

Analysis of Dominant HIV Quasispecies Suggests Independent Viral Evolution Within Spinal Granulomas Coinfected with *Mycobacterium tuberculosis* and HIV-1 Subtype C

Sivapragashini Danaviah,¹ Tulio de Oliveira,¹ Michelle Gordon,^{2,3} Shunmugam Govender,⁴ Paul Chelule,⁵ Sureshnee Pillay,¹ Thajasvarie Naicker,⁶ Sharon Cassol,⁷ and Thumbi Ndung'u^{3,8-10}

Abstract

Extrapulmonary tuberculosis (TB) is a significant public health challenge in South Africa and worldwide, largely fuelled by the HIV epidemic. In spinal TB, *Mycobacteria* infect the spinal column without dissemination to the spinal cord. The immune microenvironment, target cell characteristics, and other evolutionary forces within granulomas during HIV/TB coinfection are poorly characterized. We investigated whether spinal TB granulomas represent a sequestered anatomical site where independent HIV evolution occurs, and assessed the role of macrophages as a target cell for both HIV and mycobacteria. RNA was extracted from plasma and granulomatous tissue from six antiretroviral-naive HIV-1/spinal TB-coinfected patients, RT-PCR amplified, and the C2-V5 *env* segment was cloned and sequenced. Analysis of genetic diversity, phylogeny and coalescence patterns was performed on clonal sequences. To investigate their role in HIV sequestration, macrophages and the HIV-1 p24 protein were immune localized and ultrastructural features were studied. Intercompartment diversity measurements and phylogenetic reconstruction revealed anatomically distinct monophyletic HIV-1 clusters in four of six patients. Genotypic CCR5-tropic variants were predominant (98.9%) with conservation of putative N-linked glycosylation sites in both compartments. CD68⁺ reactivity was associated with higher tissue viral load ($r=1.0$; $p<0.01$) but not greater inpatient diversity ($r=0.60$; $p>0.05$). Ultrastructural imaging revealed the presence of bacterial and virus-like particles within membrane-bound intracellular compartments of macrophages. Spinal tuberculosis granulomas may form anatomically discrete sites of divergent viral evolution. Macrophages in these granulomas harbored both pathogens, suggesting that they may facilitate the process of viral sequestration within this compartment.

Introduction

ONE OF THE MOST DEVASTATING COMPLICATIONS of the worldwide HIV-1/AIDS epidemic has been the dramatic increase in the incidence of active tuberculosis (TB) at pul-

monary and extrapulmonary sites.¹ HIV infection contributed to 1.1 million new TB infections and approximately 320,000 TB-related deaths in 2012 alone; 75% of these occurred in Africa.¹ This enormous disease burden represents a serious challenge to TB control programs in resource-constrained

¹Africa Centre for Health and Population Studies, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal (UKZN), Durban, South Africa.

²Department of Virology, Nelson R. Mandela School of Medicine, UKZN, Durban, South Africa.

³KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), UKZN, Durban, South Africa.

⁴Department of Orthopedics, Nelson R. Mandela School of Medicine, UKZN, Durban, South Africa.

⁵School of Public Health, Sefako Makgatho Health Sciences University, Medunsa, South Africa.

⁶Optics and Imaging Centre, Nelson R. Mandela School of Medicine, UKZN, Durban, South Africa.

⁷MRC Inflammation and Immunity Unit, Department of Immunology, University of Pretoria, Pretoria, South Africa.

⁸HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, UKZN, Durban, South Africa.

⁹Max Planck Institute for Infection Biology, Chariteplatz, Berlin, Germany.

¹⁰The Ragon Institute of MGH, MIT and Harvard University, Cambridge, Massachusetts.

regions where access to combination antiretroviral therapy (ART) and large-scale antituberculosis prophylaxis is still suboptimal and logistically challenging. HIV-1-induced immunodeficiency leads to increased risk of newly acquired TB and adverse clinical outcomes.² Furthermore, HIV replication, diversity, persistence in tissue compartments and reservoirs complicate clinical outcomes and pose a threat to therapeutic management and cure strategies.^{3,4}

HIV-1 diversity is a function of its error-prone reverse transcriptase, replication rate, recombination, and host selective pressures.⁵ Following transmission, HIV-1 spreads to various anatomical sites where the site-specific microenvironment regulates its evolutionary kinetics.^{6,7} HIV-1 replication in remote or sequestered anatomical sites may have clinical consequences by creating a source of virus for transmission, drug resistance (due to poor drug penetration), tissue-specific clinical deterioration, and establishment of long-lived viral reservoirs.^{8,9} The role of coinfections such as TB, and related immune and cellular microenvironment remodeling in promoting independent viral evolution at tissue sites of infection, is poorly understood. We do know that these sites have clinical relevance as they may prevent access of infected cells to ART and appropriate host immune responses.³ Anatomical compartments representing discrete sites of independent viral evolution include the central nervous system, genital tract, kidney epithelium, and lymph nodes.¹⁰⁻¹⁴ Whether the same is true within TB coinfecting granulomas is currently unclear and inconclusive.¹⁵⁻¹⁷ Understanding the dynamics of viral evolution within this unique highly compartmentalized but immunologically active environment is important to virus eradication strategies. The nature of the viral variants, their response to treatment, and the mechanisms that govern their progression to sequestration may play critical roles in clinical patient management.

The host immune response to TB is not fully characterized but involves primarily macrophages and CD4⁺ and CD8⁺ T cells.^{2,18} Histopathology studies of pulmonary and pleural TB have been performed¹⁸ but exploratory investigations of the spine are rare.^{19,20} None of these studies, however, fully explores the role of specific cell types, such as macrophages, as drivers and/or sites of divergent HIV evolution.

In this study we sought to investigate whether the extradural spinal TB granuloma formed an anatomical site of HIV sequestration by analyzing clonal sequences generated from the blood and spinal granuloma biopsies of coinfecting patients. Our investigation revealed evidence of divergent viral evolution within spinal TB granulomas. We also explored the immune microenvironment with specific focus on macrophages given that TB and HIV are intracellular pathogens that target macrophages. Immunolocalization studies revealed the presence of HIV-infected macrophages while electron microscopy provided evidence of HIV and TB particles within an intracellular endosomal compartment.

Materials and Methods

Study subjects, clinical assessment, and specimen collection

The Institutional Review Board of the University of KwaZulu-Natal approved the study protocol (Ref. #H112/02). All participants or their legal guardians provided informed consent. Blood and matched granuloma tissue were

obtained from six HIV-1/spinal tuberculosis coinfecting patients undergoing vertebral excision and anterior spinal decompression to resolve associated neurological deficits at King George V Hospital (now King Dinuzulu Hospital) in Durban, South Africa. Given that extrapulmonary TB with HIV coinfection is AIDS defining (WHO stage 4) all patients were classified accordingly. Cerebrospinal fluid (CSF) was not sampled since there was no clinical justification to sample the CSF given that the *Mycobacterium tuberculosis* (Mtb) infection had not disseminated and breached the blood-brain barrier. All patients received preoperative nutritional support to achieve safe hemoglobin and albumin levels (>100 g/liter and >300 g/liter, respectively) and standard antituberculosis therapy (600 mg rifampicin, 400 mg isoniazid, 1,500 mg pyrazinamide, 1,500 mg and 1,200 mg ethambutol daily) for a mean period of 8 weeks (range = 5–24 weeks). All patients were antiretroviral (ARV) drug naive since ART had not been rolled out as part of the national policy at the time of sample collection.

The excised granulomatous tissue was processed immediately for histology, bacterial culture diagnostics, and molecular assays. A venous blood sample was collected for quantification of HIV-1 viral load (Nuclisens HIV-1 QT kit, Organon Teknika) and T-lymphocyte subsets (Beckman Coulter TetraOne method). For tissue viral load analysis (Nuclisens HIV-1 QT kit), HIV RNA was extracted from 10 mg of tissue. Tissue was weighed and then homogenized in lysis buffer (Nuclisens isolation kit, Biomerieux) prior to RNA extraction using the MiniMag Magnetic Extraction protocol (Biomerieux) and HIV RNA was quantified with the Nuclisens HIV-1 QT Kit (Biomerieux). Viral loads were expressed as cp/ml of blood for plasma samples and cp/mg of tissue in the case of the granulomas.

Immunolocalization of CD68⁺/p24⁺ cells

CD68⁺ cells (macrophages/monocyte lineages; Clone KP1, Dako; dilution 1:200) and HIVp24 antibody (Clone Kal-1, Dako, Denmark; dilution 1:10) were immunolocalized to 5- μ m-thick sections of granulomatous tissue using an indirect immunoperoxide-staining protocol (Dako Envision Dual Staining kit, Dako). Visualization and capture of positive staining cells utilized the Soft Imaging System (SIS). The region of interest in each section at the same initial magnification ($\times 40$) was selected based on typical features of a granuloma, namely, a central core of necrosis containing macrophages and multinucleated giant cells surrounded by lymphocytes. Additional biopsies were fixed, processed, and embedded using conventional techniques for transmission electron microscopy (TEM). Ultrathin sections (50 nm) were stained according to standard protocols and viewed with the Jeol JEM-1011 transmission electron microscope and iTEM imaging software.

PCR amplification and cloning

Paired plasma and tissue samples were available for sequencing for six patients (three adults, three children). HIV-1 RNA templates, extracted using the MiniMag extraction protocol, were reverse transcribed (Superscript III reverse transcriptase; Invitrogen, San Diego, CA) and a 621-bp segment of the *env* gene (7,026 to 7,647 by HXB2 numbering) was amplified as previously described.²¹ Purified amplicons (Qiagen, Valencia, CA) were cloned into the pCR2.1-TOPO

TA vector (Invitrogen) and positive plasmids (5–10 clones) were purified and sequenced (Big-Dye terminator V3.1).

Sequence editing, alignment, and analysis

All HIV-1 *env* chromatograms were imported into Geneious v6 (www.geneious.com), visually edited, and accepted for analysis if quality scores were >90%. To exclude potential contamination, newly derived sequences were compared to subtype reference strains (Los Alamos database; http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html) and to HIV-1 sequences previously amplified in our laboratory. Consensus sequences generated in Geneious were codon aligned in Clustal W²² and manually edited in SeAL (<http://tree.bio.ed.ac.uk>). Phylogenetic trees were constructed for the entire dataset against HIV-1 reference sequences and for the plasma and granuloma sequences from individual patients. Maximum likelihood (ML) and Bayesian trees were reconstructed in RaxML²³ and MrBayes²⁴ respectively using appropriate models estimated in ModelTest²⁵ and an estimated gamma heterogeneity alpha parameter. The ML tree topology was optimized in RaxML and the reliability of the internal nodes was estimated using 1,000 bootstrap replicates (ML trees) or four Markov Chain Monte Carlo (MCMC) chains with 10⁶ generations and a 10% burning (Bayesian trees). Results were visualized using Tracer parameters where an effective sample size (ess) >200 suggested good mixing and sampling of the trees. Consensus trees and posterior support for internal nodes were generated in TreeAnnotator (www.beast2.org/wiki/index.php/TreeAnnotator) and annotated in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

Analysis to confirm viral compartmentalization and migration

Assessment of viral compartmentalization was performed using tree-based and diversity-based algorithms in DIVEIN (<http://indra.mullins.microbiol.washington.edu/DIVEIN/>) and HyPhy.²⁶ We performed the intracompartiment diversity

and the two-sample diversity tests (*z*-test and *t*-test) in DIVEIN. In HyPhy we performed two tree-based tests of compartmentalization: the Slatkin–Maddison (SM) test to calculate the probability of as many or fewer migrations from one compartment to the other occurring by chance, the branch count correlation coefficient (*rb*), and the branch length correlation (*r*). We also employed the distance-based Hudson nearest-neighbor test (S-nn; TN93 model, 500 resampling). All sequences were screened for signature patterns indicative of viral tropism (CCR5 or CXCR4 usage) using the online Geno2Pheno tool (www.geno2pheno.org).²⁷

Statistical analysis

All statistical analyses were undertaken in Stata v11. A 5% (*p* < 0.05) level of significance was accepted for all analyses unless otherwise stated.

Results

Clinical and demographic findings

All patients (*n* = 6) were diagnosed with active spinal TB based on presumptive clinical and radiological findings. Their demographic and clinical information are summarized in Table 1. Data on when these patients acquired their HIV infection were not available. Because patients did not present with symptoms suggesting other viral infections related diagnostic tests were not performed. Since mycotic infections of the spine have a clinical presentation similar to spinal TB, patient samples were tested for evidence of fungal infection and were found to be negative. Tissue viral loads were high and well in excess of 5 log/mg in all patients. There was no correlation between tissue and plasma viral load measurements (Table 1).

Phylogenetic tree-based analysis of divergence

A total of 94 sequences, generated from tissue and plasma (50 plasma-derived and 44 tissue-derived sequences) of six

TABLE 1. DEMOGRAPHIC, CLINICAL CHARACTERISTICS, AND BASIC LABORATORY FINDINGS OF STUDY POPULATION

Patient Id.	Sex	Age (years)	CD4 count (cells/μl or %) ^a	CD8 count (cells/μl or %) ^a	Plasma viral load (log ₁₀ cp/ml)	Tissue viral load (log ₁₀ cp/mg)	Culture identification	Clinical details ^b
TB12	Female	2	21*	40*	4.2	8.1	No growth	No neurology (Frankel E)
TB25	Female	3	23*	50*	4.3	ND	No growth	Spastic paraplegia
TB32	Male	2	12*	30*	6.4	9.2	Mtb	Advanced AIDS; oral thrush, enlarged and inflamed lymph nodes
TB16	Male	28	ND	ND	4.5	6.5	No growth	Paraplegic; complete immobility (Frankel A); incontinent
TB20	Female	25	100	661	5.7	5.4	Mtb	ND
TB38	Female	43	ND	ND	4.9	8.2	Mtb	ND
Mean ± SD (range)								
Adults		32 ± 15.9	—	—	5.0 ± 0.5	6.7 ± 1.1		
Children		2.3 ± 2.1	18.6 ± 6.1*	40 ± 16.2*	4.9 ± 1.2	8.7 ± 0.8		

^aCD4 and CD8 measurements for adults (≥16 years old) are presented as absolute values and for children (<16 years old) are presented as percentages as indicated by an asterisk (*).

^bClinical data are presented where available.

ND, no data available; Mtb, *Mycobacterium tuberculosis*.

patients, were analyzed. Our objectives were first, to reconstruct patient sequences in relation to reference sequences, and second, to determine the clustering and gene flow patterns of sequences derived from the two anatomic compartments of individual patients. Phylogenetic reconstruction of clonal and published HIV-1 reference sequences (Subtypes A to K) confirmed that all patient sequences were HIV-1 subtype C (Fig. 1A). Tissue-derived and plasma-derived sequences from each patient clustered together, distinct from those of other participants, indicating that there was no interpatient contamination.

Phylogenetic reconstruction of individual patient sequences demonstrated two distinct clustering patterns, the first of discrete monophyletic clusters of tissue-derived and plasma-derived sequences, and the second of intermingling (Fig. 1B–G). In four of six patients (Fig. 1B–E), plasma-derived and tissue-derived sequences clustered in discrete branches. The longer branch lengths observed, coupled with

high internal branch support (posterior probability scores >0.9 and bootstrap values $>80\%$), strongly suggested divergent evolution within these compartments. Tree topologies were suggestive of a founder effect where a single viral variant entered and, over time, expanded into a unique monophyletic cluster within spinal granulomas. In these four cases, all computational tests of compartmentalization (SM, R, r-b, Snn, and intracompartment distance) concurred with statistical significance ($p < 0.01$; Table 2 and Fig. 1B–E). Together, these findings confirmed the separation of plasma-derived and tissue-derived sequences into distinct monophyletic populations in these patients (Fig. 1B–E).

The remaining two datasets, both from children <5 years of age, illustrated the second clustering pattern where plasma-derived and tissue-derived sequences were intermingled with unrestrained migration of HIV-1 variants between compartments (Fig. 1F and G). The tests of compartmentalization for these two study participants (Table 2; Fig. 2E and F)

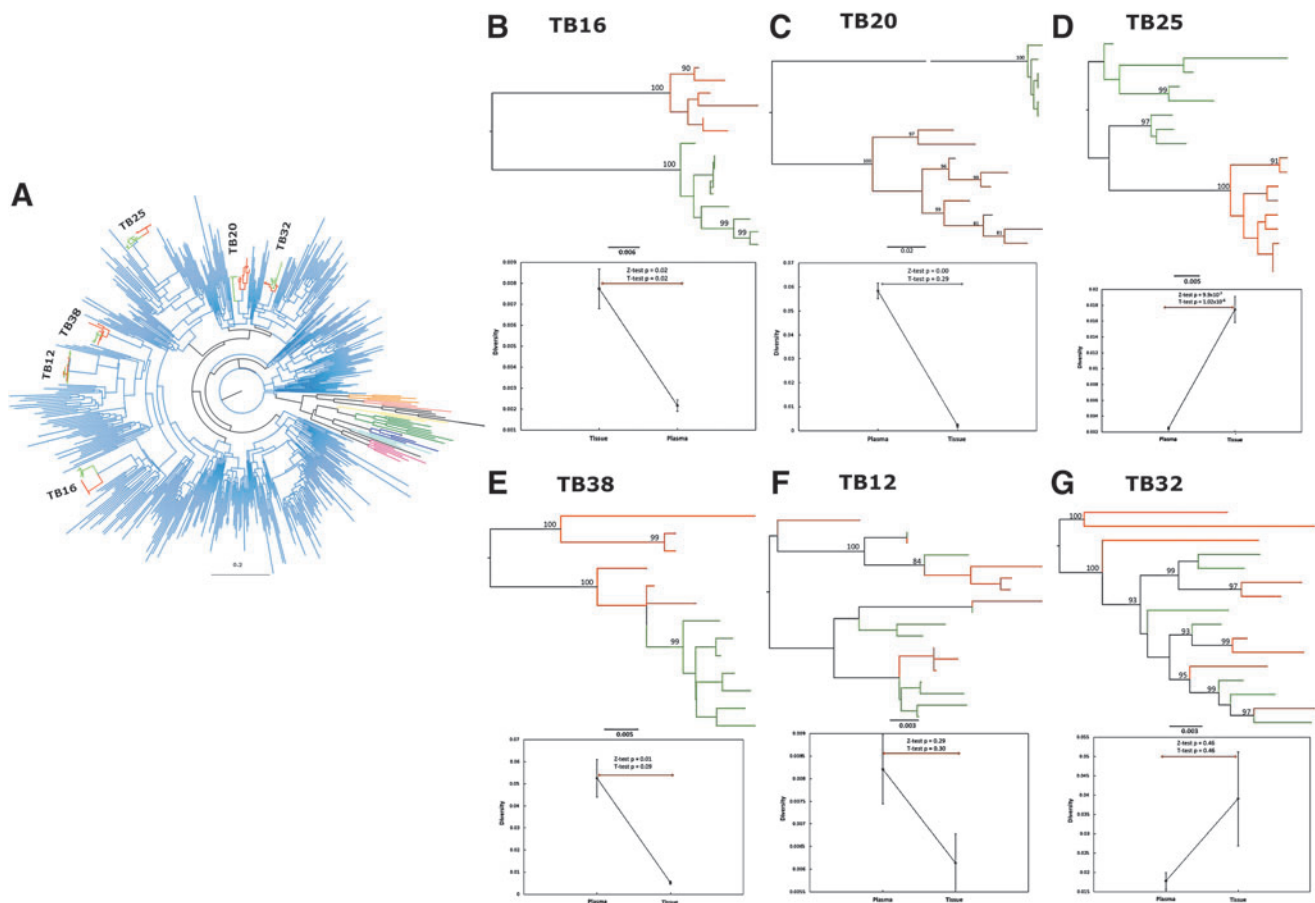


FIG. 1. (A) A maximum likelihood tree of clonal plasma (red) and tissue-derived (green) sequences from individual patients clustering within the subtype C lineages (blue). Reference sequences of subtypes A (pink), B (purple), D (light blue), F (dark green), G (black), H (orange), J (salmon), and K (yellow) are also represented. The phylogenetic reconstruction was performed in RaxML using the HKY85 model with gamma distribution. (B–G) Phylogenetic reconstructions illustrating maximum likelihood trees constructed in RaxML of individual patient plasma-derived (red) and tissue-derived (green) sequences with branch support indicated are represented. (B–E) Two distinct patterns of clustering are observed where monophyletic clustering is shown [(B) TB16; (C) TB20; (D) TB25; (E) TB38)] and intermingling of variants is illustrated [(F) TB12 and (G) TB32]. The panels below each phylogenetic tree are graphic representations of intracompartment diversity measures as estimated in DIVEIN, which illustrates the median diversity and IQR. Two-sample diversity tests (T) were conducted and illustrated are p -values derived from z -tests and t -tests comparing intracompartment diversity of plasma-derived with tissue-derived sequences.

TABLE 2. SUMMARY OF TESTS OF COMPARTMENTALIZATION COMPRISING TREE-BASED (*) AND DIVERSITY-BASED (#) ALGORITHMS

Patient number	Intracompartment diversity ^{#,a}			SM ^{*,b}	R ^{*,c}	r-b ^{*,d}	Hudson ^{#,c} S-nn	CCR5 usage ^{f,g}	
	Plasma (± SD)	Tissue (± SD)	p-value (z-test; t-test)					Plasma (no. and % clones)	Tissue (no. and % clones)
TB12	0.008 ± 0.001	0.006 ± 0.001	0.29; 0.30	4×10^{-5}	0.002	0.002	0.262	9 (100)	9 (100)
TB25	0.002 ± 0.001	0.017 ± 0.002	<u>9.9 × 10⁻⁷</u> ; <u>1.02 × 10⁻⁶</u>	6.7×10^{-5}	0.002	0.002	0.000	8 (100)	8 (100)
TB32	0.002 ± 0.0002	0.040 ± 0.012	0.46; 0.46	0.022	0.002	0.03	0.000	9 (100)	4 (100)
TB16	0.002 ± 0.0002	0.008 ± 0.001	<u>0.02</u> ; <u>0.02</u>	0.0005	0.002	0.002	0.000	6 (100)	9 (100)
TB20	0.058 ± 0.003	0.002 ± 0.001	<u>0.00</u> ; <u>0.29</u>	0.0003	0.002	0.002	0.000	6 (77)	9 (100)
TB38	0.052 ± 0.008	0.005 ± 0.001	<u>0.006</u> ; <u>0.09</u>	0.001	0.004	0.002	0.000	6 (100)	7 (100)

^aIntracompartment pairwise distance (DIVEIN).

^bSlatkin-Maddison test (HyPhy).

^cBranch length correlation coefficient (*r*, HyPhy).

^dBranch count correlation coefficient (*rb*; HyPhy).

^eHudson nearest neighbor (Snn; HyPhy).

^fCoreceptor usage is indicated as percentage number of sequences.

^gCoreceptor usage potential was analyzed using the online tool Geno2Pheno (www.geno2pheno.org) that uses sequence data and support vector machine (SVM) classifiers to predict coreceptor usage.

Tests of compartmentalization were executed in DIVEIN and HyPhy software packages indicating statistical support for divergence and anatomical compartmentalization.

Statistical significance is indicated by *p* values where values <0.05 are *underlined* and <0.005 appear in *bold*.

confirmed our visual observations in the phylogenetic trees; we therefore concluded that there was intermixing in these patients.

Analysis of viral tropism

Since key viral determinants of cell tropism and coreceptor usage are located within the *env* V3 loop,²⁸ we investigated whether there were V3 loop amino acid differences between plasma-derived and tissue-derived sequences (Table 2). The biochemical properties (charge, size, hydrophobicity) at the amino acid positions determining the folding patterns of the V3 loop were similar and indicative of predominantly CCR5-utilizing variants in plasma and tissue. Only two plasma sequences from TB20 differed, where a 2-amino acid insertion resulted in a shift in putative coreceptor usage from CCR5 to CXCR4 (Table 2).

Immunohistology and ultrastructural morphology

Macrophages play an important role, both as target cells and mediators of the immune response, in HIV and TB disease pathogenesis. We therefore explored their possible role in the complex host-pathogen interaction within the anatomically remote spinal granuloma (Fig. 2A and B). While the overall number of CD68⁺ cells infiltrating the granulomas (indicated by the area of reactivity) was comparable, the level of CD68 expression (indicated by the intensity of the stain) differed and correlated with greater tissue viral load ($\rho=1.0$; $p<0.01$) and greater inpatient genetic distance ($\rho=0.60$; $p=0.2$; Fig. 2B). These results suggest that increased site-specific macrophage activity is associated with higher HIV replication rates and viral loads in spinal TB-granulomas, although a causal relationship cannot be established.

For three of the six patients enough tissue samples were available for further IHC analysis of HIV and macrophage

colocalization. Analysis of CD68⁺ cells and HIV-1 p24 antigen by immunohistochemistry within granulomas revealed that in three of the six patients selected for sequence analysis (TB12, TB 16, and TB 25) there was clear evidence of HIV p24-CD68 colocalization (Fig. 2C), with two of these patients (TB 16 and TB 25) possessing tissue-compartmentalized viral sequences. Finally, our investigations of ultrastructural features of coinfection and pathology revealed, under TEM, bacterial (Fig. 2D) and virus-like particles (Fig. 2E) within membrane-bound intracellular compartments in cells identified as macrophages.

Discussion

HIV-1-induced immune suppression has led to an increase in disseminated mycobacterial infections worldwide. Additionally, anatomical and cellular HIV reservoirs are of great clinical interest because they may impact pathogenesis, ART-mediated virus suppression, or strategies for HIV eradication.²⁹ In this study we investigated the impact of anatomic sequestration of HIV, within a tuberculous spinal granuloma on viral evolution. Using phylogenetic reconstruction and computational measures of compartmentalization³⁰ we provide the first evidence to suggest divergent HIV evolution in spinal TB coinfecting granulomas. These findings were supported by the fact that we noted no correlation between tissue and plasma viral load measurements, which suggests that viral variants in the tissue either accumulated slowly over time independent of plasma populations or are undergoing rapid tissue-specific expansion. We further demonstrated a possible role of macrophages as an intracellular compartment for viral sequestration and divergent evolution at the site of coinfection.

Our findings parallel those of Collins *et al.*¹⁵ that demonstrated divergent HIV evolution in pleural TB granulomas. In both instances, physical separation appears to promote viral

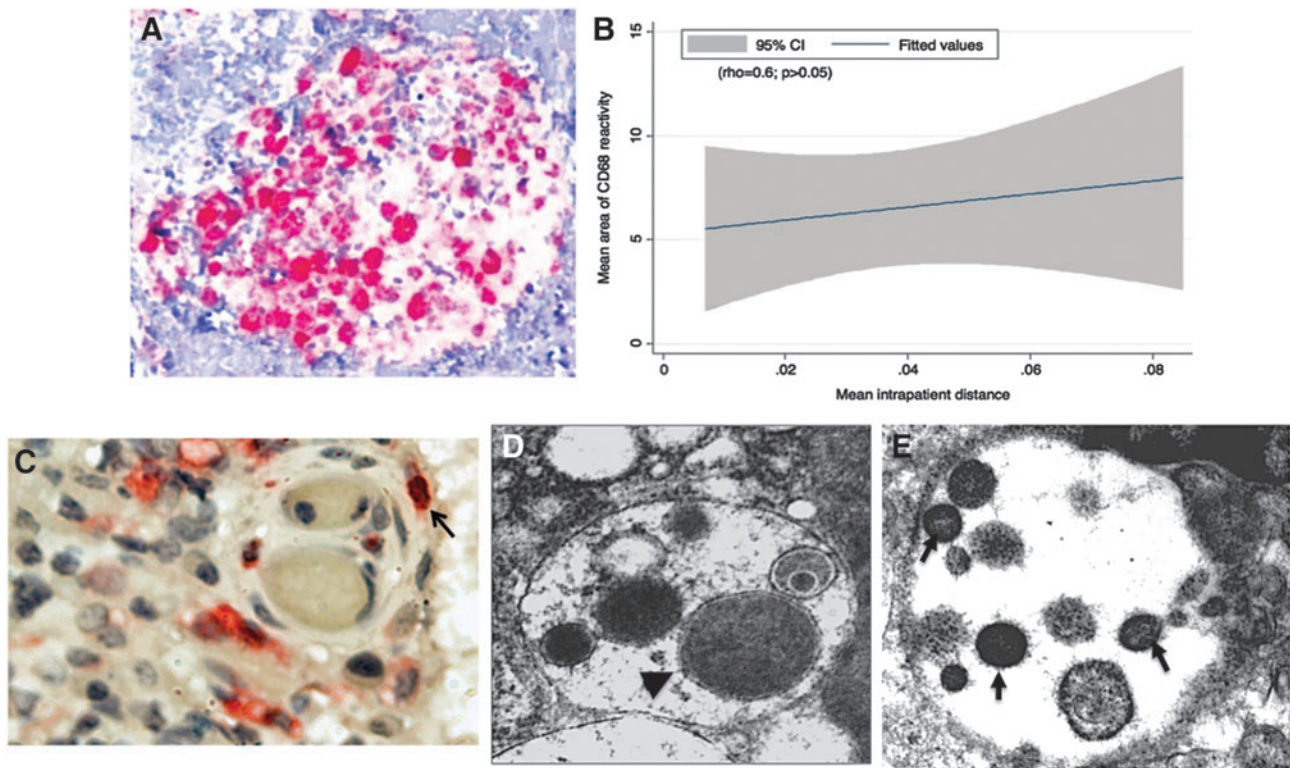


FIG. 2. (A) A micrograph image of a typical granuloma illustrating the region of interest used for our analysis of CD68 immunolocalization (initial magnification, $\times 40$). CD68-positive cells appear in *pink* and nuclei in *blue*. This image illustrates a typical granuloma with positively stained (*pink*) macrophages counted for semiquantitative analyses of intensity and area of immunolocalization. (B) A graphic illustration of the association ($\rho=0.6$; $p>0.05$; 95% CI) between the area of CD68 immunoreactivity and the overall mean inpatient genetic distance of plasma-derived and tissue-derived sequences. (C) A light micrograph and (D, E) transmission electron micrographs (TEM) of the spinal TB granulomas. Localization of macrophages (*pink*) and HIV p24 (*black*) appear in (C) (initial magnification $\times 40$) with colocalized CD68⁺/p24⁺ within the same cells indicated by an *arrow*. (D) A TEM showing a bacterial cell (*arrowhead*) within an intracellular membrane-bound compartment. (E) Virus particles (*arrows*) were also observed, under TEM, within a similar membrane-bound intracellular compartment of a cell displaying macrophage morphology [(D, E) initial magnification $\times 50$].

evolution within TB-infected tissue. Unlike pulmonary tissue where migration of immune cells, both HIV infected and uninfected, is continuous,¹⁷ the influx of immune cells to the spinal tissue is unlikely to have preceded TB infection. An influx of HIV-infected immune cells and free virions would explain the evidence of a “founder effect” that we observed through phylogenetic reconstruction. Furthermore, the close contact between HIV-infected lymphocytes and TB-infected macrophages may facilitate enhanced HIV replication or even cell-to-cell HIV spread.³¹ Despite high tissue viral loads, intracompartments diversity was low, consistent with the hypothesis of clonal expansion of HIV variants from a founder virus that migrated into the spinal TB granuloma. Furthermore, we showed greater viral diversity and evolutionary distance between plasma-derived and tissue-derived viruses, particularly when comparing adults with children; this is a reflection perhaps of the immature immune systems of children or more recent HIV acquisition where lower diversity is a characteristic of recent infection and early viral kinetics.

The immune response and physiology of children differ significantly from adults; however, our findings suggest that the phenomenon of viral sequestration within spinal TB granulomas may not be impacted by the age of the patient but

may be influenced by the duration of infection. We do not know the precise timing of infection of either the adults or children but we speculate that adults may on average have been infected longer considering that disease progression is accelerated in children compared with adults³² and the advanced clinical disease (low CD4 and high VL) among adults in this cohort.

HIV population dynamics follow a strong temporal structure where sequences from samples taken from an individual at the same time point cluster together.¹² It may be argued that a similar phenomenon is reflected in our findings of monophyletic clustering. However, the tree topologies and longer branch lengths we observed may equally be indicative of the impact of complex phylodynamic determinants such as immune selection pressure and site-specific population dynamics between latent/reactivated and productive viremia. Moreover, we demonstrated migration from the tissue to the plasma in two of six patients, both children (<5 years of age), suggesting that the contribution to the systemic viral population from the tissue may be limited. In contrast, Collins *et al.*¹⁵ noted frequent, unidirectional migration events from pleura to blood representative of a direct mechanism for greater systemic HIV heterogeneity.

The role of the V3 loop in viral tropism, cytopathicity, replication efficiency, and fusion is known.²⁸ CCR5 exploiting variants predominate, even in the context of *Mycobacteria* coinfections.³³ Our findings established CCR5-utilizing variants in both plasma and tissue in all but two sequences; this is consistent with previous studies of HIV-1 subtype C infections showing minimal coreceptor switch from CCR5 to CXCR4 even in the context of late stage AIDS and coinfections such as pulmonary TB.²⁸ Phenotypic studies may be warranted since genotype does not always accurately predict coreceptor utilization and subtle differences in usage of CD4 and coreceptors have been noted with CSF-derived and other viral isolates.^{34,35}

Given the prominent role of macrophages in the anti-TB immune response we explored their composition in the granulomas and found evidence of HIV-infected macrophages. In this study, we present electron micrographs of both HIV and TB within membrane-bound intracellular compartments. To the best of our knowledge, this is the first reported evidence of viral particles in endosomal compartments in spinal TB/HIV-coinfecting tissue. We further demonstrated an association between viral diversity and macrophage infiltration of spinal granulomas suggesting that macrophages may be a possible cellular target of HIV infection in granulomas. This hypothesis is plausible for four reasons: (1) macrophages show susceptibility to HIV infection, (2) infiltration of differentiated immune cells to the site of infection, (3) long-lived archived viruses demonstrated within macrophages, and (4) SIV models showing HIV infection of monocytes and macrophages. In the first instance, susceptibility to HIV infection is increased in differentiated macrophages, which are abundant in TB granulomas, compared to undifferentiated monocyte-derived macrophages (MDM).³⁶ Second, the influx of activated immune cells including differentiated macrophages to the site of infection regardless of HIV status and the concomitant HIV-related T cell depletion¹⁹ mean that an environment permissive to exploiting macrophages may be created in spinal TB granulomas. However, there remains controversy around this paradigm with conflicting reports showing that CD4 depletion either has an impact³⁷ or has no impact³⁸ on HIV infection of myeloid cells. Third, long-lived HIV lineages have previously been correlated with macrophage-targeting HIV variants in the CNS.^{7,35} Finally, it is noteworthy that myeloid cells (macrophages, monocytes, and dendritic cells) have been shown to become infected with HIV following phagocytosis of infected T cells in the SIV model of infection.³⁸

A limitation of our study is the small sample size and the limited number of clones analyzed per compartment. The challenges in amplifying the HIV genome from tissue restricted our use of limiting dilution techniques such as single genome amplification, which have been shown to mitigate the problem of variant resampling within quasispecies.^{39–41} However, it has also been demonstrated that the problems of variant resampling, population structure differences, and sequencing bias can be overcome or reduced if a large enough number of clones is analyzed.⁴² Although we analyzed only an average of seven clones per compartment in our study, we employed rigorous quality checks at both the laboratory and analysis stages of our study to ensure that we did not confound the interpretation of viral population diversity. In this

study, we did not explore the contribution of the CSF-derived variants to the viral population within these granulomas. In our study and previous clinical case reports it was shown that Mtb infection of the spine is extradural both in HIV-infected and HIV-uninfected patients⁴³ and penetration of the meninges into the spinal cord is rare,⁴⁴ thus maintaining the integrity of the blood–brain barrier. The possibility of viral trafficking between the granuloma and the CSF site cannot be ruled out, and this aspect needs to be explored in future investigations. Investigating the spinal granuloma as a site of HIV divergence also requires investigation of viral divergence in the context of ART, which we were unable to explore in the present study. Future prospective investigations will include HIV-infected patients on ART.

Conclusions

We show, in this small study, that spinal granulomas may form a site of independent viral evolution. If confirmed in larger studies, this may have implications for viral suppression and eradication strategies in sequestered sites based on whether antiretroviral therapy can penetrate these sites. We also show the possible involvement of macrophages in HIV replication within granulomas and raise the intriguing question of whether HIV exploits the survival strategies of TB to sequester within macrophages. Further studies to confirm these findings and to explore the accessibility of this anatomical niche by antiretroviral drugs and its implications for clinical disease progression, treatment, and eradication strategies are warranted.

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All clonal sequences are available from the authors on request and are also available on GenBank (accession numbers: FJ394222–FJ394329).

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Thumbi Ndung'u
HIV Pathogenesis Programme
University of KwaZulu-Natal
719 Umbilo Road
Durban, 4013
South Africa
E-mail: ndungu@ukzn.ac.za