent A; Life Technologies, Carlsbad, California, USA). For CSF cell staining, the total volume of CSF obtained from the patient (ranging from 3 to 30 ml) was centrifuged at 750*g* for 10 min, resuspended in 1 ml R10 (RPMI 1640 containing penicillin/streptomycin, 10% fetal calf serum, supplemented with 1.0 mg/ml L-glutamine), and live nucleated cells were enumerated with a nucleocounter (Chemometec, Allerod, Denmark). The range of the nucleocounter was $5 \times 10^3 – 2 \times 10^6$ cells/ml and as several samples were outside this range, we were unable to convert proportions to absolute numbers in this study. One-third of the total number of nucleated cells were aliquoted into fluorescence-activated cell sorting (FACS) tubes, washed with Dulbeco's phosphate-buffered saline (DPBS) and stained for 20 min with the same panel of antibodies listed above. Cells were washed and fixed as above, but the lysis step was omitted.

For intracellular cytokine staining experiments, the primary stain included anti-CD3 APC (Beckman Coulter), anti-CD56 PC7 (Beckman Coulter) and anti-CD16 PB. Cells were incubated in the presence of GolgiStop (Becton Dickinson), to prevent extracellular cytokine secretion. Following fixation, cells were incubated for 15 min, washed, and then permeabilized and stained with anticytokine antibodies by adding Reagent B (Life Technologies), anti-CXCL10 PE and anti-TNF-α AF700. After a further 15 min, cells were washed with DPBS.

Flow cytometry data were collected on a BD LSRII and analyzed using FlowJo v10.0.6 (Tree Star, Ashland, Oregon, USA). At least 5000 $CD45^+$ leukocytes were collected for each CSF sample, and 3×10^6 events were collected for each whole blood specimen. Fluorescence minus one gating strategies were used to determine gating boundaries as described [22]. The gating strategy is shown in Supplementary Figure 1, http://links.lww.com/QAD/A470.

Statistical analyses

For comparisons between paired specimens, from the same individual at different time-points, or at the same time-point but from blood and CSF, a nonparametric matched-pairs Wilcoxon signed rank test was performed. This method ignores data points where the pair is incomplete and, thus, is robust to missing data for the four individuals for whom samples were unavailable at completion of antifungal therapy induction. Statistical

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

analyses were conducted in GraphPad Prism v5 (GraphPad, La Jolla, California, USA).

Results

Proportions of immunoregulatory natural killer cells (CD56^{bright}) are expanded in the cerebrospinal fluid of patients with cryptococcal meningitis and HIV

Cytolytic and cytokine-secretory roles are performed by different NK-cell subsets that partially overlap in function: low expression of CD56 (CD56^{dim}) demarcates cytolytic NK cells and high expression of CD56 (CD56^{bright}) identifies a cytokine-secreting subset that is thought to be less mature [23]. At both enrolment and after completion of antifungal therapy, the CSF was enriched with CD56^{bright} immunoregulatory NK cells compared with blood (at enrolment, median 20 vs. 5.4%, median change $\Delta = 13.32\%$, $P = 0.0004$, Fig. 1a), and had fewer CD56^{dim} NK cells (at enrolment, median 64 vs. 86.1%, median $\Delta = 20.6\%$, $P < 0.0001$). The ratio of CD56^{bright}/CD56^{dim} NK cells was significantly higher in the CSF compared with blood (Fig. 1b). Neither the absolute proportions nor the ratio of CD56^{bright} and CD56^{dim} NK cells was significantly modified following 14 days of antifungal therapy (Fig. 1b).

Expansion of an anergic subset of NK cells with low/absent CD56 expression (CD56^{neg}) has been observed in the blood of patients with advanced HIV disease [24]. Notwithstanding the use of classical methods as opposed to recently described methods using CD7 expression to enhance specificity of NK-cell gating [25], we did not observe differences in the proportion of CD56^{neg} NK cells in the CSF relative to blood (Supplementary Figure 2, http://links.lww.com/QAD/A470). In both blood and CSF, the median proportion of CD56^{neg} NK cells was 6–7%.

In both blood and cerebrospinal fluid, immunoregulatory (CD56^{bright}) natural killer cells and cytolytic (CD56^{dim}) natural killer cells differed in their expression of CXCR3

To investigate whether immunoregulatory NK cells differed from cytotoxic NK cells in CSF, we compared their expression of chemokine receptors. Eisenhardt *et al.* [26] recently demonstrated that CXCR3 expression in extravascular tissues demarcated specific NK-cell subsets that play a role in infection. Moreover, CXCR3 is the receptor for proinflammatory chemokines: CXCL9 (MIG), CXCL-10 (IP-10), and CXCL-11 (I-TAC) [27]. Based on our prior discovery of an increasing gradient of CXCL-10 from blood to CSF in the participants of this study [28], we speculated that this chemokine could mediate chemotaxis of CXCR3-expressing NK cells into the CNS.

At enrolment in blood, we found a greater proportion of CD56^{bright} NK cells expressing CXCR3 than CD56^{dim} NK cells (median 5.4 vs. 2.2%, median $\Delta = 3.2\%$, $P < 0.0001$; Fig. 2a). In contrast, in CSF, a significantly greater proportion of CD56^{dim} NK cells expressed CXCR3 than CD56^{bright} NK cells (median 4.2 vs. 2.4%, median $\Delta = 1.5\%$, $P = 0.0011$). Furthermore, we found that differential CXCR3 expression on CD56^{bright} and CD56^{dim} NK cells extended to comparisons between blood and CSF compartments (Fig. 2b and 2c). Among CD56^{bright} NK cells, the proportion expressing CXCR3 was significantly greater in blood than CSF at enrolment (median 7.8 vs. 2.1%, median $\Delta = 4.6\%$, $P = 0.0002$), but after 14 days of antifungal therapy, the proportion of CXCR3^{pos} CD56^{bright} NK cells in blood declined (median 7.8 vs. 4.8%, median $\Delta = 2.8\%$, $P = 0.009$). By completion of antifungal therapy induction, there was no difference between blood and CSF in the proportion of CD56^{bright} NK cells expressing CXCR3 (Fig. 2b). The proportion of CXCR3^{pos} CD56^{bright} NK cells in CSF did not change over the period of antifungal therapy induction.

In contrast, among CD56^{dim} NK cells, the proportion expressing CXCR3 was significantly greater in CSF than blood at both enrolment and after completion of antifungal therapy (median 8.6 vs. 3.4%, median $\Delta = 3.2\%$ at enrolment, $P = 0.001$; median 7.9 vs. 2.5%, median $\Delta = 5.36\%$ at completion of antifungal therapy, $P = 0.0005$; Fig. 2c). This difference in CXCR3 expression was maintained in CSF over the period of antifungal therapy induction. However, in blood, the proportion of CD56^{dim} NK cells expressing CXCR3 declined (median 3.4% at enrolment vs. 2.3% at completion of antifungal therapy, median $\Delta = 1.2\%$, $P = 0.03$).

The proportion of CXCR3^{pos}, CD56^{bright}, and CD56^{dim} NK cells in blood following 4 weeks of cART did not differ from that at completion of antifungal therapy (Supplementary Figure 3A and B, http://links.lww.com/QAD/A470).

We also examined expression of CX3CR1 on the various NK-cell subsets, as CX3CR1-expressing NK cells have been reported to be involved in modifying autoimmune CNS disease pathogenesis [29]. At enrolment, in CSF, there was a higher proportion of CD56^{dim} NK cells expressing CX3CR1 than CD56^{bright} NK cells (median 10.6 vs. 2.9%, median $\Delta = 4.8\%$, $P = 0.0003$), but there was no difference observed in blood. Both at enrolment and at completion of antifungal therapy induction, a larger proportion of CD56^{bright} NK cells in blood expressed CX3CR1 than those in CSF (median 15.2 vs. 2.8%, median $\Delta = 14.3\%$, $P = 0.001$; and median 10.4 vs. 1.4%, median $\Delta = 6.5\%$, $P = 0.009$, respectively, Fig. 2b). The proportion of CX3CR1-expressing CD56^{dim} NK cells did not differ between blood and CSF regardless of

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

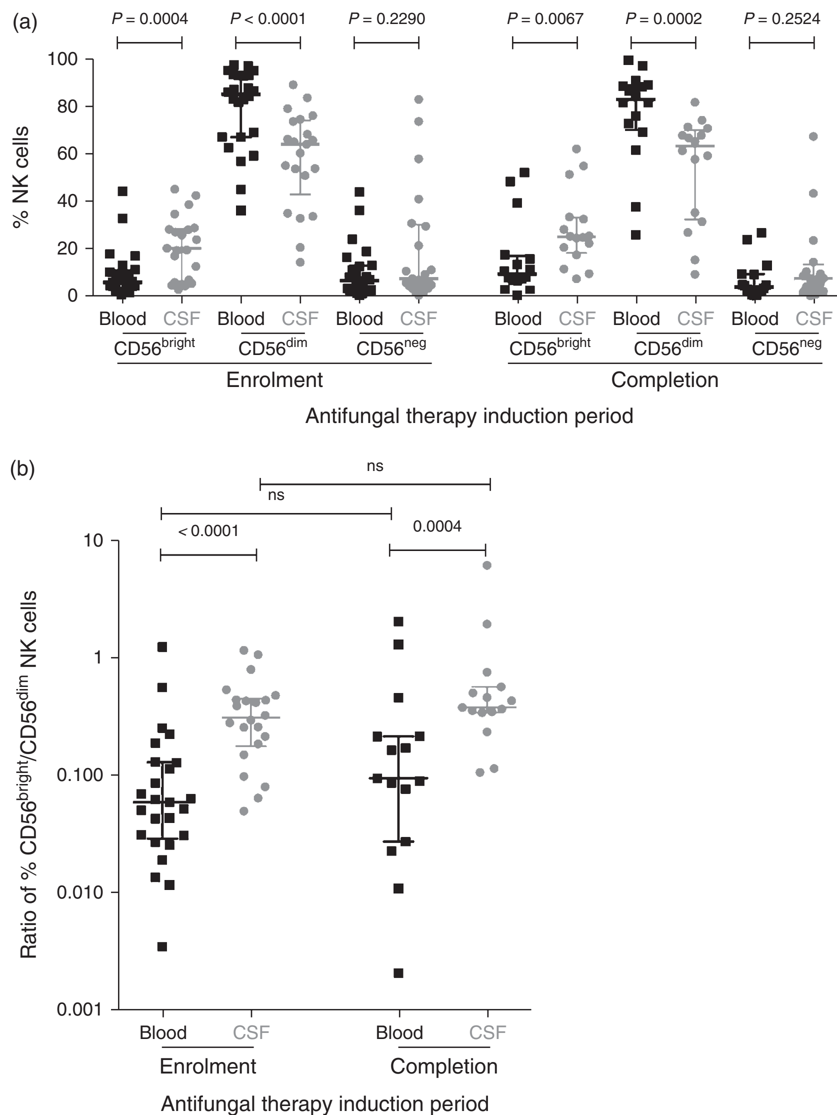

Fig. 1. Absolute proportions and ratios of natural killer-cell subsets at enrolment and completion of induction phase of antifungal therapy in blood and cerebrospinal fluid. At enrolment and after completion of antifungal therapy induction, cerebrospinal fluid (CSF) was enriched with CD56^{bright} natural killer (NK) cells, had fewer CD56^{dim} and similar frequencies of CD56^{neg} NK cells relative to blood (a) and hence the ratio of %CD56^{bright} (immunoregulatory) NK cells to %CD56^{dim} (cytotoxic) NK cells was higher in CSF than blood at enrolment and after completion of antifungal therapy induction in HIV patients with cryptococcal meningitis (b). Medians (horizontal lines) and interquartile ranges (whiskers) are shown in each graph. Measurements in blood denoted by black squares (■) and in CSF denoted by gray circles (•). NS, not significant.

time-point or antifungal therapy. In summary, these data demonstrate that NK cells differ in expression of CXCR3 and to a lesser extent CX3CR1 chemokine receptors according to compartment and subset.

Cytotoxic and immunoregulatory natural killer cells in cerebrospinal fluid were more activated than natural killer cells in blood

Activation is a necessary precursor of both CD56^{dim} and CD56^{bright} NK-cell activity. To gain insight into the role of these NK cells in cryptococcal meningitis pathogenesis, we examined the proportion of activated cells in each subset by measuring cell-surface expression of CD69, an early marker of lymphocyte activation. CD56^{bright} and CD56^{dim} NK cells in CSF were more activated than blood NK cells (Fig. 3) at enrolment (median 48.1 vs. 14.3%, median $\Delta = 37.7\%$, $P < 0.0001$; and median 54.2 vs. 19.7%, median $\Delta = 33.3\%$, $P < 0.0001$, respectively) and after completion of antifungal therapy induction (median 52.9 vs. 7.9%, median $\Delta = 43.2\%$, $P = 0.0001$;

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

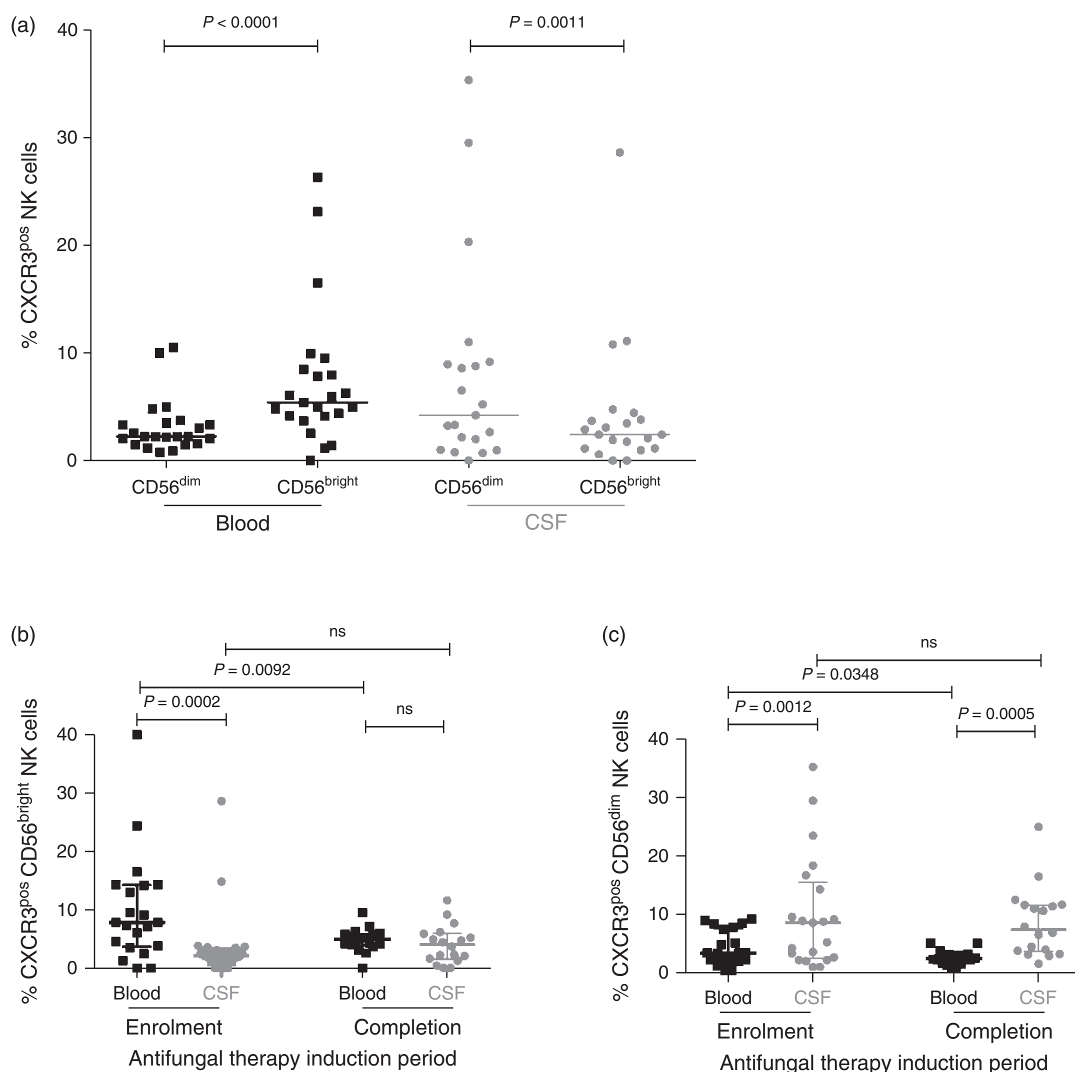

Fig. 2. Chemokine receptor 3 expression on all natural killer cells at enrolment and by subset at enrolment and completion of induction phase of antifungal therapy in blood and cerebrospinal fluid. CXCR3 expression differed by natural killer-cell subset ($CD56^{bright}$, $CD56^{dim}$) and compartment. In blood, at enrolment, the proportion of natural killer (NK) cells expressing CXCR3 was higher among $CD56^{bright}$ NK cells than among $CD56^{dim}$ NK cells, but the opposite was observed in cerebrospinal fluid (CSF) (a). The proportion of $CD56^{bright}$ NK cells expressing CXCR3 was significantly higher in blood at enrolment but declined following antifungal therapy. By completion of antifungal therapy, the proportion in blood was similar to CSF (b). In contrast, the proportion of $CD56^{dim}$ NK cells expressing CXCR3 was significantly higher in CSF than blood at enrolment and completion of antifungal therapy (c). Medians (horizontal lines) and interquartile ranges (whiskers) are shown in each graph. Measurements in blood denoted by black squares (■) and in CSF denoted by gray circles (●).

and median 46.5 vs. 9.95%, median $\Delta = 31.6\%$, $P = 0.0003$, respectively). Although antifungal therapy did not significantly reduce the proportion of activated NK cells in blood, or the proportion of activated $CD56^{bright}$ NK cells in CSF, after completion of antifungal therapy induction, the proportion of activated $CD56^{dim}$ NK cells in CSF was significantly reduced (Fig. 3). After completion of antifungal therapy induction, the proportion of activated blood NK cells was positively associated with the plasma HIV viral load ($r = 0.65$, $P = 0.007$). Consistent with previous reports [30,31], cART commencement was associated with a significant decline in the proportions of $CD69^{pos}$ NK cells in both $CD56^{bright}$ (median 16.1 vs. 5.7%, $P < 0.0001$) and $CD56^{dim}$ blood NK-cell subsets (median 10.3 vs. 6.7%, $P = 0.03$, Supplementary Figure 2C and 2D, http://links.lww.com/QAD/A470).

Immunoregulatory natural killer cells in cerebrospinal fluid expressed higher levels of CXCL10 and lower levels of tumor necrosis factor α than natural killer cells in blood prior to commencing antifungal therapy

Next, we examined whether the chemokine and cytokine secretion profiles of $CD56^{bright}$ NK cells in CSF differed from those in blood. CSF levels of the

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

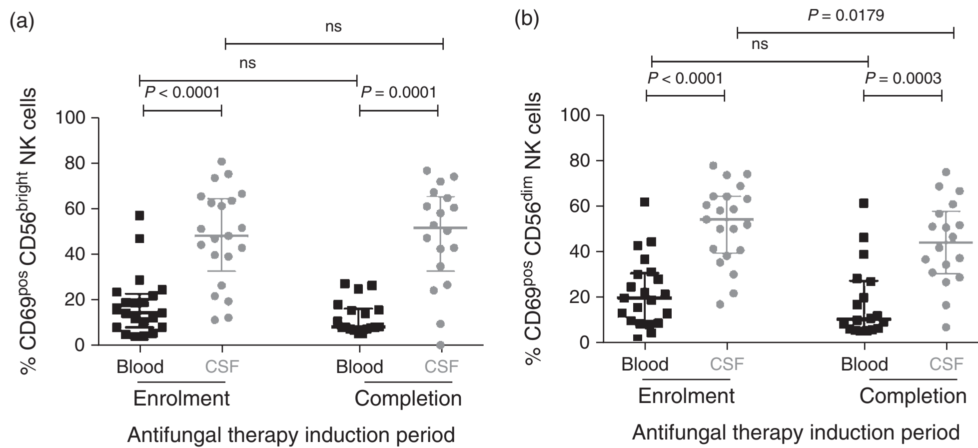

Fig. 3. Natural killer-cell activation at enrolment and completion of the induction phase of antifungal therapy in blood and cerebrospinal fluid according to natural killer-cell subset. CD56^{bright} (a) and CD56^{dim} (b) natural killer (NK) cells were more activated in cerebrospinal fluid (CSF) than in blood at enrolment and completion of antifungal therapy, and activation was only partially reduced by antifungal therapy induction. Medians (horizontal lines) and interquartile ranges (whiskers) are shown in each graph. Measurements in blood denoted by black squares (■) and in CSF denoted by gray circles (●).

chemokine CXCL-10 (IP-10) levels in CSF correlate directly with neuronal injury in CNS HIV disease [32]. Similarly, the amounts of proinflammatory chemokines and cytokines, including CXCL-10 and TNF-α, correlate with clinical outcomes in cryptococcal meningitis and HIV coinfection prior to starting cART [33]. Thus, to quantify differences in functional responses during HIV and cryptococcal meningitis coinfection, we compared intracellular cytokine profiles of CXCL-10 and TNF-α in NK cells. We obtained paired blood and CSF samples at enrolment from five participants and performed intracellular cytokine staining for CXCL-10 and TNF-α. The proportion of CD56^{bright} NK cells in CSF expressing CXCL-10 tended to be higher than in blood (median 57.6 vs. 35.7% median $\Delta = 19.8\%$, $P = 0.06$, Fig. 4). Conversely, the proportion of CD56^{bright} NK cells in CSF expressing TNF-α tended to be lower than in blood (median 64.2 vs. 42.3%, median $\Delta = 19.84\%$, $P = 0.06$, Fig. 4).

Cerebrospinal fluid was enriched for nonclassical monocytes in cryptococcal meningitis prior to antifungal therapy

NK cells engage in a bidirectional communication with other innate and adaptive immune cells. During neuroinflammation, monocytes are a major cell type that is recruited to the CNS [34], unlike other tissues that recruit neutrophils. Therefore, we also evaluated our flow cytometric data to quantify monocyte subsets in CSF and blood.

Relative to blood, CSF was enriched for nonclassical 'proinflammatory' monocytes at enrolment (median 3.12 vs. 0.78%, median $\Delta = 1.83\%$, $P = 0.001$, Fig. 5a). Correspondingly, the proportion of classical monocytes was lower in CSF than blood (median 30 vs. 64%, median $\Delta = 59.8\%$, $P = 0.0007$). There was also a trend toward a greater proportion of intermediate monocytes in CSF than blood (median 18.1 vs. 13.5%, median $\Delta = 6.5\%$, $P = 0.07$).

Antifungal therapy restores proportions of classical monocytes in cerebrospinal fluid

Comparison of the proportions of the three major monocyte subsets in CSF at enrolment and after completion of antifungal therapy demonstrated that the proportion of classical monocytes significantly increased

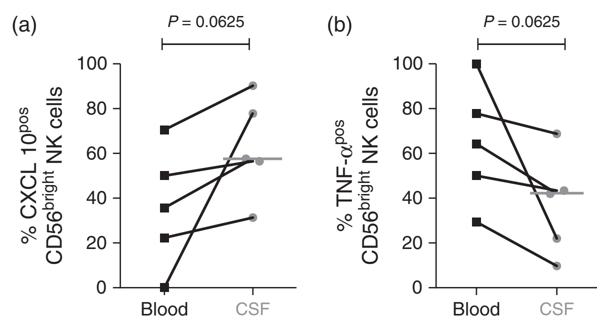

Fig. 4. Functional capacity of natural killer cells in blood and cerebrospinal fluid at enrolment to induction phase antifungal therapy. At enrolment, the proportion of CD56^{bright} natural killer (NK) cells producing CXCL-10 was higher in cerebrospinal fluid (CSF) than in blood (a), but the proportion producing tumor necrosis factor α (TNF-α) was lower in CSF than in blood (b; $n = 5$). Results are expressed as the percentage of cytokine-producing cells. Measurements in blood denoted by black squares (■) and in CSF denoted by gray circles (●).

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

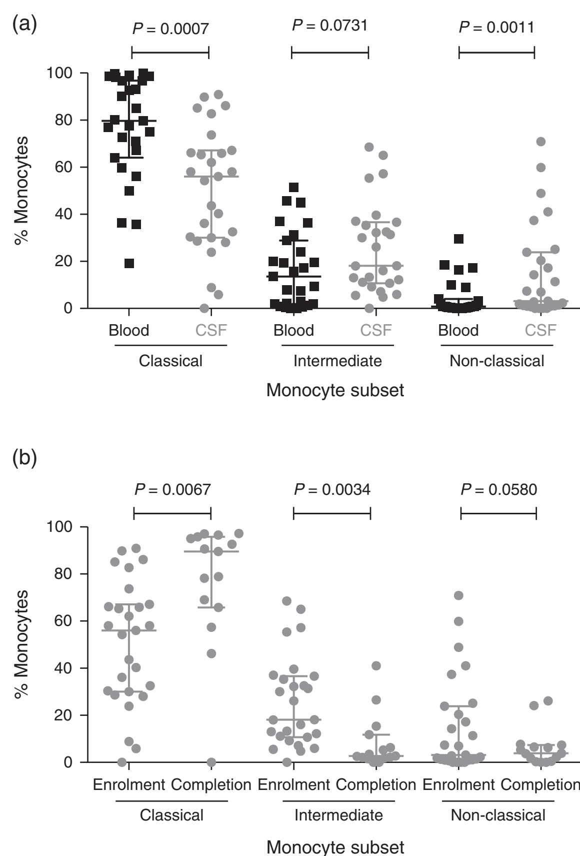

Fig. 5. Monocyte subsets in blood and cerebrospinal fluid at enrolment and in cerebrospinal fluid only at enrolment and completion of induction phase of antifungal therapy. The proportions of nonclassical and intermediate monocytes in cerebrospinal fluid (CSF) declined following antifungal therapy induction, whereas the proportion of classical monocytes increased. At enrolment, CSF was enriched with nonclassical monocytes ($CD14^{lo}CD16^{hi}$) and intermediate monocytes ($CD14^{hi}CD16^{lo}$), but had significantly lower proportions of classical monocytes ($CD14^{hi}{}_{i}CD16^{neg}$) (a). After completion of antifungal therapy induction, the proportions of classical monocyte in CSF were increased and the proportions of intermediate and nonclassical monocytes in CSF were reduced (b). Results are expressed as the percentage of total monocytes. Medians (horizontal lines) and interquartile ranges (whiskers) are shown in each graph. Measurements in blood denoted by black squares (■) and in CSF denoted by gray circles (●).

over time (median 36.1 to 89.5%, median $\Delta = 23.4\%$, $P = 0.006$, Fig. 5b). In contrast, the proportion of intermediate monocytes significantly decreased (median 13 vs. 2.69%, median $\Delta = 8.4\%$, $P = 0.003$), while the proportion of nonclassical monocytes also declined, but the difference did not achieve statistical significance (median 2.78 vs. 3.85%, median $\Delta = 2.5\%$, $P = 0.06$). In comparison, there were no significant differences between proportions of monocyte subsets in blood at enrolment and after 14 days of antifungal therapy (data not shown).

Discussion

Here we assessed NK cells and monocytes in CSF and blood in patients with HIV-cryptococcal meningitis prior to and following antifungal therapy induction and cART. We found that markers of activation and/or function expressed by NK cells and monocytes were compartmentalized in the CNS relative to blood. These findings suggest that immunoregulatory NK cells and nonclassical monocytes may play a role in cryptococcal meningitis pathogenesis. Such changes might contribute to adverse outcomes after commencing cART, such as cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS).

Consistent with previous reports of the phenotype of NK cells in extravascular tissues [2] and during CNS infections [35], we found a higher proportion of immunoregulatory ($CD56^{bright}$) NK cells in CSF than in blood. Homing of plasmablasts and T cells to the CNS compartment has been shown to be mediated by CXCR3 [36,37], a receptor for CXCL10. We previously reported that there was a higher concentration of CXCL-10 in CSF than in blood in this cohort [28]. CX3CL1 has also been reported to mediate migration of NK cells to the CNS during experimental allergic encephalomyelitis [29]. We observed differences between blood and CSF in the proportions of both $CXCR3^{pos}$ and $CX3CR1^{pos}$ NK cells. We, therefore, speculate that the enhanced CXCR3 and/or CX3CR1 expression on $CD56^{bright}$ NK cells in blood that we observed may have equipped these cells to enter the CNS compartment in response to a CXCL-10 or CX3CL1 gradient. Tracking chemokine expression on particular cells to test this hypothesis was beyond the scope of our work, and remains to be tested.

It is notable that the proportion of CXCR3-expressing NK cells in the CSF was not affected by antifungal therapy induction. This subset has been reported to have impaired cytotoxic and cytokine-secretory capacity in hepatitis C virus infection [26]. The maintenance of this population of NK cells in CSF may have implications in the development of adverse clinical sequelae such as C-IRIS. Understanding of role of this subset in cryptococcal meningitis infection will benefit from further study.

We extended previous reports of NK cells in the CNS by assessing their cytokine profiles and activation status over time. As predicted, our findings were consistent with previous reports of decreased activation of NK cells in blood of HIV-infected adults following cART initiation and the reduction of HIV burden [30,31]. However, we observed a partial decline in NK-cell activation only in the $CD56^{dim}$ subset in the CNS following 2 weeks of antifungal therapy. We attributed the maintenance of NK-cell activation in the CNS to either residual pathogen burden in the CNS [17] or an intrinsic high threshold for deactivation.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Our preliminary discovery that immunoregulatory NK cells secreted more CXCL-10 in CSF than in blood, suggested that they were preferentially promoting a proinflammatory environment in the CNS compartment. The observation that the immunoregulatory NK cells in CSF produced less TNF-α than in blood may be a result of interaction with dendritic cells or with cryptococci [38]. Taking into consideration that NK cells also generate strong interferon-γ responses to *Cryptococcus neoformans* [5] and that interferon-γ levels in CSF are one of the leading predictors of clinical outcomes in cryptococcal meningitis pathogenesis [33], they could be candidates for immune modulation to improve clinical outcomes in this disease. However, we examined only a small number of patients and further studies are needed.

In contrast to NK cells, we observed a rebalancing of monocyte subsets following antifungal therapy induction. After 14 days of antifungal therapy, the proportions of intermediate monocyte subsets in CSF declined, whereas the proportion of classical monocytes increased. We attribute this change to differences in functional roles during clearance of *Cryptococcus*. Unlike classical monocytes, nonclassical and intermediate monocytes secrete large amounts of TNF-α and IL-1β, are expanded during many infectious diseases [39–41], including HIV [42], and are preferentially recruited to sites of inflammation [16]. Restoration of monocyte subset distribution with antifungal therapy suggests that reducing antigen burden is sufficient to restore monocyte homeostasis in the CNS compartment.

Although we discovered novel changes in NK cells and monocyte phenotypes in the CNS compartment during treated cryptococcal meningitis disease, our findings have limitations. Because we were unable to quantify absolute numbers of cells in either CSF or in blood, we cannot infer whether absolute numbers of specific subsets were altered. With the exception of antifungal therapy induction, we were unable to examine the association between innate immunological events in CSF or blood and clinical outcomes. Nor can we definitely demonstrate whether these observations are specific to HIV/cryptococcal meningitis or may be observed in other forms of meningoencephalitis with or without HIV infection. We examined the effect of cART in only nine patients. Larger studies are required to establish the clinical relevance of our findings. Nevertheless, our data provided new insights into regulation of compartmentalized immune responses during treated cryptococcal meningitis disease in adults with advanced HIV.

In summary, our findings suggest that NK cell and monocyte responses to cryptococci are compartmentalized in patients with cryptococcal meningitis and HIV coinfection. Furthermore, they highlight a potential role of immunoregulatory NK cells and different monocyte subsets in cryptococcal meningitis pathogenesis. Prospective studies of CNS-resident NK cells and monocytes, and their association with clinical outcomes, such as C-IRIS, are warranted.

Acknowledgements

The authors would like to thank the staff of the HIV Pathogenesis Programme (HPP) at the University of KwaZulu-Natal (Durban, South Africa) for their assistance in processing clinical samples.

V.N. and C.C. conceived and conducted the experiments described in this study. R.D., S.O., and A.L. provided technical support for experiments. M.Y.S., J.H.E., T.N., S.R., M.A.F., and W.H.C. provided overall oversight of the clinical cohort accrual, follow-up, and experimental procedures. M.A.F. and W.H.C. provided critical advice throughout the conduct of the study. All the authors read, commented, and approved the final version of this article.

This study was supported by the South African HIV/AIDS Research Platform (SHARP), the REACH initiative grant 2007, and US National Institutes for Health FIC K01-TW007793. V.N. was supported by LIFELab and the Columbia University-South Africa Fogarty AIDS International Training and Research Program (AITRP, grant #D43 TW000231). C.C.C. was supported by an Australian Postgraduate Award 2009, Australian National Health and Medical Research Council (NHMRC) Postgraduate Scholarship 2010–2012. S.R.L. is a NHMRC Practitioner Fellow. T.N. holds the South African Research Chair in Systems Biology of HIV/AIDS and is a Howard Hughes Medical Institute International Early Career Scientist. Additional training was supported by the South African National Research Foundation KISC Award.

Conflicts of interest

There are no conflicts of interest.

References

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. **Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS.** *AIDS* 2009; **23**:525–530.
2. Fehniger TA, Cooper MA, Nuovo GJ, Cella M, Facchetti F, Colonna M, *et al*. **CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity.** *Blood* 2003; **101**:3052–3057.
3. Mavilio D, Lombardo G, Benjamin J, Kim D, Follman D, Marcenaro E, *et al*. **Characterization of CD56–/CD16+ natural killer (NK) cells: a highly dysfunctional NK subset expanded in HIV-infected viremic individuals.** *Proc Natl Acad Sci U S A* 2005; **102**:2886–2891.
4. Hidore MR, Nabavi N, Sonleitner F, Murphy JW. **Murine natural killer cells are fungicidal to Cryptococcus neoformans.** *Infect Immun* 1991; **59**:1747–1754.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

5. Marr KJ, Jones GJ, Zheng C, Huston SM, Timm-McCann M, Islam A, *et al*. **Cryptococcus neoformans directly stimulates perforin production and rearms NK cells for enhanced anticryptococcal microbicidal activity.** *Infect Immun* 2009; **77**:2436–2446.
6. Kawakami K, Koguchi Y, Qureshi MH, Yara S, Kinjo Y, Uezu K, *et al*. **NK cells eliminate Cryptococcus neoformans by potentiating the fungicidal activity of macrophages rather than by directly killing them upon stimulation with IL-12 and IL-18.** *Microbiol Immunol* 2000; **44**:1043–1050.
7. Ransohoff RM, Kivisakk P, Kidd G. **Three or more routes for leukocyte migration into the central nervous system.** *Nat Rev Immunol* 2003; **3**:569–581.
8. Poli A, Kmiecik J, Domingues O, Hentges F, Blery M, Chekenya M, *et al*. **NK cells in central nervous system disorders.** *J Immunol* 2013; **190**:5355–5362.
9. Ho EL, Ronquillo R, Altmeppen H, Spudich SS, Price RW, Sinclair E. **Cellular composition of cerebrospinal fluid in HIV-1 infected and uninfected subjects.** *PLoS One* 2013; **8**:e66188.
10. Zhang AL, Colmenero P, Purath U, Teixeira de Matos C, Hueber W, Klareskog L, *et al*. **Natural killer cells trigger differentiation of monocytes into dendritic cells.** *Blood* 2007; **110**:2484–2493.
11. Michel T, Hentges F, Zimmer J. **Consequences of the crosstalk between monocytes/macrophages and natural killer cells.** *Front Immunol* 2012; **3**:403.
12. Wong KL, Yeap WH, Tai JJ, Ong SM, Dang TM, Wong SC. **The three human monocyte subsets: implications for health and disease.** *Immunol Res* 2012; **53**:41–57.
13. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, *et al*. **The proinflammatory CD14+CD16+ DR++ monocytes are a major source of TNF.** *J Immunol* 2002; **168**:3536–3542.
14. Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F. **Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans.** *Infect Immun* 2009; **77**:120–127.
15. Tascini C, Vecchiarelli A, Preziosi R, Francisci D, Bistoni F, Baldelli F. **Granulocyte-macrophage colony-stimulating factor and fluconazole enhance anticryptococcal activity of monocytes from AIDS patients.** *AIDS* 1999; **13**:49–55.
16. Shi C, Pamer EG. **Monocyte recruitment during infection and inflammation.** *Nat Rev Immunol* 2011; **11**:762–774.
17. Chang CC, Dorasamy AA, Gosnell BI, Elliott JH, Spelman T, Omarjee S, *et al*. **Clinical and mycological predictors of cryptococcosis-associated immune reconstitution inflammatory syndrome (C-IRIS).** *AIDS* 2013; **27**:2089–2099.
18. Republic of South Africa Department of Health. South African National Antiretroviral Treatment Guidelines. 2010.
19. Svenningsson A, Andersen O, Edsbagge M, Stemme S. **Lymphocyte phenotype and subset distribution in normal cerebrospinal fluid.** *J Neuroimmunol* 1995; **63**:39–46.
20. Neuenburg JK, Cho TA, Nilsson A, Bredt BM, Hebert SJ, Grant RM, *et al*. **T-cell activation and memory phenotypes in cerebrospinal fluid during HIV infection.** *J Acquir Immune Defic Syndr* 2005; **39**:16–22.
21. Margolick JB, McArthur JC, Scott ER, McArthur JH, Cohn S, Farzadegan H, *et al*. **Flow cytometric quantitation of T cell phenotypes in cerebrospinal fluid and peripheral blood of homosexual men with and without antibodies to human immunodeficiency virus, type I.** *J Neuroimmunol* 1988; **20**:73–81.
22. Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR. **Interpreting flow cytometry data: a guide for the perplexed.** *Nat Immunol* 2006; **7**:681–685.
23. Moretta L. **Dissecting CD56dim human NK cells.** *Blood* 2010; **116**:3689–3691.
24. Brunetta E, Hudspeth KL, Mavilio D. **Pathologic natural killer cell subset redistribution in HIV-1 infection: new insights in pathophysiology and clinical outcomes.** *J Leukoc Biol* 2010; **88**:1119–1130.
25. Milush JM, Long BR, Snyder-Cappione JE, Cappione AJ 3rd, York VA, Ndhlovu LC, *et al*. **Functionally distinct subsets of human NK cells and monocyte/DC-like cells identified by coexpression of CD56, CD7, and CD4.** *Blood* 2009; **114**:4823–4831.
26. Eisenhardt M, Glassner A, Kramer B, Korner C, Sibbing B, Kokordelis P, *et al*. **The CXCR3(+)CD56Bright phenotype characterizes a distinct NK cell subset with antifibrotic potential that shows dys-regulated activity in hepatitis C.** *PLoS One* 2012; **7**:e38846.
27. Billottet C, Quemener C, Bikfalvi A. **CXCR3, a double-edged sword in tumor progression and angiogenesis.** *Biochim Biophys Acta* 2013; **1836**:287–295.
28. Chang CC, Omarjee S, Lim A, Spelman T, Gosnell BI, Carr WH, *et al*. **Chemokine levels and chemokine receptor expression in blood and the CSF of HIV-infected patients with cryptococcal meningitis and C-IRIS.** *J Infect Dis* 2013; **208**:1604–1612.
29. Huang D, Shi FD, Jung S, Pien GC, Wang J, Salazar-Mather TP, *et al*. **The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system.** *FASEB J* 2006; **20**:896–905.
30. Le Guillou-Guillemette H, Renier G, Vielle B, Abgueguen P, Chennebault JM, Lunel F, *et al*. **Immune restoration under HAART in patients chronically infected with HIV-1: diversity of T, B, and NK immune responses.** *Viral Immunol* 2006; **19**:267–276.
31. Alter G, Teigen N, Ahern R, Streeck H, Meier A, Rosenberg ES, *et al*. **Evolution of innate and adaptive effector cell functions during acute HIV-1 infection.** *J Infect Dis* 2007; **195**:1452–1460.
32. Letendre SL, Zheng JC, Kaul M, Yiannoutsos CT, Ellis RJ, Taylor MJ, *et al*. **Chemokines in cerebrospinal fluid correlate with cerebral metabolite patterns in HIV-infected individuals.** *J Neurovirol* 2011; **17**:63–69.
33. Boulware DR, Bonham SC, Meya DB, Wiesner DL, Park GS, Kambugu A, *et al*. **Paucity of initial cerebrospinal fluid inflammation in cryptococcal meningitis is associated with subsequent immune reconstitution inflammatory syndrome.** *J Infect Dis* 2010; **202**:962–970.
34. Terry RL, Getts DR, Deffrasnes C, van Vreden C, Campbell IL, King NJ. **Inflammatory monocytes and the pathogenesis of viral encephalitis.** *J Neuroinflammation* 2012; **9**:270.
35. Hamann I, Dorr J, Glumm R, Chanvillard C, Janssen A, Millward JM, *et al*. **Characterization of natural killer cells in paired CSF and blood samples during neuroinflammation.** *J Neuroimmunol* 2013; **254**:165–169.
36. Marques CP, Kapil P, Hinton DR, Hindinger C, Nutt SL, Ransohoff RM, *et al*. **CXCR3-dependent plasma blast migration to the central nervous system during viral encephalomyelitis.** *J Virol* 2011; **85**:6136–6147.
37. Stiles LN, Hosking MP, Edwards RA, Strieter RM, Lane TE. **Differential roles for CXCR3 in CD4+ and CD8+ T cell trafficking following viral infection of the CNS.** *Eur J Immunol* 2006; **36**:613–622.
38. Murphy JW, Zhou A, Wong SC. **Direct interactions of human natural killer cells with Cryptococcus neoformans inhibit granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha production.** *Infect Immun* 1997; **65**:4564–4571.
39. Castano D, Garcia LF, Rojas M. **Increased frequency and cell death of CD16+ monocytes with Mycobacterium tuberculosis infection.** *Tuberculosis (Edinb)* 2011; **91**:348–360.
40. Fingerle G, Pforte A, Passlick B, Blumenstein M, Strobel M, Ziegler-Heitbrock HW. **The novel subset of CD14+/CD16+ blood monocytes is expanded in sepsis patients.** *Blood* 1993; **82**:3170–3176.
41. Soares G, Barral A, Costa JM, Barral-Netto M, Van Weyenbergh J. **CD16+ monocytes in human cutaneous leishmaniasis: increased ex vivo levels and correlation with clinical data.** *J Leukoc Biol* 2006; **79**:36–39.
42. Han J, Wang B, Han N, Zhao Y, Song C, Feng X, *et al*. **CD14(high)CD16(+) rather than CD14(low)CD16(+) monocytes correlate with disease progression in chronic HIV-infected patients.** *J Acquir Immune Defic Syndr* 2009; **52**:553–559.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.