Bronchoalveolar neutrophils, interferon gamma-inducible protein 10 and interleukin-7 in AIDS-associated tuberculosis

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Summary
During advanced AIDS tuberculosis (TB) often presents atypically with smear-negative and non-cavitary disease, yet immune features associated with this change are poorly characterized. We examined the local immune response in a cohort of Tanzanian AIDS-associated TB patients who underwent bronchoalveolar lavage. TB infection was confirmed in bronchoalveolar lavage (BAL) fluid by culture, probe and polymerase chain reaction (PCR). Among TB patients CD4 count correlated positively with the extent of cavitary disease as well as BAL TB load (qPCR CT). TB patients had significantly higher granulocyte–macrophage colony-stimulating factor (GM-CSF) than non-TB patients, and those with non-cavitary TB had significantly higher BAL interferon gamma-inducible protein (IP-10) and interleukin (IL)-7 than those with cavities. BAL neutrophils were as prevalent as monocytes/macrophages or epithelial cells, and immunohistochemistry revealed that neutrophils, monocytes/macrophages, and epithelial cells were major sources of the IP-10 and IL-7. These data suggest a dysregulated cytokine profile may contribute to the TB of advanced AIDS.

Keywords: cavity, GM-CSF, IL-7, IP-10, neutrophils, tuberculosis

Introduction
Mycobacterium tuberculosis is the world’s most deadly bacterial pathogen [1]. Tuberculosis (TB) rates and mortality are higher in HIV infected individuals [2] and TB accelerates HIV replication and heterogeneity [3,4]. The tendency for TB to present atypically during AIDS, without the hallmark of upper lobe cavitary lung disease, is well established [5–8]. A proposed explanation for the lack of lung cavitation with advanced AIDS is the loss of a CD4-mediated interferon (IFN)-γ or delayed-type hypersensitivity (DTH) response to drive the granuloma and cavitation process [9,10]. Unfortunately, supporting data that evaluate the cytokine profile from AIDS-associated TB patients are few, particularly from the bronchoalveolar compartment, and particularly from sub-Saharan Africa where the disease burden is highest. We therefore sought to examine the immune characteristics of AIDS patients with well-documented TB infection across a range of CD4 counts and radiographic presentations.

Materials and methods
Patient population and bronchoscopy
Informed consent was obtained from all participants and the University of Virginia (UVA) Human Investigation Committee and the Kilimanjaro Christian Medical Centre (KCMC) Ethics Committee reviewed and approved the project. Bronchoscopy with bronchoalveolar lavage was performed by standard procedure using a flexible fiberoptic bronchoscope (Olympus p45, Tokyo, Japan) wedged into involved segmental bronchi. In the case of diffuse lung involvement, the scope was wedged into one of the segmental bronchi of the right middle lobe.
Detection of TB

Each bronchoalveolar lavage (BAL) fluid was assayed by acid-fast bacilli (AFB) smear (at KCMC), culture (2 ml on solid and liquid media at UVA), polymerase chain reaction (PCR) (at UVA, see below) and by Ziehl–Neelsen stain of BAL cell block (at UVA, see below). TB infection was defined by positive culture with confirmation of *M. tuberculosis* complex by DNA probe (*n* = 13). The TB-negative group (*n* = 21) was defined as AFB smear, culture and PCR negativity. Eight patients were excluded from further analysis because their TB status was uncertain (five were smear-positive but culture-negative and PCR-negative; three were smear-negative and culture-negative but PCR-positive). Four patients were excluded because their BAL grew non-tuberculous mycobacteria and we could not rule out overgrowth of TB. Among the non-TB cases, 10 had the bronchoscopic appearance of Kaposi’s sarcoma. Other diagnostic tests were performed as per the attending physician’s orders but were not revealing.

TB PCR

PCR for the IS6110 gene was performed on BAL fluid using the assay and primers of Pounder et al. [11]. Briefly, 2 ml of the BAL fluid was centrifuged at 16 000 *g* for 5 min and 200 µl of PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) was added to the pellet. The mixture was vortexed, boiled for 15 min and centrifuged at 16 000 *g* for 5 min to remove cellular debris. PCR of 5 µl of extracted DNA was performed in a total volume of 25 µl containing 12.5 µl of iQ SYBR Green Supermix 2× (Bio-Rad, Hercules, CA, USA), iTaq DNA polymerase (50 U/ml), 6 mM MgCl₂, 20 nM SYBR Green I, 0.5 µM of each primer, 100 ng *M. tuberculosis* DNA for positive control and water for negative control. Cycling conditions entailed 1 cycle at 95°C for 15 min followed by 40 cycles at 95°C for 10 s, 58°C for 20 s and 76°C for 20 s.

Radiographic analysis

All patients had chest radiographs (CXR) read in blinded fashion by a radiologist (C. F. K.). CXR scores were determined as described previously [12]. The extent of cavitation was estimated by measuring the diameter of each cavity (mm) on CXR, assuming cavities were roughly spherical (4/3 πr³), and summing the volumes of all cavities.

CD4 count

CD4 count was measured using the Coulter Manual CD4 Count kit (Beckman Coulter, Hialeah, FL, USA).

Cytokine measurements

Five ml of BAL fluid was added to dithiothreitol (DTT) (0-05% final concentration) to dissolve mucus and filtered with 0.22 µm filters (Millex GP, Millipore, Cork, Ireland). Fluid was then concentrated in Amicon Ultra-15 filters (10 000 MWCO; Millipore) and assayed for 22 cytokines/chemokines using the human 22-plex cytokine kit (Upstate, Charlottesville, VA, USA) on a Luminex-100 instrument, according to the manufacturer’s instructions. For transforming growth factor (TGF)-β1 quantification, samples were acid-activated to pH 1.5 with 1 N HCl for 60 min prior to assay. To adjust for BAL dilution, all cytokine data were normalized to the total protein content in the fluid as determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA).

Histology and immunohistochemistry (IHC)

Ten ml of BAL fluid was stored in RNALater (Ambion, Austin, TX, USA) at −70°C and cell blocks were made by pelleting the material, fixing in 10% formalin and embedding in paraffin. Tissue sections were stained with haematoxylin and eosin, Ziehl–Neelsen and IHC was performed for inducible protein (IP)-10 and interleukin (IL)-7. All pathology was scored blindly by a pathologist (L. M.). For each sample a cellularity score was determined for epithelial cells, monocytes/macrophages, lymphocytes and neutrophils as follows: 0 = none, 1 = few, 2 = scattered, 3 = many. Ziehl–Neelsen-stained cell blocks were viewed at 1000× and scored for AFB positivity as follows: 0 = none, 1 = rare, 2 = few, 3 = scattered throughout. For IHC, 3-µm-thick paraffin sections were obtained using a Leica Microtome, mounted on superfrost/PLUS glass slides and deparaffinized to deionized water. Samples were incubated overnight at 4°C using IP-10 and IL-7 antibodies (R&D systems, Minneapolis, MN, USA), with dilutions ranging from 5 µg/ml to 125 µg/ml. Samples were rinsed in phosphate-buffered saline (PBS) and incubated with a biotinylated secondary antibody for 30 min at room temperature. Samples were then incubated with the ABC kit (Vector, Burlingame, CA, USA) for 30 min at room temperature, rinsed in PBS and stained with 3,3′ diaminobenzidine.

(DAB) substrate (Vector) before counterstaining. An IHC score was determined for each cell population (epithelial cells, monocytes/macrophages, lymphocytes and neutrophils) according to the following scale: 0 = weak, majority of cells not staining; 1 = intermediate; 2 = strong, majority of cells staining.

Statistics

Means and medians were compared using *t*-test and Mann–Whitney test, respectively. Correlations were measured using the Pearson linear correlation. All *P*-values are two-tailed. Data shown are mean ± standard deviation (s.d.) unless indicated otherwise.
Results

Utility of BAL PCR for detection of TB infection

The endoscopy unit at the KCMC is one of few centres in the region equipped for bronchoscopy and serves as a referral centre for patients with chest disease of unknown aetiology. We enrolled and obtained consent from 46 such patients known to be HIV-positive and performed TB diagnostics and cytokine analysis on BAL. TB infection was identified in 13/46 (28%) by culture with confirmation of *M. tuberculosis* by DNA probe. Against this gold standard, BAL PCR exhibited better sensitivity and specificity than BAL AFB smear (100%/91% versus 54%/85%). Furthermore, real-time PCR Ct (cycle threshold) exhibited the expected quantitative correlation with time to positive culture (Ct versus days to positivity; \( r = 0.68, P = 0.01 \)) and AFB score (Ct versus AFB score; \( r = 0.67, P = 0.02 \)).

CD4 count correlates with cavitary disease and endobronchial TB burden

There were no significant differences between the TB-positive and TB-negative populations in terms of age, gender, CXR score \([4.8 \pm 1.4 \text{ versus } 7.4 \pm 0.9, P = \text{n.s.}] \) or CD4 count \([194 \pm 41 \text{ versus } 146 \pm 31, P = \text{n.s.}] \). Among TB patients, average CD4 count was higher in patients with cavities as detected by CXR (Fig. 1a). Additionally, CD4 count correlated positively with cavitary volume and BAL Tb burden (qPCR Ct; Fig. 1b,c).

BAL cytokine/chemokine response in AIDS-associated TB

Scant data are available on the BAL response during AIDS-associated TB. Most reports derive from HIV-negative TB in developed countries and findings have been extremely variable [13–15]. First, we examined the cell blocks prepared from the BAL fluid. As expected, lymphocytes were scant (cellularity score 0.6 ± 0.5), but we were surprised to find a substantial amount of neutrophils, many of which contained intracellular *M. tuberculosis* bacilli (Fig. 2; cellularity scores 2.1 ± 0.9 for neutrophils, 2.3 ± 0.7 for monocytes/macrophages and 2.0 ± 0.6 for epithelial cells, respectively, \( P = \text{n.s.} \)). We next performed a broad characterization of 23 chemokines and cytokines in BAL fluid using the Luminex assay, with all cytokine data normalized for dilution against the total protein content in the fluid. The analysis showed that the TB-positive group exhibited higher BAL granulocyte–macrophage colony-stimulating factor (GM-CSF) levels than TB-negative individuals (the definition of TB-negativity required AFB smear, culture and PCR negativity; \( P = 0.007 \)), while all other cytokines/chemokines were not changed statistically (Table 1). Upon comparing the immune profile within the TB group, individuals with non-cavitary TB had significantly higher BAL IL-7 and IP-10 than those with cavitary disease (\( P = 0.02 \)). Other cytokines/chemokines were not associated significantly with radiographic findings or CD4 count. Finally, immunohistochemistry of BAL cell blocks revealed that IP-10 was produced by epithelial cells, monocytes/macrophages and neutrophils but not lymphocytes (IHC positivity score 1.5 ± 0.5, 1.6 ± 0.5, 1.2 ± 0.8 and 0.1 ± 0.4, respectively; \( n = 12, P = \text{n.s.} \) between epithelial cells, monocytes/macrophages and neutrophils). Meanwhile, IL-7 was produced predominantly by epithelial cells and monocytes/macrophages (IHC score 1.5 ± 0.7 and 1.3 ± 0.6 versus 0.7 ± 0.6 and 0.0 ± 0.0 for neutrophils and lymphocytes; \( n = 11, P < 0.05 \) comparing epithelial cells and monocytes/macrophages versus neutrophils or lymphocytes). For both IP-10 and IL-7, the absence of lymphocyte staining may reflect their paucity, given HIV infection.

Discussion

The importance of this work lies in the characterization of the immune profile at the site of infection from a substantial number of HIV-infected, probe-confirmed TB patients from sub-Saharan Africa. Many studies from developing world sites perform only smear or culture but not probe or PCR for confirmation of *M. tuberculosis* complex. This would have led to a significant rate of erroneous assignment to both the
**Fig. 2.** Inducible protein (IP)-10 and interleukin (IL)-7 in AIDS-associated tuberculosis. Formalin-fixed, paraffin-embedded cell blocks were prepared from bronchoalveolar lavage (BAL) fluid and immunohistochemistry performed with anti-IP-10 and anti-IL-7 primary antibody. Positive staining macrophages (arrows) and neutrophils (arrowheads) are shown. No background staining was observed without IP-10 (lower left) or IL-7 antibody (not shown). Ziehl–Neelsen (ZN) stain shows *Mycobacterium tuberculosis* bacilli both inside neutrophils (arrowhead) and extracellularly (arrow). All photomicrographs come from one of the seven patients with non-cavitary tuberculosis.

**Table 1.** Comparison of bronchoalveolar lavage (BAL) cytokine/chemokines between HIV-infected patients with cavitary tuberculosis (TB), non-cavitary TB and without tuberculosis.

| Cytokine/chemokine | TB cavitary (n = 6) | TB non-cavitary (n = 7) | Non-TB (n = 21) | P (TB versus non-TB) |
|---------------------|---------------------|------------------------|-----------------|----------------------|
| IL-1α               | 205·3 ± 69·6        | 112·9 ± 43·4           | 143·3 ± 33·1    | n.s.                 |
| IL-1β               | 91·8 ± 46·5         | 116·1 ± 52·7           | 93·0 ± 24·6     | n.s.                 |
| IL-2                | 6·3 ± 1·5           | 4·6 ± 1·4              | 5·7 ± 0·9       | n.s.                 |
| IL-3                | 1·1 ± 1·1           | n.d.                  | 1·9 ± 1·2       | n.s.                 |
| IL-4                | 3·7 ± 1·2           | 2·9 ± 1·2              | 2·6 ± 0·6       | n.s.                 |
| IL-5                | 10·3 ± 2·3          | 10·7 ± 1·7             | 8·8 ± 1·0       | n.s.                 |
| IL-6                | 73·0 ± 23·0         | 306·9 ± 94·3           | 136·7 ± 43·0    | n.s.                 |
| IL-7                | 28·0 ± 5·9          | 57·5 ± 7·3             | 32·0 ± 3·7      | n.s.                 |
| IL-8                | 456·5 ± 107·3       | 1272·2 ± 493·7         | 730·4 ± 137·6   | n.s.                 |
| IL-10               | n.d.                | 5·5 ± 4·5              | 1·1 ± 0·6       | n.s.                 |
| IL-12p40            | 47·3 ± 18·9         | 61·2 ± 14·3            | 47·3 ± 14·1     | n.s.                 |
| IL-12p70            | 8·0 ± 1·8           | 12·9 ± 2·8             | 10·3 ± 1·3      | n.s.                 |
| IL-13               | 8·1 ± 1·6           | 10·2 ± 1·8             | 8·7 ± 1·1       | n.s.                 |
| IL-15               | 4·8 ± 2·2           | 7·9 ± 3·8              | 7·5 ± 1·4       | n.s.                 |
| IFN-γ               | 354·7 ± 203·5       | 59·9 ± 16·2            | 63·8 ± 12·8     | n.s.                 |
| TGF-β1              | 2·2 ± 1·1           | 2·8 ± 1·5              | 1·7 ± 0·5       | n.s.                 |
| TNF-α               | 25·6 ± 20·9         | 10·3 ± 5·9             | 10·3 ± 3·5      | n.s.                 |
| Eotaxin             | 54·6 ± 17·8         | 102·1 ± 27·0           | 82·0 ± 11·9     | n.s.                 |
| GM-CSF              | 8·1 ± 1·6           | 12·9 ± 2·2             | 5·1 ± 1·0       | 0·007                |
| IP-10               | 350·5 ± 203·5       | 1709·9 ± 604·4         | 306·2 ± 75·1    | n.s.                 |
| MCP-1               | 42·6 ± 13·3         | 117·6 ± 39·7           | 47·3 ± 6·2      | n.s.                 |
| MIP-1α              | 56·8 ± 12·8         | 57·9 ± 17·5            | 46·7 ± 3·0      | n.s.                 |
| RANTES              | 37·0 ± 4·7          | 64·2 ± 14·8            | 318 ± 3·2       | n.s.                 |

Data shown as mean ± s.e. pg cytokine/mg protein. n.s. = Not statistically significant. P-values reflect t-test; however, all significant P-values were also met when comparing medians (Mann–Whitney test). n.d. = Not detected for any specimen. Transforming growth factor (TGF)-β1 data available for only six cavitary TB specimens and 19 non-TB specimens. GM-CSF: granulocyte–macrophage colony-stimulating factor; RANTES: regulated upon activation normal T cell expressed and secreted cytokine; IL: interleukin; IFN: interferon; TNF: tumour necrosis factor; IP: inducible protein; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein.
TB-positive and TB-negative groups in our population. As a noteworthy aside, we feel that PCR directly on BAL, while technically demanding in a resource-limited setting, has tremendous advantages in terms of accuracy over smear and rapidity over culture/probe.

The comparison of cytokine responses between the TB and non-TB groups revealed a statistical increase in BAL GM-CSF in the setting of TB, a finding that has not been reported previously. One might predict that this chemokine may exert a partially protective response, given its role in TB containment and granuloma development in mice [16]. Whether additional exogenous GM-CSF would be therapeutic in active TB is unknown: in one small study clinical improvement was modest [17]. Potentially driven by the increase in GM-CSF, the BAL neutrophilic response we saw in these TB patients was notable, particularly given the abundant bacilli within the neutrophils. The role of neutrophils in TB is unresolved: intracellular killing has been demonstrated in vitro by some but not others [18,19]. In mouse models, neutrophils do not affect survival but do contribute to granuloma formation [20,21]. Taken together, we speculate that the increased GM-CSF and neutrophil response is an attempt by the host to control TB, particularly in the setting of AIDS.

The association we found between CD4 count and cavitary disease and endobronchial TB burden was not surprising. Several studies have documented this trend [5–8,10], supporting the hypothesis that CD4 cells contribute to the pathogenesis of cavitary disease and endobronchial spread. The mechanism by which this cavitation process occurs is clearly of paramount importance to the AIDS/TB epidemic due to its implication on TB transmission. We therefore sought to examine the cytokine/chemokine profile between the cavitary and non-cavitary groups. Based on limited human or mouse data we predicted a correlation between loss of cavitation and a loss of tumour necrosis factor (TNF)-α or interferon (IFN)-γ [22–24]; however, diminution of these cytokines in the non-cavitary group was modest and not statistically significant. More readily apparent was the association between the loss of cavitation and IL-7 and IP-10. To our knowledge, IL-7 has not been reported in the BAL of TB patients. One could postulate that the IL-7 is merely a marker for the advanced AIDS of the non-cavitary group, given its association with CD4 decline [25]; however, IL-7 may be relevant to HIV pathogenesis via its up-regulation of regulated upon activation normal T cell expressed and secreted cytokines (RANTES) (and thereby promotion X4 virus) [26]. The localization of IL-7 production by immunohistochemistry to bronchial epithelial cells and macrophages was not surprising, and has been reported by others [27,28].

IP-10 is an IFN-γ-inducible chemokine critical for effector T cell development and trafficking in mice [29]. Its elevation during TB has been reported [20,30]; however, a neutrophilic source has not been described. Some authors postulate a protective role for this chemokine [31], yet our findings of extremely high levels in the advanced AIDS/non-cavitary group would argue against an unequivocally protective function for IP-10. Indeed, given the positive effect of IFN-γ on IP-10 transcription (as well as that of IL-1α and TNF-α in some systems [32]), the high IP-10/low IFN-γ/low IL-1α/low TNF-α pattern of the non-cavitary group was unexpected and suggest loss of IP-10 regulation or induction by other known mediators such as Toll-like receptor ligands [33,34].

Human data are important to direct research in experimental and animal models, particularly given the complexity of AIDS-associated TB which cannot be replicated easily. Based upon our findings we question the role of GM-CSF and neutrophils during AIDS-associated TB. Additionally, the function of IL-7 on TB-specific HIV progression and of IP-10 in T cell trafficking in TB would seem to be fertile topics for research.

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