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Review

Strong anti-Epstein Barr virus (EBV) or cytomegalovirus (CMV) cellular immune responses predict survival and a favourable response to anti-tuberculosis therapy

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ABSTRACT

Background: Intact immune responses to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) represent a biologically and clinically relevant correlate of 'immunological fitness' in humans. However, there is a lack of knowledge concerning anti-EBV or anti-CMV responses in patients with pulmonary tuberculosis (TB), in whom aberrant immune responses may promote progression of clinical disease.
Methods: Venous blood samples were obtained at the time of (sputum smear positive) pulmonary TB diagnosis. A whole blood assay was performed by exposing PBMCs (peripheral blood mononuclear cells) to a panel of infectious antigens, including CMV, EBV and mycobacterial proteins. Cell culture supernatants were collected after seven days and interferon gamma (IFN-γ) was measured using a sandwich ELISA. Patients received standard first line anti-tuberculosis rifampicin (R)/isoniazid (H)/ethambutol (E)/pyrazinamide (Z) for two months followed by RH for four months.
Results: PBMCs from cured patients (after treatment completion) exhibited significantly stronger IFN-γ responses to CMV (p=0.035), EBV (p=0.006) or *Mycobacterium tuberculosis* ESAT-6 (p = 0.043) at the time of diagnosis as compared to patients who succumbed to TB during treatment. IFN-γ responses to other viral (H5N1, HSV-1) as well as other mycobacterial (Ag85A, Rv2958c, Rv0447c) antigens were not found to be significantly different among patients who were cured or those who succumbed to TB.
Conclusions: Increased cellular immune responses to CMV and EBV antigens at the time of diagnosis of pulmonary tuberculosis are associated with increased survival after a standard six months anti-TB therapy. CVM and EBV antigens may represent "intrinsic markers for immune fitness" and guide improved TB therapies including host-directed therapies.

Contents

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Introduction

Intact cellular immune responses to Epstein-Barr virus (EBV) and cytomegalovirus (CMV) reflect the general immunological fitness.[1,2] Compromised immune responses to either CMV or EBV are linked to disease progression in patients with cancer or in patients after transplantation.[1,2] Progression of *Mycobacterium tuberculosis* (*M. tb*) infection to clinical TB disease is associated with aberrant host immune responses.[3] Increased T-cell reactivity to mycobacterial purified protein derivative (PPD) has been reported in individuals without active TB, irrespective of a concomitant HIV infection as reflected by strong Th1 responses (interferon gamma (IFN-γ)) and tumour necrosis factor alpha (TNF-α) production in response to CMV, while EBV-specific IFN-γ/TNF-α responses were generally weaker than CMV or PPD responses.[4,5] The immune reactivity to EBV and CMV among patients with active pulmonary TB has not been explored, particularly in the context of survival. Using whole blood assays and enzyme-linked immunosorbent assay (ELISA), we present here the IFN-γ responses in peripheral blood of patients pulmonary TB to CMV and EBV antigens versus survival in the course of standard anti-TB therapy.

Methods

Ethical clearance was granted by the Muhimbili University of Health and Allied Health sciences (MUHAS) Senate Research and Publications committee (Ref. No. RP/AEC/VOL.XII).

Sputum smear positive pulmonary tuberculosis (PTB) patients were recruited at diagnosis and prior to initiating anti-TB therapy after obtaining written informed consent. The following selection criteria applied: 18 years and above; confirmed for pulmonary TB by *M. tb* smear positivity ; with or without HIV co-infection; without previous TB episode; visiting one of the three study sites in Dar-es-Salaam: Mnazi mmoja health centre, Mwananyamala or Amana municipal hospitals. For the whole blood assay, venous blood drawn at TB diagnosis was diluted 1:1.5 in RPMI medium supplemented with 10% foetal calf serum (FCS) and 1% antibiotics and cultured in micro-titre plates containing a panel of twelve infectious antigens, each at 1 μg/ml (Table 1). The plates were incubated at 37 °C with 5% CO_2 over a 7-day period, after which supernatants were harvested and stored at −80 °C. IFN-γ content was measured in the supernatants using a commercially available ELISA kit (Mabtech, Sweden).

Table 1
List of whole blood assay antigens.

Antigen	Source pathogen	Description of the target antigen
EBNA1	Epstein-Barr virus (EBV)	Epstein-Barr nuclear antigen 1, involved in negative regulation of MHC class antigen processing and presentation
CMV pp65	Cytomegalovirus (CMV)	CMV tegument protein (UL83) involved in immune regulation in the host
PPD	*Mycobacterium tuberculosis* (*M. tb*) H37Ra	Purified cellular protein extract of an attenuated *M. tb* strain (H37Ra)
ESAT-6	*M. tb* H37Rv	Pore-forming toxin secreted by pathogenic mycobacteria, B- and T-cell target
Ag85A	*M. tb* H37Rv	Mycolyl transferase, a dominant secreted antigen, serves as B- and T-cell target
Rv2957	*M. tb* H37Rv	Cytoplasmic glycosyltransferase involved in mycobacterial cell wall synthesis and maintenance
Rv2958c	*M. tb* H37Rv	Cytoplasmic glycosyltransferase involved in mycobacterial cell wall synthesis and maintenance
Rv0447c	*M. tb* H37Rv	Cytoplasmic cyclopropane-fatty-acyl-phospholipid synthase involved in mycobacterial cell wall synthesis and maintenance
HSV-1 antigen	Herpes simplex virus 1 (HSV-1)	HSV-1 glycoprotein (gG)
H5N1 antigen	Influenza virus	Recombinant H5N1 Influenza-A Virus Vietnam 1203/04
HIV env	Human immunodeficiency virus (HIV)	gp160 protein involved in viral replication
HIV gag	HIV	group-specific antigen involved in viral particle assembly

Table 2
Comparison of baseline characteristics among patients with tuberculosis according to vital status at the end of treatment with anti-tuberculosis (n = 234).

		Survived (n = 213)	Died (n = 21)	Total (n = 234)	P
	Missing	n(%)/median	n(%)/median	n(%)/median	
% Male sex	0	144 (67,6)	11 (52,4)	155 (66,2)	0.23
Age (years)	1	36,0	35,00	36,00	0.69
CD4+ at TB diagnosis (cell/mL)	21	459.0	342	425	0.06
% HIV infected	0	81 (38,0)	15 (71,4)	96 (41,0)	0.004
% on Fluconazole treatment	43	11 (59.78)	1 (14.29)	12 (6.28)	0.370
HIV and anti-retroviral therapy	0				
HIV un-infected		132 (62,0)	6 (28,6)	138 (59,0)	0.0001
HIV infected ART* prior to anti-TB		31 (14,6)	2 (9,5)	33 (14,1)	
HIV infected ART* during anti-TB		47 (22,1)	10 (47,6)	57 (24,4)	
HIV infected no ART at any time of anti-TB		3 (1,4)	3 (14,3)	6 (2,6)	
MTB load	0				
Scanty		28 (13,145)	2 (9,52)	30 (12,82)	0.972
1+		57 (26,76)	6 (28,57)	63 (26,92)	
2+		60 (28,17)	6 (28,57)	66 (28,21)	
3+		68 (31,92)	7 (33,33)	75 (32,05)	
Smoking status	4				
Never smoked		156 (74,3)	19 (95,0)	175 (76,1)	0.115
Ever smoked		54 (25,7)	1 (5,0)	55 (26.1)	

* ARV – Antiretroviral therapy.

All patients followed the Tanzanian treatment protocol which requires combination of isoniazid, rifampicin, ethambutol and pyrazinamide for two months followed by rifampicin and isoniazid for 4 months. All patients had direct observed therapy at the most convenient clinic or with a treatment supporter. Drug adherence in our cohort was 97.3%.

Statistical analysis was performed with the SPSS software and GraphPad Prism 6 was used to create graphs. Mann-Whitney U test was used to test the median difference between IFN-γ concentration between cured/survivors and those who died during tuberculosis treatment (all-cause mortality). Reported gamma interferon results are absolute values after subtracting background reactivity of each individual against medium value. P value of less than 0.05 was considered significant.

Results

We report a total of 21 deaths which occurred during the course of follow up; most TB patients (72%) who exhibited a compromised IFN-γ response died within the first three months of anti-TB treatment. Meanwhile, 213 patients with tuberculosis were successfully treated. With exception of HIV, clinical and demographic characteristics did not differ among patients who were cured compared to those who died, particularly the two patient

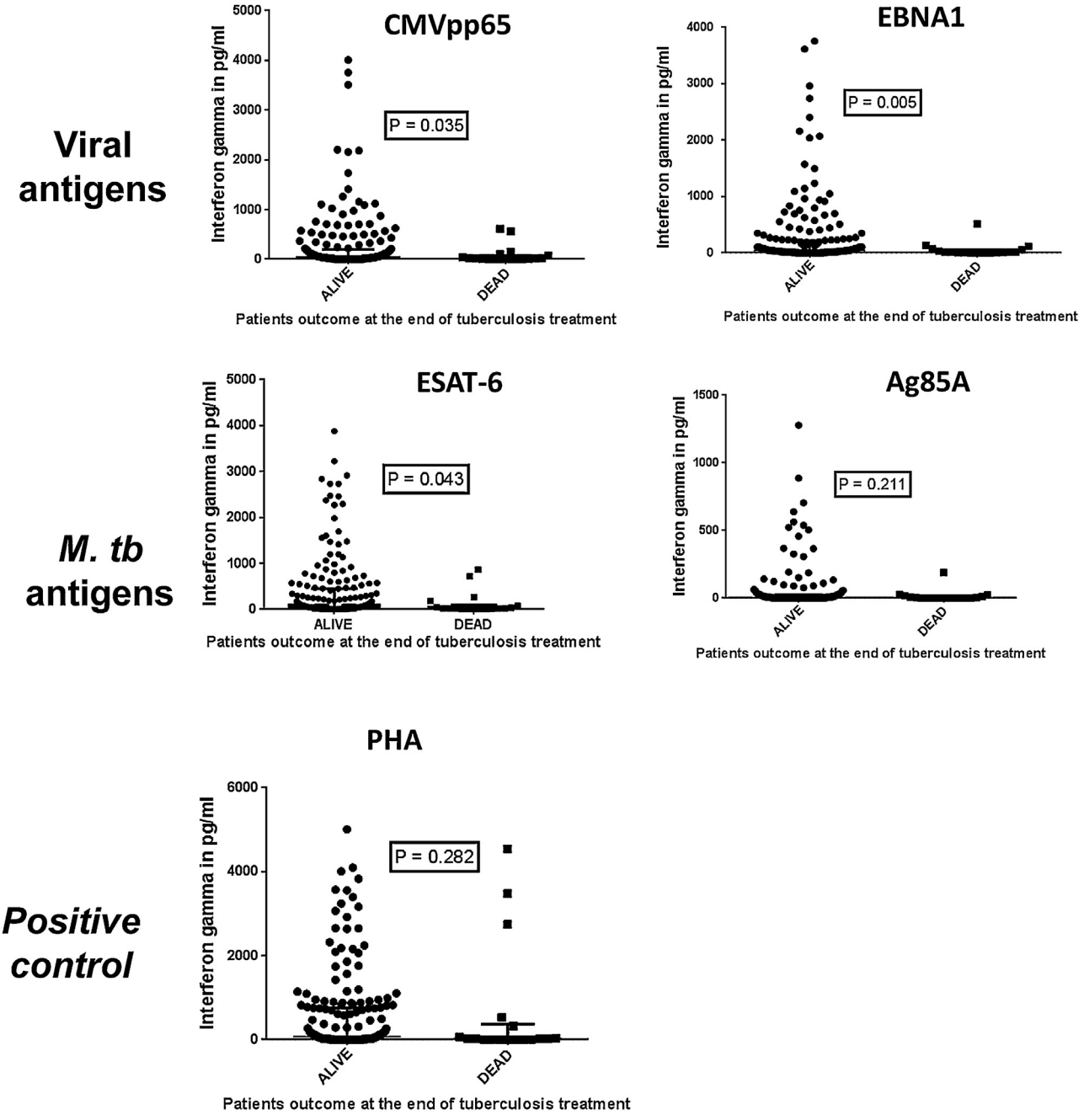

Figure 1. IFN-γ responses to viral and mycobacterial antigens in whole blood assay. Diluted venous blood from patients with pulmonary TB were exposed to a panel of viral and mycobacterial antigens over a 7-day period. Supernatants were harvested after 7 days for IFN-γ measurement by sandwich ELISA. Shown are means and standard error of the mean (SEM) (n = 234 pulmonary TB patients).gr1

groups who exhibited similar mycobacterial load (Table 2). Among those patients who succumbed, 15 had HIV co-infection while 6 had no HIV infection (Table 2).

Statistically significant differences in IFN-γ responses in peripheral blood between patients who died versus those who survived and were cured of TB were observed concerning the following antigens: ESAT-6 (0.043), CMV pp65 (p = 0.035) and EBNA1 (p = 0.006) (Figure 1). We observed a similar trend as we dichotomised IFN-γ responses by HIV infection; however, significantly higher IFN-γ responses to CMV (p = 0.004), EBV (p = 0.05) and ESAT6 (p = 0.003) were demonstrated among (surviving) patients co-infected with TB/HIV, as compared to TB/HIV patients who died.

Other infectious antigens with a similar trend, but no statistical difference were Rv2958c, Rv0447c, PPD, Ag85A, herpes simplex virus 1 (HSV-1) glycoprotein and HIV env protein. Patients who survived 6 months of TB therapy produced approximately three times more IFN-γ in response to the above-mentioned antigens as compared to those who died during the course of TB therapy.

Discussion

We show, to our knowledge, for the first time that impaired cellular immune responses to ESAT-6, CMV (pp65) or EBV (EBNA-1), reflected by IFN-γ production in peripheral blood are associated with death among TB patients during anti-TB treatment. Intact T-cell responses to CMV and/or EBV are integral to general immunological fitness. EBNA-1 represents a constitutively expressed latency-associated antigen of EBV, it is also a CD4 T cell target that induces potent Th1 responses in EBV-positive individuals.[6] CMV pp65 is a dominant T-cell target that also elicits strong antibody responses; anti-CMV pp65 reactivity is considered to represent a clinically and biologically relevant biomarker to gauge the overall anti-CMV response in humans.[1]

ESAT-6 is a virulence factor expressed and secreted by pathogenic mycobacteria including *M. tb*, and a strong T-cell target.[7] The QuantiFERON-TB Gold IFN-γ-release assay for latent TB infection (LTBI) diagnosis is based on peripheral blood T-cell responses to ESAT-6 and its chaperone, CFP10,[8] while several clinical TB vaccine candidates incorporate ESAT-6 in their design.[9] Taken together, impaired cellular responses to EBV, CMV and ESAT-6 during the intensive, first, phase of anti-TB therapy is a potential predictor of poor survival among pulmonary TB patients. Although statistically not significant, reduced IFN-γ responses to the *M. tb* antigens (Rv2958c, Rv0447c and Ag85A) as well as to PPD[8] in PBMCs from patients who died during treatment, suggests that these antigens may play a role in orchestrating protective cellular immune responses in pulmonary TB.

We believe it is clinically and biologically relevant to test for EBV/CMV DNA in the blood samples of TB patients in future studies and link this information to their respective anti- EBV/CVM directed immune reactivity. Gauging immune responses directed against CMV or EBV as markers of 'immune-fitness' may aid in identifying patients who are at risk to succumb to TB, or are effectively able to combat *M. tb* – without overt organ/tissue (pulmonary) damage, which also associates with long-term aberrant inflammation and immune-exhaustion.[3,10] In order to discern whether CMV/EBV responses are a reflection of prior or acute infection, it would add value to assess EBV and CMV DNA viral load; this may be considered a limitation of this study.

Conclusion

CMV, EBV and ESAT-6 – specific IFN-γ responses in TB patients with pulmonary TB at the time point of diagnosis correlate with cure and survival during anti-TB therapy. We recommendd future studies concerning i) CVM/EBV-vectored vaccines, and ii) gauging CMV/EBV responses as prognostic markers to identify patients at high risk to succumb to TB disease and to identify time points for Host-Directed Therapies (HDTs). 'Immune competence' may be measured using anti-EBV or anti-CMV cellular immune responses in individuals using this (anti-EBV/anti-CMV) immunological 'setpoint' as a clinically and biological relevant parameter for 'T-cell fitness'.

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