Mortality in Severe HIV-TB Associates with Innate Immune Activation and Dysfunction of Monocytes

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Short title: Innate Immunity and Mortality in HIV-TB

Key points:

Mortality remains unacceptably high in patients hospitalized with HIV-associated tuberculosis. Among these patients, half had TB mycobacteraemia and death was associated with higher concentrations of immune activation and anti-inflammatory markers and impaired ex vivo innate immune responses to bacterial antigens.

ABSTRACT

BACKGROUND

Case fatality rates among hospitalised patients diagnosed with HIV-associated tuberculosis remain high, and tuberculosis mycobacteremia is common. We aimed to define the nature of innate immune responses associated with 12-week mortality in this population.

METHODS

This prospective cohort study was conducted at Khayelitsha Hospital, Cape Town, South Africa. Hospitalised HIV-infected tuberculosis patients with CD4 counts <350 cells/μL were included; tuberculosis blood cultures were performed in all. Ambulatory HIV-infected patients without active tuberculosis were recruited as controls. Whole blood was stimulated with *E. coli* derived lipopolysaccharide, heat-killed *S. pneumoniae* and *M. tuberculosis*. Biomarkers of inflammation and sepsis, intracellular (flow cytometry) and secreted cytokines (Luminex) were assessed for associations with 12-week mortality using Cox-proportional hazard models. Secondly, we investigated associations of these immune markers with tuberculosis mycobacteremia.

RESULTS

Sixty patients were included (median CD4 count 53 cells/μL (interquartile range 22-132)), sixteen (27%) died after a median of 12 (interquartile range 0-24) days. Thirty-one (52%) grew *M. tuberculosis* on blood culture. Mortality was associated with higher concentrations of procalcitonin, activation of the innate immune system (% CD16+CD14+ monocytes, interleukin-6, tumour necrosis factor-α and colony stimulating factor 3), and anti-inflammatory markers (increased interleukin-1RA and lower monocyte and neutrophil

responses to bacterial stimuli). Tuberculosis mycobacteremia was not associated with mortality, nor with biomarkers of sepsis.

CONCLUSIONS

Twelve-week mortality was associated with greater pro- and anti-inflammatory alterations of the innate immune system, similar to those reported in severe bacterial sepsis.

Key words: HIV, tuberculosis, mortality, innate immunity, mycobacteraemia

INTRODUCTION

Tuberculosis (TB) remains the most frequent cause of hospitalisation and death in HIV-infected patients [1]. Mortality is particularly high among HIV-infected patients who start TB treatment in hospital, ranging from six to 32% [2].

The causes of this high mortality (despite receiving TB treatment) are not well defined. Post-mortem examinations report disseminated TB as a primary cause of death in many HIV-infected persons [3, 4]. However, TB mycobacteremia –a frequent finding in febrile HIV-infected inpatients [5, 6] – has not been consistently associated with mortality [5, 7, 8].

Although there is no published literature to date on immunological changes accompanying mycobacteremia, bacterial sepsis has been studied extensively. Bacterial sepsis is characterized by imbalanced host responses with both pro-inflammatory and immune suppressive features [9], including monocyte deactivation, neutrophil dysfunction and increased production of interleukin (IL)-10 [10], potentially contributing to a heightened risk for secondary infections [9, 11].

In HIV-associated TB (HIV-TB), similar changes have been described; reduced innate responses upon stimulation *in vitro* with lipopolysaccharide (LPS) and heat-killed *Mycobacterium tuberculosis* (*Mtb*) have been associated with death and poor outcome [12], and failure of cellular immune recovery has been associated with death after ART initiation in TB patients [13, 14].

We hypothesized that hospitalised HIV-TB patients, compared with HIV-infected outpatients without TB (controls), would have elevation of sepsis biomarkers and pro-inflammatory cytokines as well as evidence of increased anti-inflammatory signalling and decreased innate

immune responses to bacterial stimuli, and that these features would be associated with mortality. Secondly, we hypothesized that TB mycobacteremia would be associated with these immunological features and mortality. Additionally, we tested whether *ex vivo* treatment with recombinant interferon (IFN)-ɣ (a potential host-directed therapy) could restore monocyte responses to LPS.

METHODS

Study design & population

We conducted a prospective cohort study in Khayelitsha, Cape Town, South Africa. Khayelitsha has a TB notification rate of 1065/100000 person-years (City of Cape Town, unpublished data), and antenatal HIV seroprevalence of 34%[15].

Non-pregnant HIV-infected patients with CD4 counts < 350 cells/μL, diagnosed with TB on admission to Khayelitsha Hospital were recruited between June and October 2014. Patients were excluded if they were transfused or had received more than one dose of TB treatment within the preceding month. Patients with microbiologically proven rifampicin-susceptible TB were included. Selection bias was minimised by using a random selection procedure.

HIV-infected outpatients with CD4 counts < 350 cells/μL without active TB were recruited at Ubuntu clinic (controls). To ensure more appropriate matching, only those control patients with CD4 counts < 150 cells/μL were included in the final analyses.

Outcomes and definitions

We aimed to determine immunologic changes associated with the primary outcome 12-week mortality. Secondly, we assessed associations of TB mycobacteremia, defined as at

least one MycoFlytic blood culture growing *Mtb,* with immunologic profile and outcome. Sepsis definitions were adapted from published criteria (Supplementary Table 1) [9].

Procedures

A detailed description of ethical aspects and data collection is provided in the Supplementary Methods. All participants had the following tests performed: Xpert® MTB/RIF assay on sputum and urine, TB culture on sputum, chest X-ray, full blood count and differential, chemistry, HIV-viral load and CD4 count. MycoFlytic blood cultures were performed for hospitalised patients. Control patients were excluded if a TB symptom screen or any TB diagnostic test was positive. Hospitalized patients were contacted telephonically at 4 weeks and clinically reviewed at 12 weeks.

Whole blood was stimulated for six hours. Anti-(myco-)bacterial responses were tested using *E. coli*-derived LPS, heat-killed *Streptococcus pneumoniae* and heat-killed *Mtb* strain H37Rv. The *ex vivo* effect of IFN-ɣ was assessed in a co-stimulation assay with LPS.

Samples were stained with surface and intracellular markers (Supplementary Table 2) and acquired on a BD LSR Fortessa Flow Cytometer. Data were analysed in FlowJo version 10 (Ashland, OR, USA). Gating strategies are illustrated in the Supplementary Figure 1. Concentrations of 12 cytokines (Supplementary Table 2) were measured in culture supernatants using a Luminex multiplex assay.

Statistical analysis

Data were analyzed using SPSS Version 22 (IBM, Chicago, IL, USA), GraphPad PRISM Version 6 (San Diego, CA, USA) and R (Vienna, Austria).

Medians were compared among groups using the appropriate statistic tests. Variables were investigated for associations with mortality in a Cox-proportional hazards model. *A priori* defined potential confounders were age, sex, ART status, HIV-viral load and CD4 count, and Luminex plate number; a variable was retained in the model if introduction led to a >10% change of the effect measure.

All reported q-values were calculated using Benjamini-Hochberg procedures for multiple-testing correction[16]; p-values <0.05 and q-values <0.10 were regarded significant.

Patients for whom data were available for all stimulation conditions were included (n=37) for principal component analysis (PCA); 90 variables were included per patient. Using a Shapiro test, all variables with p<0.05 were log-transformed and scaling was done. Data missing due to technical difficulties (112/3300 values; 3.4%) was imputed using the K-nearest-neighbor technique.

RESULTS

Participants

From 124 HIV-infected patients with probable TB who were enrolled, sixty participants with confirmed rifampicin-susceptible TB were included in this analysis. Twenty-seven HIV-infected control patients were included (Supplementary Figure 2). All HIV-TB patients fulfilled criteria for sepsis, 38/60 (63.3%) had severe sepsis, and 23/60 (38.3%) had septic shock (Table 1). TB treatment was initiated in all patients, a median of one day (IQR 0.5-2.5 days) after recruitment. Co-trimoxazole prophylaxis was initiated in hospital in 28 (46.7%) of patients, and 54 (90.0%) received broad-spectrum antibiotics (mostly ceftriaxone). Most patients were on ART at enrolment (31/60, 52%; Table 1).

Clinical presentation and outcomes

Compared to controls, HIV-TB patients had higher HIV viral loads, and were more often anaemic (Table 1). 35/41 (85%) HIV- TB patients who could produce sputum had a positive sputum Xpert® MTB/RIF or culture for *Mtb*, 31/60 (52%) patients grew *Mtb* on blood culture, 15/35 (43%) had a positive urine Xpert® MTB/RIF, and 16 patients had positive cultures or Xpert® MTB/RIF from other sites. 28/60 (47%) of patients had standard bacterial blood cultures performed prior to receiving broad spectrum antibiotics; none grew pathologic bacteria. By 12 weeks, 16 HIV-TB patients had died (26.7%) a median of 12 (IQR 0-24) days after enrolment (Supplementary Table 3); none were lost to follow-up. All deaths occurred in hospital. Four patients had suspected bacterial sepsis when they died, and 11 deaths were attributed to disseminated TB; one of them had disseminated TB and features suggesting bacterial sepsis.

Compared to HIV-TB patients who survived, patients who died were older and presented more often with severe sepsis or septic shock (Table 1). Mycobacteremia was not associated with mortality (crude hazard ratio (cHR 0.74, 95% CI 0.3-2.0, p = 0.55; adjusted HR (aHR) 0.80, 95% CI 0.3-2.2, p = 0.64), nor was time to TB treatment in days (cHR 0.87, 95% CI 0.69-1.11, p = 0.26; aHR 0.82, 95% CI 0.63-1.07, p = 0.14). HIV-TB patients who died had significantly higher concentrations of procalcitonin compared to survivors (Table 1).

Immune phenotype in HIV- TB versus controls

In unstimulated samples, patients with HIV-TB had higher percentages of TNF-α+ monocytes compared to controls (Fig 1), and higher supernatant concentrations of CSF-3 and IFN-ɣ, whereas supernatant concentrations of IL-1β and IL-8 were lower (Table 2).

Whole blood of HIV-TB patients showed a significantly reduced production of pro-inflammatory cytokines following stimulation compared to controls (Fig 1, Table 2). In response to LPS, percentages of IL-6+ and TNF-α+ monocytes were lower (Fig 1), as were percentages of IL-6+ and TNF-α+ neutrophils (q= 0.003, q=0.003), and supernatant concentrations of CSF-2 and pro-inflammatory cytokines IL-12p40, IL-1β, IL-6, IL-8 and TNF-α (Table 2). Supernatant IFN-ɣ and anti-inflammatory IL1-RA concentrations were higher in HIV-TB patients (Table 2).

In *S. pneumoniae* stimulations, percentages of IL-6+ and TNF-α+ monocytes, and IL-6+ absolute monocyte counts were lower in HIV-TB patients (Fig 1), as were percentages and absolute counts of IL-6+ and TNF-α+ neutrophils (q=0.003, q=0.003, q=0.003 and q=0.01). HIV-TB patients had higher supernatant concentrations of CSF-3, IFN-ɣ and IL-1RA, and lower concentrations of CSF-2, IL-12p40, IL-1β, IL-6 and TNF-α (Table 2).

In *Mtb* stimulations, percentages and absolute counts of IL-6+ and TNF-α+ monocytes were similar in HIV-TB patients and controls (Fig 1). Percentages of IL-6+ and TNF-α+ neutrophils were lower in HIV-TB patients (q=0.01 and q=0.07), whereas concentrations of CSF-3, IFN-ɣ and IL-1RA were higher (Table 2).

Pro- and anti-inflammatory changes in hospitalized HIV-TB patients who died versus survivors

Immune activation was associated with mortality. Patients who died had higher percentages of CD16+CD14+ monocytes and higher supernatant concentrations of CSF-3 in unstimulated samples compared to patients who survived (Fig 1, Table 2). Higher percentages of activated CD16+CD14+ monocytes (Fig 2(A)) were independently associated with mortality, as were supernatant concentrations of CSF-3, IL-1RA, IL-6 and TNF-α (Fig 2).

Compared to survivors, patients who died had lower absolute counts of IL-6+ and TNF-α+ producing monocytes in response to LPS and *S. pneumoniae* (Fig 1). Lower supernatant IL-1β and IL-6 concentrations and higher CSF-3 concentrations were measured in *S. pneumoniae* stimulations of patients who died (Table 2). Impaired monocyte and TNF-α and IL-6 production in response to *S. pneumonia*e and lower percentages of IL-6+ neutrophils in response to LPS were independently associated with mortality (Fig 2(A), Fig 3), as were increased supernatant concentrations of CSF-3 and lower concentrations of IL-1β and TNF-α in response to *S. pneumoniae* (Fig 2(B)).

In response to heat-killed *Mtb*, patients who died had lower absolute counts of IL-6+ and TNF-α+ neutrophils. No differences were seen in *Mtb* culture supernatants (Table 2). *Mtb* responses were not associated with mortality (Fig 2).

In PCA, the first principal component (PC1) was significantly associated with mortality ($p = 0.003$) (Fig 4(A-B)). Arranging all samples by their value on PC1, an immunological signature associated with mortality was revealed (Fig 4(C)), characterized by increased production of cytokines in unstimulated conditions and impaired production of pro-inflammatory cytokines in response to all antigen stimuli.

***Mtb* mycobacteremia is associated with cytopenia but not with sepsis severity**

Mycobacteremic patients had lower platelet (median 203 vs 311*10^9/L), lymphocyte (median 0.42 vs 0.66*10^9/L) and monocyte counts (median 0.25 vs 0.42*10^9/L) compared to non-mycobacteremic patients (p=0.05, p=0.0003 and p=0.03 respectively). There were no differences in plasma concentrations of procalcitonin or lactate, percentages of CD16+CD14+ monocytes in unstimulated samples, or percentages of IL-6+ or TNF-α+ monocytes in response to stimulations (Supplementary Figure 3). Mycobacteremic patients had lower absolute counts of IL-6+ monocytes compared to non-mycobacteremic TB patients in unstimulated samples, and lower absolute counts of both IL-6+ and TNF-α+ monocytes in response to LPS (Supplementary Figure 3). There were no differences in supernatant cytokine concentrations (Supplementary Table 4). None of the variables were associated with mycobacterial load, expressed in days to blood culture positivity (results not shown).

No reversal of immune deficits with IFN-ɣ co-stimulation

There were no differences in neutrophil and monocyte capacity to produce IL-6 or TNF-α, nor in the concentration of any of the extracellular cytokines, when the LPS with IFN-ɣ co-stimulation was compared to LPS only.

DISCUSSION

In HIV-infected patients diagnosed in hospital with microbiologically-proven drug-susceptible TB, 12-week mortality was 27%. Over half of patients had mycobacteremia; however, this was not associated with mortality. Patients who died had an immune phenotype characterised by higher concentrations of pro-inflammatory cytokines (CSF-3, TNF-α and IL-6) and anti-inflammatory IL-1RA, an increased proportion of pro-inflammatory

CD14+CD16+ monocytes and impaired capacity of innate cells to produce pro-inflammatory cytokines in response to bacterial antigen stimuli.

Increased concentrations of pro-inflammatory cytokines CSF-3, IL-6 and TNF-α in unstimulated samples were associated with mortality. These cytokines are mainly produced by innate cells, suggesting a more activated state of the innate immune system in patients who die. This is consistent with other studies of mortality in HIV-associated TB [14], ART-naïve patients starting ART [17] and bacterial sepsis in HIV-infected patients [18, 19]. There are several plausible explanations for this association. It is possible that immune activation reflects more disseminated TB. Another possible explanation is a causal relation: higher concentrations of pro-inflammatory cytokines led to increased tissue damage, organ failure and immune exhaustion, impairing host defence to other pathogens.

Increased percentages of CD16+CD14+ monocytes were associated with mortality. This activated monocyte subset, generally produces higher amounts of pro-inflammatory cytokines and is more phagocytic compared to the classical, CD16- subset[20]. However, previous studies have shown that monocyte functionality may be impaired in active TB with CD16+CD14+ monocytes refractory to dendritic cell maturation, leading to impaired antigen-presentation and decreased secretion of IL-1β and IL-12 [21, 22]. Impaired phagocytic and antigen-presenting capacity of CD14+CD16+ cells has also been described in bacterial sepsis, and monocyte deactivation, with reduced production of TNFα in response to LPS, was associated with fatal outcome [10, 23]. Our findings indicate that expansion of the CD16+ CD14+ monocyte population is observed together with impaired total monocyte and neutrophil responses to bacterial antigens in patients who die, potentially contributing to mortality.

The immunological phenotype associated with mortality is similar to what has been described in bacterial sepsis. In bacterial sepsis, time to intra-venous broad-spectrum antibiotic treatment is associated inversely with survival [9]. We did not find such an association for time to TB treatment. Although a potential association might have been masked by the lack of exact data on time of TB treatment initiation in hours, a more likely explanation is the fact that TB treatment is administered orally and drug absorption might be hampered by intestinal tissue damage by TB or HIV.

Although invasive pneumococcal disease is one of the most frequent and lethal bacterial infections in HIV-infected patients, most studies focus on LPS responses. Interestingly, we found that impaired responses to *S. pneumoniae* were also strongly associated with mortality, indicating defects in host-defence to this pathogen.

Reduced pro-inflammatory responses of innate cells to LPS and *S. pneumoniae* in HIV-TB patients, compared to controls, suggest that TB has an immunosuppressive effect additive to HIV. CD4 count and HIV viral load were not associated with mortality, whereas the innate immune features were independently associated.

TB mycobacteremia was neither associated with mortality, nor with more severe derangement of sepsis biomarkers. Mycobacteremia was associated with cytopenia, but there were no functional differences of innate cells. Previous studies have shown that in hospitalized febrile patients, mycobacteremia was associated with mortality [5, 6, 24], whereas in other studies among HIV-TB patients with mycobacteremia it was not [7, 8]. Our findings illustrate that patients with severe HIV-TB can develop features of a sepsis syndrome even when mycobacterial blood cultures are negative.

There is increasing interest in host-directed immunotherapies for TB [25]. Recombinant IFN-ɣ has been shown to be beneficial in the treatment of cryptococcal meningitis [26] and other fungal infections [27], and a trial to investigate its application for bacterial sepsis is ongoing [28]. We found no effect of *ex vivo* co-stimulation with recombinant IFN-ɣ in restoring monocyte responses to LPS. Although *ex vivo* data cannot be directly extrapolated to *in vivo* conditions, our findings do not support investigating recombinant IFN-ɣ as a potential immunotherapy in this patient subset. Our data are supported by two clinical trials [25, 29], showing no beneficial effects of IFN-ɣ on sputum culture conversion in drug-sensitive or drug-resistant pulmonary TB. CSF-3 has been investigated as adjunctive immune therapy for bacterial sepsis, but there was no significant survival benefit [30]. Our data of increased concentrations of CSF-3, rather than deficiency, in patients who die does not support investigation of CSF-3 for host-directed therapy in HIV-TB either.

The early mortality [2] and prevalence of mycobacteremia [6, 7, 24] reported here are similar to other studies in Africa and suggest our immunological findings are generalizable to other settings with a high TB and HIV burden. Limitations of our study include a limited number of bacterial blood cultures and lack of post-mortem examinations. Due to the strategy of treating patients with sepsis syndrome with broad spectrum antibiotics at primary care referral centres upon referral to hospital, only 47% of patients had bacterial blood cultures performed prior to antibiotics. Post-mortem examinations were planned, but in this study none of the families agreed to this. *Ex vivo* markers of immune exhaustion were not measured, this should be subject of future studies.

Major strengths are the variety of antigens/organisms used for our stimulations, enabling *in vitro* simulation of Gram-negative, Gram-positive and mycobacterial infections. The inclusion of an HIV-infected control group without active TB and the exclusion of patients

without microbiologically-confirmed TB facilitate conclusions on the associations of findings with severe TB specifically, minimizing misclassification bias of other diagnoses, or advanced HIV alone.

Our study has several implications for clinical care and future research. The immune profile observed in HIV-TB patients and particularly those who died, suggest that disseminated TB in the context of advanced HIV infection can significantly impair host innate responses to bacteria, possibly resulting in an immunological predisposition to bacterial superinfections [3, 4]. This study adds to our understanding of immunopathology in HIV- TB. Focusing on patients requiring hospital admission and innate immune changes, we confirm that the high mortality in this patient subset is associated with an immunological phenotype similar to bacterial sepsis: immune activation, with higher concentrations of pro-inflammatory cytokines and expansion of the CD16+CD14+ monocyte population, potentially leading to increased tissue damage, together with impairment of innate immune functional responses, with reduced production of pro-inflammatory cytokines in response to antigens of bacterial pathogens. In the future, immunomodulatory interventions proven beneficial in bacterial sepsis should also be evaluated for patients with severe HIV-TB.

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FIGURE LEGENDS

Figure 1. Monocyte responses and mortality

Median values and interquartile ranges are shown for the percentage of CD16+ monocytes in unstimulated samples (HIV-controls n=26, HIV-TB patients n=55), (A), and percentages (B) and absolute counts (C) of IL-6+ and TNF-α+ monocytes in response to LPS (HIV-controls n=25, HIV-TB patients n=55), *S. pneumoniae* (HIV-controls n=25, HIV-TB patients n=31) and *M. tuberculosis* (HIV-controls n=26, HIV-TB patients n=47) respective stimulants. Absolute counts were derived by multiplying the percentage of positive cells by the monocyte count obtained from the NHLS clinical laboratory. HIV-infected control patients (black circles), HIV-TB patients who survived (blue triangles) and HIV-TB patients who died (red squares) are shown. Kruskall-Wallis and Mann-Whitney-U tests were used for comparisons between groups; *p-value < 0.05, **p-value < 0.01, *** p-value < 0.001, ****p-value < 0.0001

Figure 2. Death hazard ratios for intra- and extracellular cytokines

(A)Adjusted hazard ratios for monocyte activation and intracellular cytokines measured in monocytes and neutrophils. Monocyte responses are in red; neutrophil responses are in yellow.

*per 1% increase; ** per 10% increase; *** per 10^{6}/L increase; **** per 0.1% increase

(B) Adjusted hazard ratios for the extracellular cytokines measured. Hazard ratios are per log2 pg/mL increase. In red pro-inflammatory cytokines mainly produced by monocytes; in yellow pro-inflammatory cytokines mainly related to neutrophil function; in purple pro-inflammatory cytokines related to T helper 1 function; in green anti-inflammatory cytokines; in dark blue growth factors.

All hazard ratios are adjusted for age, sex, CD4 count, HIV viral load, ART status and Plate Number, where applicable. q-values are shown on the left; significant associations are in bold.

Figure 3. Time to death and innate responses to *S. pneumoniae*

Kaplan-Meier curves for the survival analysis of monocyte (n=31) and neutrophil (n=37) production of IL6 and TNF-α in response to *S. pneumoniae* are shown. The population of patients with HIV-associated TB was split at the median for each respective analysis; blue lines are for the group with cytokine production above the median, in red the patients who had responses below the median.

Figure 4. Principal component analysis

(A) Values for principal component 1 (PC1) for patients with HIV-associated TB who died, versus those that survived; those patients who died had significantly higher values for PC1.
(B) Loadings of respective variables on the first principal component (PC1); variables significantly associated with clinical outcome are coloured red. Variables significantly associated with clinical outcome (Mann-Whitney U test $q < 0.10$ & $p < 0.05$) tend to have high loadings, hence contribute strongly to PC1.
(C) Heatmap showing variables associated significantly with mortality in PCA. Survivors are in blue, non-survivors in red. Increased production of pro-inflammatory cytokines in unstimulated samples appeared to be associated with mortality, as well as impaired production of pro-inflammatory cytokines in response to all antigen stimuli used.

TABLE LEGENDS

Table 1 Clinical characteristics and haematology and chemistry results

[1] q – value of comparisons between the HIV-infected control group and HIV-associated TB patients; significant differences (q <0.10) are in bold

[2] q – value of comparisons between the patient group that deceased and survived; significant differences (q <0.10) are in bold.

Chi-square tests were used for categorical data, Student's t-test for normally distributed continuous data and Mann-Whitney-U tests for non-normally distributed data. Medians and interquartile ranges are shown for all variables, unless otherwise indicated.

[§]Human Immunodeficiency Virus (HIV); †Tuberculosis (TB); *Antiretroviral therapy (ART); [‡]Viral Load (VL).

Table 2 Cytokine concentrations in culture supernatants

Median and IQRs of cytokines concentrations measured in culture supernatants of HIV-infected control patients, HIV-TB survivors and HIV-TB non-survivors, respectively. Values are in picogram per millilitre. Mann-Whitney U tests were used for non-parametric data, Students' T-tests for parametric data.

[1]q – value of comparisons between the HIV-infected control group and HIV-associated TB patients; significant differences (q <0.10) are in bold.

[2]q – value of comparisons between the patient group that deceased and survived; significant differences (q <0.10) are in bold.

* Interquartile range (IQR); [†]colony stimulating factor (CSF); [‡]interferon (IFN); [§]interleukin (IL); [||]tumour necrosis factor-α (TNF-α); **lipopolysaccharide (LPS).

Table 1 Clinical characteristics and haematology and chemistry results

	HIV§+TB†-Controls (n=14)	HIV§+TB†+Patients (n=60)	q-value[1]	Survivors (n=44)	Deceased (n=16)	q-value[2]
Demographics						
Male sex (n,%)	4/14 (29)	30/60 (50)	0.21	24/44 (55)	6/16 (38)	0.22
Age	34 (29-42)	39 (33-45)	0.25	36 (31-41)	44 (35-55)	**0.09**
ART* status (n,%)						
Naïve	8/14 (57)	31/60 (52)	0.79	25/43 (57)	6/16 (38)	0.35
*On ART**	1/14 (7)	16/60 (27)		11/43 (25)	5/16 (31)	
Defaulted	5/14 (36)	12/60 (20)		7/43 (16)	5/16 (31)	
HIV§ disease markers						
CD4 count (cells/µL)	71 (56-121)	53 (22-132)	0.26	57 (22-139)	45 (19-90)	0.63
HIV§ VL‡ (log)	4.55 (2.16-5.29)	5.62 (3.97-6.08)	**0.02**	5.68 (4.85-6.16)	4.39 (3.18-5.69)	0.17
HIV§ VL‡ undetectable (n,%)	3 (21)	5 (8.3)	0.19	3 (6.8)	2 (12.5)	0.48
TB† diagnostics						
Sputum culture/Xpert positive (n,%)	0/14 (0)	35/41 (85)	**0.0002**	26/31 (84)	9/10 (90)	0.69
Urine Xpert positive (n,%)	0/14 (0)	15/35 (43)	**0.0002**	9/26 (35)	6/9 (66)	0.22
TB† blood culture positive (n,%)	ND	31/60 (52)		24/44 (55)	7/16 (44)	0.60
Sepsis criteria						
Sepsis (n,%)	0/14 (0)	60/60 (100)	**0.0002**	44/44 (100)	16/16 (100)	ND
Severe sepsis (n,%)	0/14 (0)	39/60 (65)	**0.0002**	26/44 (59.1)	13/16 (81.3)	0.22
Septic shock (n,%)	0/14 (0)	23/60 (38)	**0.0002**	14/44 (31.8)	9/16 (56.3)	0.22
Haematology						

Hemoglobin (g/dL)	12.0 (10.1-12.9)	8.9 (6.7-10.8)	**0.002**	9.3 (7.0-11.4)	6.9 (6.4-9.8)	0.22
White cell count (*10^9/L)	3.7 (2.9-4.5)	6.4 (4.4-9.4)	**0.0002**	7.0 (4.8-10.4)	5.8 (3.9-7.6)	0.22
Neutrophils (*10^9/L)	1.2 (1.3-2.5)	5.8 (3.6-9.3)	**0.0002**	6.0 (4.2-9.5)	4.7 (2.7-6.8)	0.22
Lymphocytes (*10^9/L)	1.00 (0.88-1.43)	0.56 (0.36-0.96)	**0.002**	0.58 (0.37-1.03)	0.49 (0.29-0.78)	0.31
Monocytes (*10^9/L)	0.36 (0.33-0.42)	0.33 (0.16-0.51)	0.53	0.39 (0.19-0.58)	0.20 (0.12-0.43)	0.22
Platelets (*10^9/L)	220 (192-316)	251 (179-325)	0.75	250 (183-325)	259 (119-330)	0.78
Serum chemistry						
Glucose (mmol/L)	ND	5.3 (4.9-6.5)	ND	5.3 (5.0-6.2)	5.5 (3.9-7.8)	0.86
Lactate (mmol/L)	ND	1.9 (1.3-3.0)	ND	1.8 (1.2-2.6)	2.7 (1.5-3.8)	0.22
Procalcitonin(ug/L)	ND	2.42 (0.54-8.67)	ND	1.31 (0.36-4.98)	8.28 (3.63-61.05)	**0.07**
C-reactive protein (mg/L)	ND	143 (94-191)	ND	139 (83-191)	143 (129-230)	0.39
Albumin (g/L)	ND	23 (19-28)	ND	25 (19-28)	20 (17-24)	0.15
Creatinine (µmol/L)	ND	90 (49-131)	ND	84 (60-139)	105 (61-195)	0.63

[1] q – value of comparisons between the HIV-infected control group and HIV-associated TB patients; significant differences (q <0.10) are in bold

[2] q – value of comparisons between the patient group that deceased and survived; significant differences (q <0.10) are in bold.

Chi-square tests were used for categorical data, Student's t-test for normally distributed continuous data and Mann-Whitney-U tests for non-normally distributed data. Medians and interquartile ranges are shown for all variables, unless otherwise indicated.

[§]Human Immunodeficiency Virus (HIV); †Tuberculosis (TB); *Antiretroviral therapy (ART); [‡]Viral Load (VL).

Table 2 Cytokine concentrations in culture supernatants

	HIV+TB- Controls		HIV+TB+ Patients			HIV+TB+ Deceased		HIV+TB+ Survived		
Cytokine	**Median**	**IQR***	**Median**	**IQR***	**q-value[1]**	**Median**	**IQR***	**Median**	**IQR***	**q-value[2]**
Unstimulated	**n=14**		**n=60**			**n=16**		**n=44**		
CSF[†]-3	**51**	**39.78-86.03**	**77**	**52.4-163.5**	**0.04**	**134.7**	**75.3-341.8**	**66.86**	**48.6-143.9**	**0.09**
CSF[†]-2	14	10.65-18.82	12	10.5-13.7	0.30	11.69	10.7-14.2	12.01	10.4-13.7	1.00
IFN[‡]-A2	15	10.97-20.00	14	10.9-18.4	0.66	13.41	11.7-18.1	14.07	10.1-19.1	0.99
IFN[‡]-γ	**45**	**16.36-74.39**	**92**	**39.7-249.4**	**0.02**	168.5	81.5-246.6	72.73	35.1-269.6	0.49
IL[§]-10	23	14.77-34.57	24	14.9-29.6	0.96	25.91	14.4-62.6	23.11	14.9-27.7	0.61
IL[§]-12p40	21	13.84-31.33	20	16.3-23.7	0.82	21.03	14.7-27.0	19.57	16.3-23.5	0.76
IL[§]-1RA	151	83.78-212.6	252	109.2-495.3	0.14	566.7	130.2-958.6	241.9	107.2-423.8	0.30
IL[§]-1β	**6**	**3.75-29.79**	**3**	**2.4-4.7**	**0.01**	3.19	2.5-4.4	3.14	2.34-5.0	0.93
IL[§]-6	91	34.59-442.9	50	24.4-106.8	0.16	55	41.3-128.2	45.09	20.4-90.8	0.35
IL[§]-8	**1826**	**922.5-4095**	**619**	**276.8-1563**	**0.01**	579.4	279-1314	658.8	220.1-1781	0.89
TNF-α[II]	111	69.52-291.7	93	62.7-153.9	0.48	125.1	72.7-202	88.18	54.9-137.2	0.30
LPS**	**n=14**		**n=60**			**n=16**		**n=44**		
CSF[†]-3	363	259.5-514.8	393	206.1-614.0	0.89	439.5	227.6-1235	370.6	202.8-557.3	0.66
CSF[†]-2	**18**	**12.46-23.33**	**13**	**11.4-14.8**	**0.02**	12.69	11.4-15.4	13.28	11.4-14.8	0.79
IFN[‡]-A2	16	14.30-24.19	15	11.9-19.1	0.31	14.63	11.9-17.6	14.94	11.7-20.8	0.93
IFN[‡]-γ	**38**	**29.85-74**	**87**	**51.3-217.4**	**0.02**	160.2	83.6-229.4	76.97	44.4-212	0.31
IL[§]-10	236	199.9-493	225	83.2-445.2	0.50	170.9	65.4-341.1	236	112.9-635.3	0.31
IL[§]-12p40	**130**	**63.49-274**	**38**	**25.2-82.8**	**0.01**	33.82	23.5-71.5	42.07	25.8-89.0	0.61
IL[§]-1RA	**768**	**388.5-1511**	**2219**	**993.7-4478**	**0.01**	1961	500-4498	2302	1137-4607	0.66

IL[§]-1β	**2740**	**1772-4494**	**337**	**61.2-1024**	**0.0001**	149.6	43.3-451	416.3	89.3-1102	0.24
IL[§]-6	**10432**	**3015-12118**	**7860**	**2664-9437**	**0.07**	2941	1829-9386	8372	3442-9475	0.31
IL[§]-8	7229	3150-9331	4079	1788-9546	0.29	3229	1778-9309	4891	1793-9684	0.76
TNF-α[II]	**7452**	**3634-10339**	**1669**	**823.4-4490**	**0.01**	1197	589.6-2734	2506	961.6-5070	0.21
S. pneumoniae	**n=14**		**n=37**			**n=11**		**n=26**		
CSF[†]-3	**81**	**65-122**	**149**	**88.1-240.6**	**0.04**	**237.5**	**138.0-483.5**	**103**	**78.1-171**	**0.08**
CSF[†]-2	**21**	**14-27**	**13**	**11.4-14.8**	**0.0001**	12.69	9.7-13.3	12.69	11.5-14.9	0.76
IFN[‡]-A2	16	12--21	14	11.5-19.4	0.50	13.76	12.5-22.5	13.64	11.0-18.6	0.81
IFN[‡]-γ	**35**	**25-82**	**118**	**49.6-211.0**	**0.02**	150.3	56.5-182.5	97.6	45.9-224.3	0.80
IL[§]-10	39	32-102	41	25.3-72.2	0.77	34.88	21.3-76.8	42.75	25.7-63.8	0.89
IL[§]-12p40	**43**	**27-60**	**25**	**19.9-35.0**	**0.04**	25.44	18.2-30.0	25.53	20.7-37.7	0.76
IL[§]-1RA	**422**	**168-488**	**801**	**499.3-1880**	**0.0001**	1030	424.2-3361	786.9	522.3-1789	0.76
IL[§]-1β	**955**	**786-1252**	**131**	**21.2-265.6**	**0.0001**	**20.55**	**9.3-61.5**	**152**	**47.7-311.5**	**0.08**
IL[§]-6	**7491**	**2114-8801**	**1581**	**339.5-3673**	**0.01**	**834.6**	**169.6-1586**	**2031**	**409.5-4748**	**0.08**
IL[§]-8	10000	3544-12138	9067	2890-11003	0.52	4028	1394-10033	9415	4394-11286	0.35
TNF-α[II]	**5158**	**2965-7742**	**1270**	**480.0-3063**	**0.01**	488.2	248.8-1021	2074	750.7-3263	0.20
M. tuberculosis	**n=14**		**n=54**			**n=14**		**n=40**		
CSF[†]-3	**107**	**64-275**	**404**	**178.4-902.1**	**0.01**	383.1	305.9-924.2	445	135.4-929.1	0.89
CSF[†]-2	32	23-45	21	13.6-32.3	0.10	17.73	12.7-20.8	23.61	14.4-39.3	0.21
IFN[‡]-A2	16	13-21	15	12.2-19.9	0.42	14.6	12.0-19.2	14.74	12.3-20.5	0.89
IFN[‡]-γ	**37**	**18-68**	**138**	**55.2-289.0**	**0.01**	149.7	62.2-226.1	119.2	54.5-293.3	0.96
IL[§]-10	125	61-194	93	47.4-306.9	0.93	54.45	44.2-148.4	104.8	62.9-365.8	0.30
IL[§]-12p40	28	17-42	25	17.5-37.2	0.89	22.32	16.3-30.1	25.94	18.1-38.7	0.49
IL[§]-1RA	**295**	**139-554**	**844**	**315.7-1517**	**0.01**	1051	192.9-2238	816.9	369.9-1448	0.96

IL[§]-1β	705	389-1305	415	48.6-1203	0.48	202.9	29.6-411.2	686.4	67.8-2329	0.19
IL[§]-6	5744	2441-7976	5848	1528-10229	0.77	1972	1097-8467	7954	2044-10663	0.26
IL[§]-8	9121	2805-11520	9303	3516-11278	0.73	8351	5566-10254	9798	3430-11789	0.66
TNF-α[II]	2103	1508-3971	2296	755.0-6655	0.74	1153	548.6-2473	2793	844.6-8411	0.20

Median and IQRs of cytokines concentrations measured in culture supernatants of HIV-infected control patients, HIV-TB survivors and HIV-TB non-survivors, respectively. Values are in picogram per millilitre. Mann-Whitney U tests were used for non-parametric data, Students' T-tests for parametric data.

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[2]q – value of comparisons between the patient group that deceased and survived; significant differences (q <0.10) are in bold.

* Interquartile range (IQR); [†]colony stimulating factor (CSF); [‡]interferon (IFN); [§]interleukin (IL); [II]tumour necrosis factor-α (TNF-α);

**lipopolysaccharide (LPS).

Figure 1.

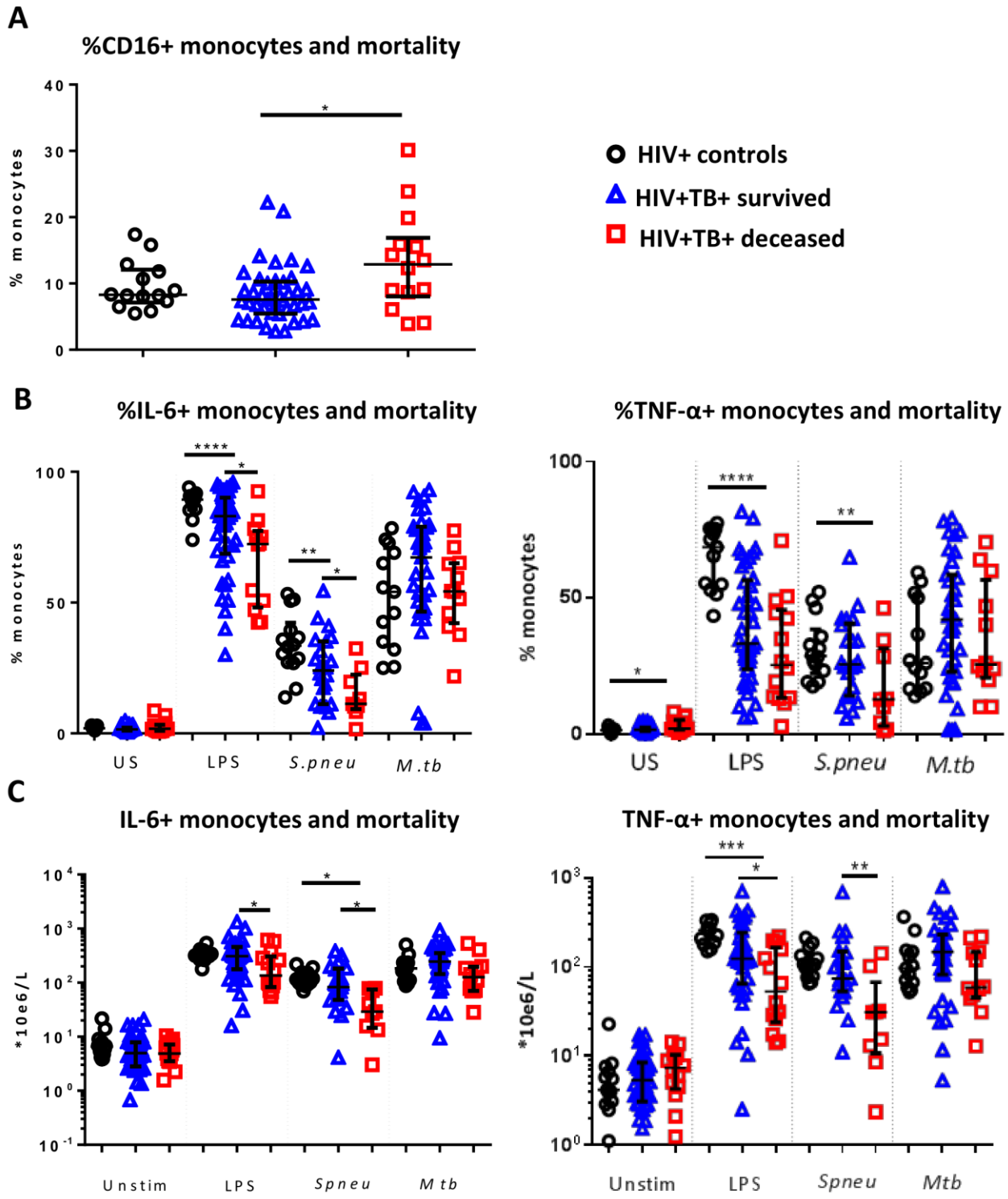

A
%CD16+ monocytes and mortality
% monocytes
HIV+ controls
HIV+TB+ survived
HIV+TB+ deceased
B
%IL-6+ monocytes and mortality
%TNF-α+ monocytes and mortality
% monocytes
US
LPS
S.pneu
M.tb
C
IL-6+ monocytes and mortality
TNF-α+ monocytes and mortality
*10e6/L
Unstim
LPS
Spneu
Mtb

Figure 2.

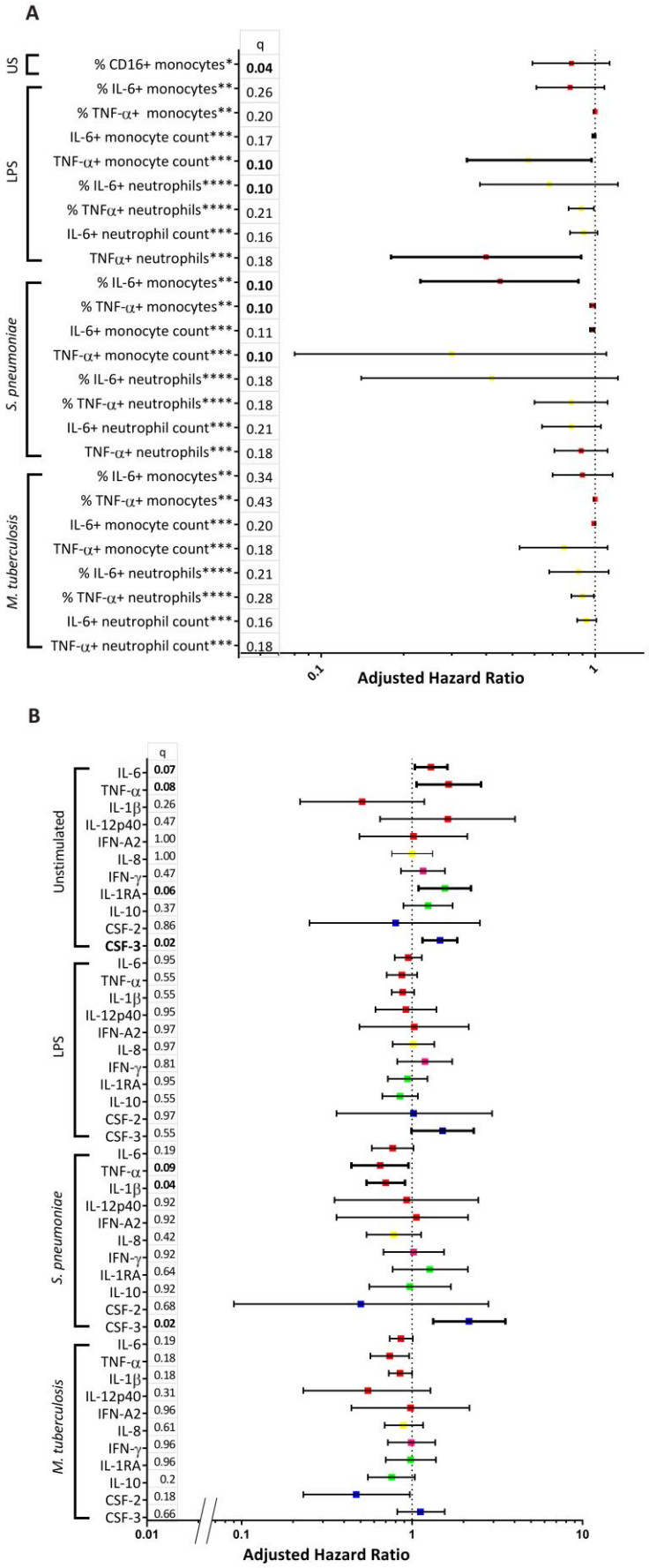

A
q
US
% CD16+ monocytes* 0.04
% IL-6+ monocytes** 0.26
LPS
% TNF-α+ monocytes** 0.20
IL-6+ monocyte count*** 0.17
TNF-α+ monocyte count*** 0.10
% IL-6+ neutrophils**** 0.10
% TNFα+ neutrophils**** 0.21
IL-6+ neutrophil count*** 0.16
TNFα+ neutrophils*** 0.18
S. pneumoniae
% IL-6+ monocytes** 0.10
% TNF-α+ monocytes** 0.10
IL-6+ monocyte count*** 0.11
TNF-α+ monocyte count*** 0.10
% IL-6+ neutrophils**** 0.18
% TNF-α+ neutrophils**** 0.18
IL-6+ neutrophil count*** 0.21
TNF-α+ neutrophils*** 0.18
M. tuberculosis
% IL-6+ monocytes** 0.34
% TNF-α+ monocytes** 0.43
IL-6+ monocyte count*** 0.20
TNF-α+ monocyte count*** 0.18
% IL-6+ neutrophils**** 0.21
% TNF-α+ neutrophils**** 0.28
IL-6+ neutrophil count*** 0.16
TNF-α+ neutrophil count*** 0.18
0.1
1
Adjusted Hazard Ratio
B
q
Unstimulated
IL-6 0.07
TNF-α 0.08
IL-1β 0.26
IL-12p40 0.47
IFN-A2 1.00
IL-8 1.00
IFN-γ 0.47
IL-1RA 0.06
IL-10 0.37
CSF-2 0.86
CSF-3 0.02
LPS
IL-6 0.95
TNF-α 0.55
IL-1β 0.55
IL-12p40 0.95
IFN-A2 0.97
IL-8 0.97
IFN-γ 0.81
IL-1RA 0.95
IL-10 0.55
CSF-2 0.97
CSF-3 0.55
S. pneumoniae
IL-6 0.19
TNF-α 0.09
IL-1β 0.04
IL-12p40 0.92
IFN-A2 0.92
IL-8 0.42
IFN-γ 0.92
IL-1RA 0.64
IL-10 0.92
CSF-2 0.68
CSF-3 0.02
M. tuberculosis
IL-6 0.19
TNF-α 0.18
IL-1β 0.18
IL-12p40 0.31
IFN-A2 0.96
IL-8 0.61
IFN-γ 0.96
IL-1RA 0.96
IL-10 0.2
CSF-2 0.18
CSF-3 0.66
0.01
0.1
1
10
Adjusted Hazard Ratio

Figure 3.

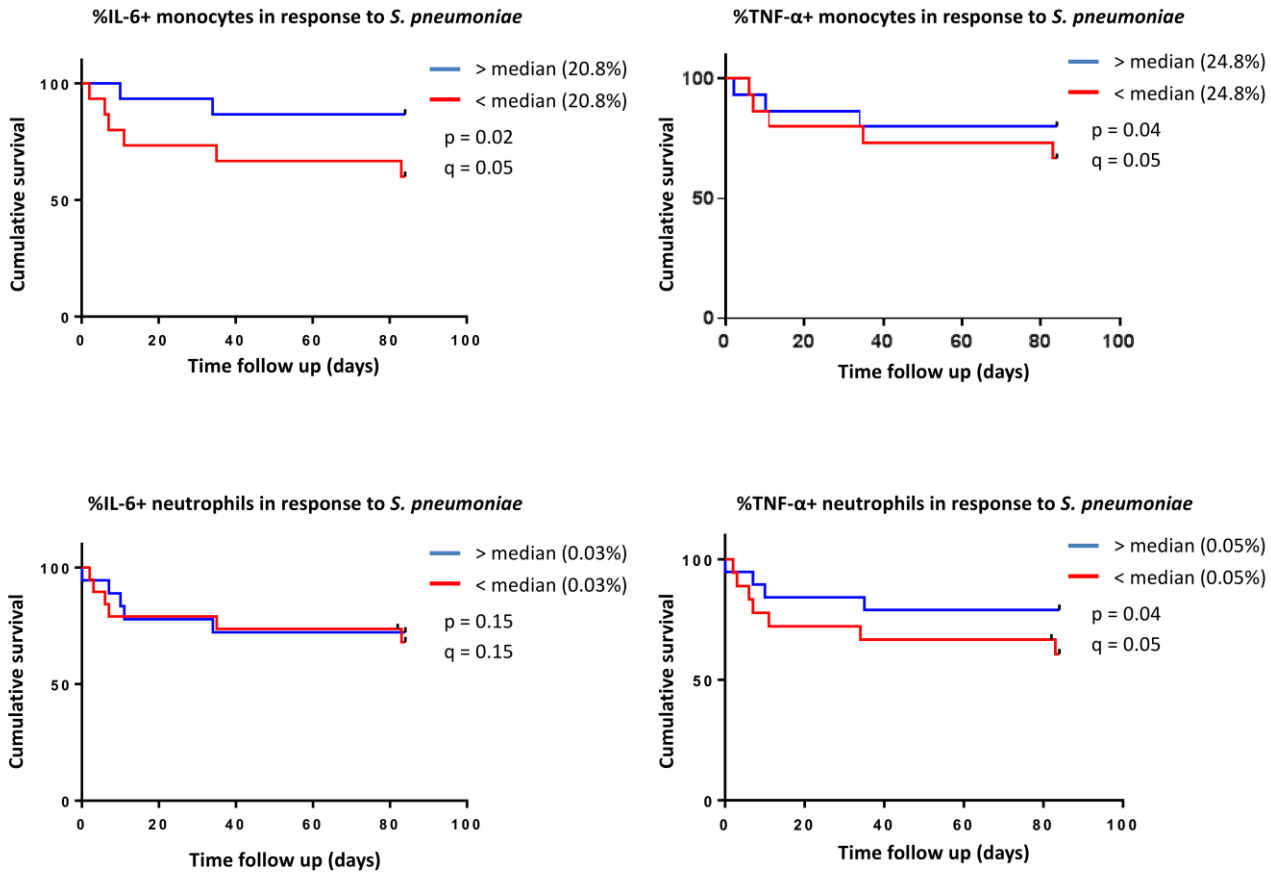
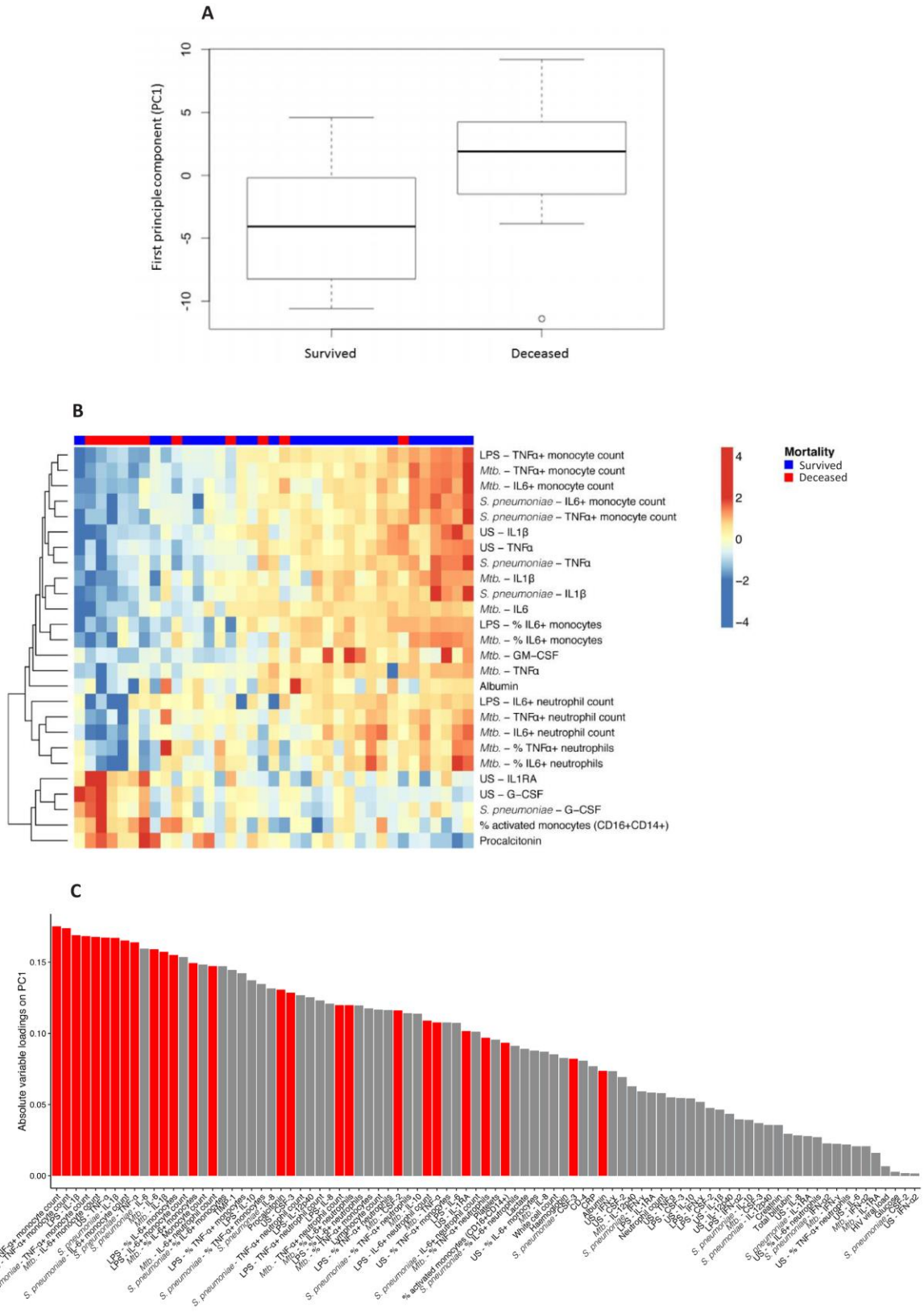

%IL-6+ monocytes in response to S. pneumoniae
> median (20.8%)
< median (20.8%)
p = 0.02
q = 0.05
Cumulative survival
Time follow up (days)
%TNF-α+ monocytes in response to S. pneumoniae
> median (24.8%)
< median (24.8%)
p = 0.04
q = 0.05
Cumulative survival
Time follow up (days)
%IL-6+ neutrophils in response to S. pneumoniae
> median (0.03%)
< median (0.03%)
p = 0.15
q = 0.15
Cumulative survival
Time follow up (days)
%TNF-α+ neutrophils in response to S. pneumoniae
> median (0.05%)
< median (0.05%)
p = 0.04
q = 0.05
Cumulative survival
Time follow up (days)

Figure 4.


A
First principle component (PC1)
10
5
0
-5
-10
Survived
Deceased
B
LPS – TNFα+ monocyte count
Mtb. – TNFα+ monocyte count
Mtb. – IL6+ monocyte count
S. pneumoniae – IL6+ monocyte count
S. pneumoniae – TNFα+ monocyte count
US – IL1β
US – TNFα
S. pneumoniae – TNFα
Mtb. – IL1β
S. pneumoniae – IL1β
Mtb. – IL6
LPS – % IL6+ monocytes
Mtb. – % IL6+ monocytes
Mtb. – GM–CSF
Mtb. – TNFα
Albumin
LPS – IL6+ neutrophil count
Mtb. – TNFα+ neutrophil count
Mtb. – IL6+ neutrophil count
Mtb. – % TNFα+ neutrophils
Mtb. – % IL6+ neutrophils
US – IL1RA
US – G–CSF
S. pneumoniae – G–CSF
% activated monocytes (CD16+CD14+)
Procalcitonin
4
2
0
−2
−4
Mortality
Survived
Deceased
C
Absolute variable loadings on PC1
0.15
0.10
0.05
0.00