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Short Communication

Differential RD-1-specific IFN-γ host responses to diverse *Mycobacterium tuberculosis* strains in HIV-uninfected persons may be explained by genotypic variation in the ESX-1 region

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ABSTRACT

Objectives: Between-person variability in T-cell-specific interferon-gamma release assay (IGRA) responses and discordance between IGRA test formats are poorly understood.
Methods: We evaluated the IFN-γ responses (QuantiFERON-TB Gold-In-Tube [QFT-GIT] and TSPOT-TB) stratified according to the *Mycobacterium tuberculosis* spoligotype of the culture isolate obtained from the same patients with confirmed active tuberculosis (n = 91). We further analysed differences within the RD-1-encoding ESX-1 region between the different strain types using whole genome sequencing.
Results: In HIV-uninfected patients, TSPOT.TB and QFT-GIT IFN-γ responses were 5-fold ($p < 0.01$) and 2-fold higher ($p < 0.05$) for those infected with family 33 compared to the LAM strain (additionally, TSPOT.TB responses were 5.6-fold [$p < 0.05$] and 2.6-fold higher [$p < 0.05$] for the patients infected with the family 33 versus the X strain and Beijing versus the LAM strain, respectively). Multivariate analysis revealed that strain type (determined by spoligotyping) was independently associated with the magnitude of the IGRA response (varied by IGRA test type) and this is likely explained by variability in the ESX-1 region of *Mycobacteriumtuberculosis* (determined by next-generation sequencing).
Conclusions: These data have implications for the understanding of between-person heterogeneity in IGRA responses, *Mycobateriumtuberculosis*-specific host immunity, and the discordance between different IGRA test formats.

Introduction

There is increasing evidence that members of the Mycobacterium complex are differentially virulent both *in vivo* and *in vitro* (Aguilar et al., 2010, de Jong et al., 2008). The molecular mechanisms that underpin these differences remain unclear, however, the 6-kDa early secretory antigenic target (ESAT-6) system-1 (ESX-1) is likely involved as it is essential for *Mycobacterium tuberculosis* (*M.tb*) virulence (Gey Van Pittius et al., 2001).

We investigated whether *M.tb* strains differentially modulate host IFN-γ responses in a tuberculosis (TB) endemic setting. We further analysed genetic differences within the ESX-1 region of an independent set of strains using whole genome sequencing (WGS).

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This is the first study to show that there are multiple strain-specific genetic differences in the ESX-1 region that differentially regulate host IFN-γ responses.

Methods

Study site, population and diagnostic tests

We recruited 645 patients with suspected TB from three study sites in Cape Town, South Africa. Informed written consent was obtained from patients 18 years or older and who presented to a peri-urban primary-care TB clinic. Patients were enrolled who had one or more symptom suggestive of pulmonary TB (n = 645) according to defined WHO criteria (Bassett et al., 2010, Getahun et al., 2011), were able to expectorate two sputum specimens and had not received anti-TB treatment within the previous 60 days. Sputum was obtained for liquid culture and culture positivity was used as the reference standard for TB. Each patient received a HIV test after counselling. Whole blood was collected by venepuncture and stimulated immediately with the region of difference 1 (RD-1) antigens for use in the TSPOT.TB (Oxford Immunotech, UK) and QFT-GIT (Qiagen, Germany) interferon-gamma release assays (IGRAs) as indicated previously (Theron et al., 2012).

Strain typing and sequence analysis

Spoligotyping was performed according to the Kamerbeek method (Kamerbeek et al., 1997).

Statistics

See online data supplement.

Results

Patients and samples

The study overview and baseline characteristics of the 645 patients is shown in Figure S1 and Table S1, respectively.

Comparison of IFN-γ responses according to M.tb *spoligotype*

The IFN-γ responses for TSPOT.TB and QFT-GIT of the 91 patients stratified by strain type are depicted in Figure 1 and S2. There were differences in the IFN-γ responses for TSPOT.TB (p = 0.005) and QFT-GIT (p = 0.032) in HIV-uninfected patients (n = 51) harbouring different *M.tb* strains (Figure 1). The differences in IFN-γ readouts for TSPOT.TB were independently strain-specific and could not be explained by confounding factors such as age, sex, smear grade, and race as determined by multivariate and univariate analysis (Table 1 and S2). Similarly, differences in IFN-γ readouts for QFT-GIT were significantly associated with strain and not with age, sex or smear grade and were strain-specific. However, the QFT-GIT analysis showed that race was also significantly associated with differences in IFN-γ readouts. We further show that the strain-induced IFN-γ responses did not change with culture time to positivity, mean Xpert MTB/RIF C_T value and chest radiological scores (Figure S3, S4 and S5).

Sequence investigation of the ESX-1 region

We assessed the genetic diversity between the Beijing, X, and LAM strains in the ESX-1 region using WGS data from a convenience dataset of 104 isolates collected in the Cape Town, Western Cape to potentially explain the differences in IFN-γ readouts between the different strains (these isolates were not from the same patients where IGRA testing was performed).

A discriminant analysis of principal components was performed to investigate if we could correctly identify the strain types using mutations in these regions using a subset of 16 single nucleotide polymorphisms (SNPs) that occurred in our dataset and 429 previously described isolates from a global collection of strains; 2 principal components were retained (Table S4) (Coll et al., 2014). This model correctly typed over 99% (428/429; Table S6) of the global isolates. It thus appears that mutations in the ESX-1 region are strain-specific and allow for the identification of Beijing, X and LAM strains.

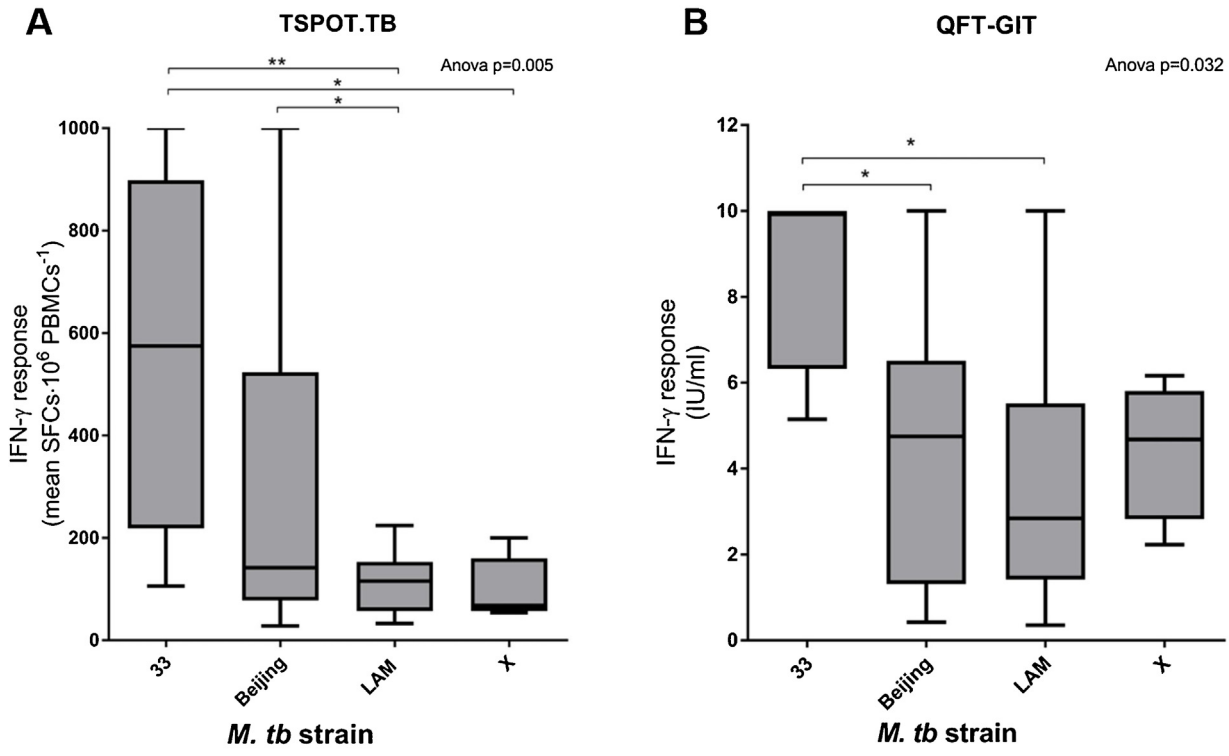
Discussion

Collectively these data suggest that the strain type, and possibly the ESX-1 genotype, modulates the RD-1 antigen specific responses in humans. This is the first study to outline strain-specific multiple mutations in the ESX-1 gene and link this to RD-1-specific Th1 responses in TB patients from an endemic setting. Our data are in keeping with animal models that show differential virulence of *M.tb* strains *in vivo* (Manca et al., 2001).

We show that the Beijing strain is associated with the most mutations in the ESX-1 region (compared to *M.tb* H37Rv) compared to the LAM and X strains (for example, we found mutations in the *mprB* and *espA* genes) which may partly explain the differences in the host immune responses observed (Gey Van Pittius et al., 2001).

The LAM strain was associated with the most missense mutations in the *eccCb1* and *espB* genes, which are pro-virulent and essential for the secretion of ESAT-6/CFP-10 (Brodin et al., 2006). It is well established that EspA/EspC and the EspA/EspB dimers are important for secretion of ESX-1 proteins and virulence (Fortune et al., 2005). Thus, mutations in *espB*, as demonstrated here for the LAM strain, may express dysfunctional EspB, resulting in defective ESX-1 secretion and reduced virulence.

There are several limitations to our study. We utilised all of the DNA from the isolates for spoligotyping and, as a result, none was available for sequencing. We therefore applied next generation WGS to a convenience set of samples from the same geographical location to further explore our hypothesis. We show that the mutations within the ESX-1 region of the individual strains are conserved using a global collection of isolates (Figure 1C), and as a result, the immunomodulatory capacity of the individual strains included in the WGS versus the IGRA analyses are likely to be similar. We could not make any inference about genetic changes in the ESX-1 region of family 33 as WGS data was unavailable for this strain. However, family 33 is quite rare in our setting representing 6.7% of 904 South African isolates collected between 2006 and 2016 (Togo et al., 2017). Spoligotyping was not performed on all the clinical isolates where TSPOT.TB and QFT-GIT were performed due to unavailability of the culture isolates including, limited material for initial bio-banking, contamination, or failure to grow. By contrast, some available isolates (n = 51) were not spoligotyped because TSPOT.TB and QFT-GIT were not available for these samples (indeterminate results *etc.*). However, these samples were unlikely to have influenced our findings because a sensitivity analysis revealed that there were no demographic differences (*i.e.* race, HIV-status, smear-status) between the groups (included versus excluded from the analysis). The strain-specific IFN-γ IGRA responses were only observed in the HIV-uninfected samples and not the HIV-infected samples. Multi-variant analysis revealed that HIV positivity could influence IGRA responses and this is most likely due to depletion of the IFN-γ producing CD4+ T-cells during HIV infection.



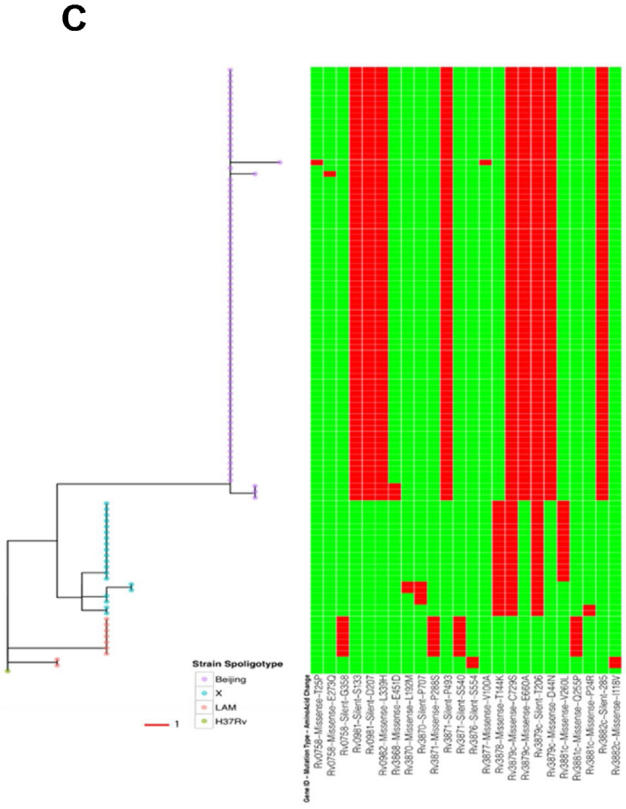


Figure 1. Differential host IFN-γ responses in HIV-uninfected patients may be explained by genotypic differences in the ESX-1 region. PBMCs were isolated from 51 HIV-uninfected patients and stimulated with the *M.tb*-specific antigens, and (**A**) TSPOT.TB and (**B**) QFT-GIT were performed as described by the manufacturer. The mean TSPOT.TB IFN-γ response was 5-fold higher in patients infected with the family 33 strain as compared to the LAM strain (564 SFCs/106 PBMCs vs 111 SFCs/106 PBMCs; $p < 0.01$). Similarly, the mean TSPOT.TB IFN-γ response was 5.6-fold higher in patients infected with the family 33 strain as compared to the X strain (564 SFCs/106 PBMCs vs 100 SFCs/106 PBMCs; $p < 0.05$). The mean TSPOT.TB IFN-γ response was 2.6-fold higher in patients infected with the Beijing strain compared to the LAM strain (292 SFCs/106 PBMCs vs 111 SFCs/106 PBMCs; $p < 0.05$). Interestingly, there were no differences in IFN-γ responses observed for HIV-uninfected patients infected with Beijing versus the LAM or the X strain for QFT-GIT. The differences in IFN-γ responses observed for TSPOT.TB versus QFT-GIT could be as a consequence of QFT-GIT utilizing an additional antigen (TB7.7). We found that patients infected with family 33 (8.7 IU/ml) strains have a 2-fold increase in mean IFN-γ expression compared to patients infected with the LAM (3.9 IU/ml; $p <$

Table 1
Multivariate regression analyses showing strain and race specific associations with a heightened IGRA response in culture-positive, HIV-uninfected active TB cases (n = 43) from an endemic setting. Please see Supplementary Table 2 and 3 for additional univariate and multivariate analyses.

		TSPOT.TB		QFT-GIT	
Term	Variable	Sensitivity estimate (95% CI)	p-Value	Sensitivity estimate (95% CI)	p-Value
Age	Years	−2.52 (−9.45, 4.40)	0.464	−0.05 (−0.14, 0.031)	0.209
Sex	Male	109.04 (−91.64, 309.72	0.277	−0.68 (−2.88, 1.52)	0.533
Race	Mixed	93.23 (−125.08, 311.54)	0.391	2.82 (0.54, 5.10)	**0.017**
Smear grade*	Scanty	−102.87 (−438.88, 233.15)	0.537	−2.88 (−7.44, 1.68)	0.206
	1+	8.56 (−232.04, 249.17)	0.943	−1.93 (−4.38, 0.51)	0.116
	2+	64.17 (−155.89, 284.22)	0.557	0.13 (−2.46, 2.72)	0.921
	3+	−14.64 (−292.38, 263.09)	0.915	0.72 (−2.56, 3.99)	0.658
Strain type	Beijing	171.11 (−30.17, 372.37)	**0.093**	−0.64 (−2.82, 1.53)	0.552
	Family 33	447.34 (152.50, 742.18)	**0.004**	4.20 (0.91, 7.49)	**0.014**
	X	−90.902 (−421.74, 239.94)	0.580	−0.05 (−0.14, 0.031)	0.918

* Smear grade was classified according to standard WHO criteria: Scanty = 1–9 acid fast bacilli (AFB)/100 oil immersion fields. +1 = 10–99 AFB/100 oil immersion fields. +2 = 1–10 AFB/50 oil immersion fields. +3 = >10 AFB/20 oil immersion fields.

In summary, RD-1-specific IFN-γ responses are associated with the specific infecting *M.tb* strain in patients from a TB endemic setting. This is likely explained by strain-specific genomic variability of the ESX-1 region. These data have implications for understanding host immunity to *M.tb*, interpreting diagnostic test results and the variability in the magnitude of IGRA responses, and the discordance between different IGRA test formats.

Conflict of interest

No conflict of interest to declare.

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Ethical approval

The study was approved by the University of Cape Town Health Sciences Ethics Committee (HREC #307/2014).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.04.053.

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0.05) or Beijing (4.2 IU/ml; $p < 0.05$) strains for QFT-GIT. Differences in strain-specific IFN-γ responses for both TSPOT.TB and QFT-GIT were only observed in HIV-uninfected patients and not in HIV-infected patients (Figure S2 A and B). The horizontal bar shows the median magnitude of the responses while the error bars represent the interquartile range. *, **, and *** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.005$, respectively. (**C**) A neighbour joining tree constructed using the inter-isolate Manhattan distances in the ESX-1 region. The red scale bar is equivalent to one SNP difference. The tree is rooted to H37Rv (AL123456.3). The tabular bar graph to the right shows the presence (red) or absence (green) of a particular mutation in each strain.