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Dominant TNF-α$^+$ *Mycobacterium tuberculosis*–specific CD4$^+$ T cell responses discriminate between latent infection and active disease

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Rapid diagnosis of active *Mycobacterium tuberculosis* (*Mtb*) infection remains a clinical and laboratory challenge. We have analyzed the cytokine profile (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2)) of *Mtb*-specific T cells by polychromatic flow cytometry. We studied *Mtb*-specific CD4$^+$ T cell responses in subjects with latent *Mtb* infection and active tuberculosis disease. The results showed substantial increase in the proportion of single-positive TNF-α *Mtb*-specific CD4$^+$ T cells in subjects with active disease, and this parameter was the strongest predictor of diagnosis of active disease versus latent infection. We validated the use of this parameter in a cohort of 101 subjects with tuberculosis diagnosis unknown to the investigator. The sensitivity and specificity of the flow cytometry–based assay were 67% and 92%, respectively, the positive predictive value was 80% and the negative predictive value was 92.4%. Therefore, the proportion of single-positive TNF-α *Mtb*-specific CD4$^+$ T cells is a new tool for the rapid diagnosis of active tuberculosis disease.

Cellular immunity, particularly of CD4$^+$ T cells, IFN-γ and TNF-α, has a central role in the control of and protection against *Mycobacterium tuberculosis* (*Mtb*) infection[1,2]. Diagnosis of *Mtb* infection remains complex and requires several clinical, radiological, histopathological, bacteriological and molecular parameters. IFN-γ release assays measure responses to antigens (for example, 6-kDa early secretory antigenic target (ESAT-6) or 10-kDa culture filtrate antigen (CFP-10)) expressed by *Mtb* itself and discriminate between infection by *Mtb* and immunity induced by vaccination with Bacille Calmette-Guérin (BCG)[3,4] but not between active disease and latent infection[5,6].

Studies in the field of antiviral immunity have shown that polyfunctional (IFN-γ$^+$IL-2$^+$TNF-α$^+$) profiles of virus-specific T cell responses, and not IFN-γ production alone, correlated with disease activity[7–10].

Therefore, we have used the same strategy, polychromatic flow cytometry, to functionally characterize *Mtb*-specific T cells in subjects with latent *Mtb* infection or active tuberculosis disease and tested the hypothesis that different cytokine profiles of pathogen-specific T cells may discriminate between active tuberculosis disease and latent *Mtb* infection.

We enrolled an initial cohort of 283 individuals with known diagnosis of *Mtb* infection in Switzerland and termed it the 'test cohort' (**Supplementary Fig. 1**). Subjects were selected on the basis of positive IFN-γ ELISPOT responses against CFP-10, ESAT-6 or both. Among the 283 subjects, active tuberculosis disease was diagnosed in 11 subjects on the basis of clinical signs (for example, cough, weight loss and lymphadenopathy), sputum stain for acid-fast bacilli (AFB), culture and PCR for *Mtb* and chest radiography[6] (the Online Methods and **Supplementary Table 1** contain detailed clinical parameters). The remaining 272 participants were diagnosed with latent *Mtb* infection. We first assessed the magnitude of *Mtb*-specific T cell responses by IFN-γ ELISPOT after stimulation with pools of peptides encompassing CFP-10 or ESAT-6 proteins. In agreement with previous studies[11,12], *Mtb*-specific T cell responses were similar in subjects with latent infection and active disease (**Fig. 1a**).

We then assessed the functional profile of *Mtb*-specific T cell responses by polychromatic flow cytometry and a panel of markers including a viability marker and antibodies specific for CD3, CD4, CD8, IL-2, TNF-α and IFN-γ. Owing to blood specimen availability or quality (see flowchart in **Supplementary Fig. 1**), this analysis was performed in 48 subjects with latent infection and eight subjects with active disease (**Supplementary Table 1**). Within the group with latent infection, five were investigated for suspected tuberculosis disease but had negative sputum AFB staining and culture and PCR for *Mtb*. Twenty-three were health-care workers screened for *Mtb* infection as part of routine surveillance at the Centre Hospitalier Universitaire Vaudois (CHUV;

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Supplementary Fig. 1). Twenty were investigated for *Mtb* infection before the initiation of anti-TNF-α antibody treatment and had negative chest radiographs (**Supplementary Fig. 1**). In agreement with previous studies[11,12], *Mtb*-specific $CD4^+$ T cell responses in a representative subject with latent *Mtb* infection (subject L5) were mostly (>70%) polyfunctional (**Fig. 1b**), that is, producing IFN-γ, IL-2 and TNF-α. In contrast, a representative subject with active tuberculosis disease (subject A2) (**Fig. 1b**) showed a dominant (>70% of $CD4^+$ T cells) TNF-α–only response. In these two participants, the functional profile of *Mtb*-specific $CD4^+$ T cells was similar regardless of the stimulus, that is, ESAT-6 or CFP-10 peptide pools or tuberculin purified protein derivative (PPD). Of note, *Mtb*-specific T cell responses (analyzed by either IFN-γ ELISPOT or flow cytometry) from the 20 subjects analyzed before the initiation of TNF-α–specific antibody treatment were not dif-

In summary, in an opportunistically selected (on the basis of a sufficient number of mononuclear cells; **Supplementary Data 1**) subgroup of individuals subjected to detailed intracellular cytokine staining (ICS) studies, a functional profile (single-positive TNF-α *Mtb*-specific CD4$^+$ T cells) was associated with disease activity and so might be helpful for rapid diagnosis of active disease as compare to the conventional culture tuberculosis tests, which require up to several weeks.

We then calculated which parameter (that is, functional T cell subset) was the strongest predictive measure of discrimination between active disease and latent infection. For these purposes, because CFP-10 was more frequently recognized than ESAT-6 (**Fig. 1d**), we focused the analysis on CFP-10–specific CD4$^+$ T cell responses and included ESAT-6–specific CD4$^+$ T cell responses only when CFP-10 responses were negative. We observed the latter scenario in only one individual with active disease and one individual with latent infection (**Fig. 1d**).

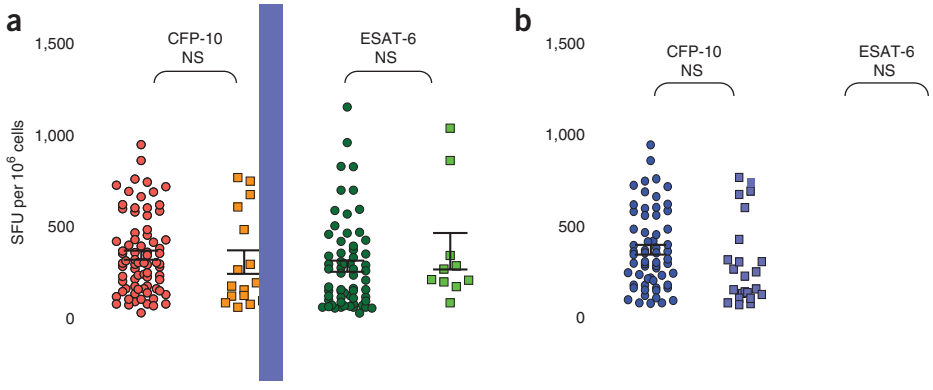
On the basis of the logistic regression analysis of the multiple functionally distinct T cell subsets, the proportion of TNF-α single-positive *Mtb*-specific CD4$^+$ T cells was the strongest predictive measure of discrimination between active disease and latent infection (area under the curve (AUC) = 0.995 (95% confidence interval: 0.984–1); odds ratio = 1.45; **Supplementary Fig. 4**). In addition, a cutoff of 37.4% of single-positive TNF-α–producing CD4$^+$ T cells was calculated as the value allowing the best (sensitivity of 100% and specificity of 96%) separation between latent infection and active disease (**Supplementary Fig. 4**).

A limitation of these results was that the laboratory investigators were not blinded to the diagnosis of latent infection or active disease. We therefore examined peripheral blood mononuclear cells from a second independent cohort termed the 'validation cohort', whose clinical status was unknown to the investigators. We tested whether the proportion of TNF-α single-positive *Mtb*-specific CD4$^+$ T cells, and particularly the cutoff at 37.4%, could discriminate between latent infection and active disease.

One hundred and fourteen participants from both Switzerland (n = 72) and South Africa (n = 42) were enrolled between 2009 and 2010 to confirm the functional profile also in persons from a setting (South Africa) where tuberculosis is prevalent (**Supplementary Fig. 5**). Participants from South Africa were enrolled from clinics in the public health sector in Cape Town and Worcester, both in the Western Cape province of South Africa. Participants from Switzerland in the validation cohort were not included in the test cohort described above. Subjects were selected on the basis of the following criteria: positive *Mtb*-specific IFN-γ ELISPOT responses, absence of *Mtb*-specific treatment, seronegative for HIV and good general health status (the Online Methods and **Supplementary Fig. 5** contain a full description of the subjects). Active tuberculosis disease diagnosis in subjects from both Switzerland and South Africa was based on clinical signs (for example, cough, weight loss and lymphadenopathy), sputum stain for AFB, culture and PCR for *Mtb* and chest radiography[6] (the Online Methods and **Supplementary Table 2** contain detailed clinical parameters). Flow cytometry analyses were performed on the 101 subjects from the validation cohort with positive *Mtb*-specific CD4$^+$ T cell responses (**Supplementary Fig. 5**).

IFN-γ ELISPOT and CD4$^+$ T cell specific cytokine expression in response to CFP-10, ESAT-6 or both were evaluated, and the data were provided to the biostatistics facility of CHUV. Later, unblinding of the *Mtb* clinical status allowed us to confirm that IFN-γ ELISPOT responses were not significantly different between latent infection and active disease (**Fig. 2a**). Of note, the magnitude of *Mtb*-specific IFN-γ ELISPOT responses (**Fig. 2b**) and the distribution of CFP-10– and/or ESAT-6–specific CD4$^+$ T cell responses among subjects with latent *Mtb* infection or active disease were similar between subjects from Switzerland and South Africa (**Fig. 2c**).

With regard to the polychromatic flow cytometric cytokine profile, 15 participants had a dominant TNF-α single-positive *Mtb*-specific CD4$^+$ T cell response, that is, >37.4%, considered predictive of active disease in the test cohort (**Supplementary Fig. 4**). After unblinding, active disease was confirmed in 12 of these 15 participants (**Fig. 3a**). Along the same line, 79 participants had polyfunctional *Mtb*-specific CD4$^+$ T cells, including a TNF-α single-positive proportion of <37.4%, which we considered predictive of latent infection in the test cohort (**Supplementary Fig. 4**). After unblinding, 73 out of these 79 participants had latent infection (**Fig. 3a**). The distribution of subjects from Switzerland and South Africa is also shown in **Figure 3b**. Notably, among the 94 aforementioned subjects (that is, 15 with a profile of active disease and 79 with a profile of latent infection), CFP-10– and ESAT-6–specific CD4$^+$ T cell responses, when both positive, were



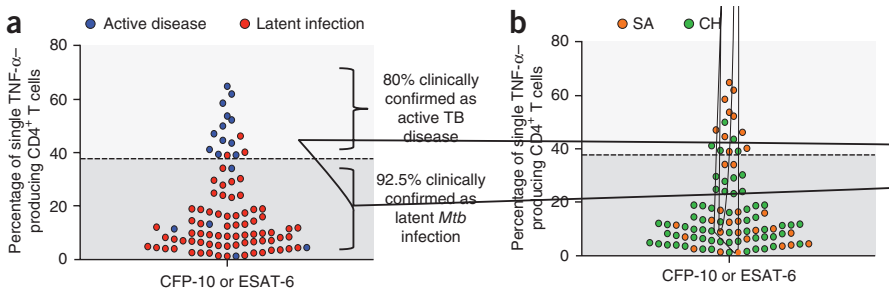


Figure 3 Percentages of CFP-10– or ESAT-6–specific single-positive TNF-α–producing CD4+ T cells of the 94 subjects from the validation cohort with concordant responses against CFP-10 and ESAT-6. Dashed line represents the cutoff of 37.4% of single-positive TNF-α. (**a**) Subjects with active disease or latent infection are identified with blue and red dots, respectively. (**b**) Subjects from South Africa (SA) or Switzerland (CH) are identified with orange and green dots, respectively.

concordant (that is, both either above or below the cut-off of 37.4% TNF-α single-positive cells). In these 94 concordant cases, the data of CFP-10–specific CD4+ T cell responses were considered for the analyses, and ESAT-6–specific CD4+ T cell responses were only included when CFP-10 responses were negative (**Fig. 3a**,**b**). Seven out of 101 (6.9%) participants showed discordant CD4+ T cell responses to ESAT-6 and CFP-10 peptide pools (one response >37.4% and the other response <37.4%) and were therefore excluded from the analysis (**Supplementary Fig. 6**). The performance of the test on the cohorts from Switzerland and South Africa was not significantly different ($P > 0.05$ for positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity), thus providing evidence that the combined analysis of Swiss and South African cohorts is valid. On the basis of the analysis on the combined cohorts, the global performance of the assay was as follows: PPV = 80%, NPV = 92.4%, sensitivity = 66.67% and specificity = 92.41% (**Supplementary Fig. 7**). Overall, the cytokine profile predicted the clinical diagnosis in 90% of cases. Of note, these values apply to subjects with detectable ICS responses. When subjects with discordant ESAT-6 and CFP-10 responses were also included in the analysis, the correct clinical diagnosis was made in 84% of subjects.

We then investigated whether the percentage of *Mtb*-specific TNF-α–producing CD4+ T cells was the parameter with the strongest predictive value of the clinical status in the validation cohort. On the basis of the logistic regression analysis of the multiple functionally distinct T cell subsets, the proportion of TNF-α single-positive *Mtb*-specific CD4+ T cells was indeed the strongest predictive measure of discrimination between active disease and latent infection (AUC = 0.825 (95% confidence interval: 0.683–0.968); odds ratio = 1.10; **Supplementary Fig. 7**). In addition, a cutoff of 38.8% (as compared to 37.4% obtained in the test cohort) of single-positive TNF-α–producing CD4+ T cells was calculated as the value allowing the best separation between latent infection and active disease (**Supplementary Fig. 7**).

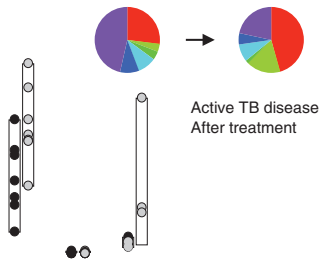
We also had the opportunity to analyze T cell cytokine profiles in five participants during untreated active tuberculosis disease and then after tuberculosis treatment (**Fig. 4**). In agreement with the above data, the percentage of single-positive TNF-α–producing CD4+ T cells was >37.4% in individuals with active tuberculosis disease. We observed a shift to a polyfunctional profile (single-positive TNF-α–producing CD4+ T cells < 37.4%) of *Mtb*-specific CD4+ T cell responses after therapy and transition to latent infection in all five participants (**Fig. 4**).

The association between different functional profiles of T cell responses and disease activity is consistent with the current paradigm in antiviral immunity[9,10], where virus-specific T cell responses endowed with only effector functions such as production of IFN-γ, TNF-α or both have been found in individuals with active virus replication and active disease. In contrast, polyfunctional responses, comprising cells producing IL-2 in addition to effector and inflammatory cytokines, have been found to be present in individuals with controlled virus replication and no signs of clinical disease[7–10].

The fundamental role of TNF-α in the control of *Mtb* infection in both humans and mice is well established[1,2,13], and this is also supported by the increased risk of *Mtb* reactivation in patients with rheumatoid arthritis receiving anti–TNF-α therapy[14,15]. However, the dominant TNF-α single-positive CD4+ T cell response observed during active tuberculosis disease may rather reflect the elevated degree of inflammation rather than of protection.

A recent study showed that a neutrophil-driven interferon-inducible gene profile correlated with active tuberculosis disease, and it was also found in about 10% of people with latent infection[16]. As about 10% of subjects with latent infection go on to develop active disease, it was suggested that this biomarker may be useful in both prognosis and diagnosis[16]. There was no evidence in that study of a T cell–driven TNF-α–inducible gene profile correlated with active tuberculosis disease. However, the transcriptional profile was assessed on total unstimulated blood cell populations. It was therefore not suitable for evaluating the transcriptional profile in T cell populations, an assay that needs to be performed on stimulated T cells[16].

Our results indicate that the analysis of cytokine profiles in *Mtb*-specific CD4+ T cells by polychromatic flow cytometry is a major immunological measure discriminating between active and latent *Mtb* infection. Therefore, polychromatic flow cytometry may be a reliable laboratory tool for the timely diagnosis of active *Mtb* infection.



METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

A.H. designed the study, performed the analyses and wrote the manuscript; V.R., F.B.E. and M.P. generated data and performed analyses; J.M.S., L.P.N., M.C., T.C., C.L.B., C.L.D. and W.A.H. recruited study participants; K.J. performed analyses; M.F. performed the statistical analyses; P.-A.B. contributed to the design of the study, performed analyses and wrote the manuscript; G.P. designed the study, supervised the analyses and wrote the paper. All authors have read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study groups. Participants (n = 283) from the test cohort were all recruited at CHUV. These samples were selected based on positive *Mtb*-specific IFN-γ ELISPOT responses routinely performed in the context of the diagnosis for *Mtb* infection at CHUV. Individuals with active tuberculosis disease had a diagnosis based on laboratory isolation of *Mtb* in mycobacterial culture from sputum, broncho alveolar lavage fluid or biopsies and/or tuberculin skin test and/or PCR (**Supplementary Table 1** contains a full clinical description of each subject). The final diagnosis was given by a clinician after validation of these criteria associated with clinical symptoms. The selection of subjects tested by flow cytometry was based on the availability of cryopreserved material. In addition, samples with low (<70%) cell recovery and viability upon thawing were discarded from the analyses, in concordance with the current guidelines in the field of intracellular flow cytometric analyses[17]. Furthermore, none of these subjects was undergoing antimycobacterium treatment at the time of the present analyses (**Supplementary Fig. 1**). Participants from the validation cohort (n = 114) were obtained from two clinical sites (**Supplementary Fig. 5**); subjects from Switzerland were all recruited from the CHUV, subjects from South Africa were recruited from clinics in the public health sectors in Cape Town and Worcester and subjects with latent infection were recruited from the South African Tuberculosis Vaccine Initiative clinical trials field site in Worcester. Inclusion criteria included positive *Mtb*-specific IFN-γ ELISPOT responses, age between 18 and 80 years old, body weight ≥50 kg, hemoglobin ≥100 g per liter, leukocyte count ≥3,000 cells mm^3 per liter, platelet count ≥75,000 cells mm^3 per liter and negative for HIV-specific antibody on the basis of a routine rapid HIV test. Individuals with active tuberculosis had a diagnosis based on laboratory isolation of *Mtb* in mycobacterial culture from sputum, broncho alveolar lavage fluid or biopsies and/or tuberculin skin test and/or PCR (**Supplementary Table 2** contains full clinical descriptions of each subject). The final diagnosis was given by a clinician after validation of these criteria associated with clinical symptoms such as cough or weight loss. Furthermore, none of these subjects was undergoing antimycobacterium treatment at the time of the present analyses. All participants gave written informed consent.The study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, University of Lausanne.

Peptides. Stimulations were performed with *Mtb*-derived peptide pools covering ESAT-6 and CFP-10. CFP-10 and ESAT-6 peptides pools are composed of 15-mers overlapping by 11 amino acids, and all peptides were HPLC purified (>80% purity). Tuberculin Purified Protein Derivative (PPD RT 23) was purchased from Statens Serum Institute.

IFN-γ ELISPOT assays. ELISPOT assays were performed per the manufacturer's instructions (Becton Dickinson). Briefly, cryopreserved blood mononuclear cells were rested for 8 h at 37 °C, and then 2×10^5 cells were stimulated with peptide pools (1 μg of each single peptide) in 100 μl of complete medium (RPMI + 10%FBS) in quadruplicate conditions as previously described[18]. Medium only was used as negative control. Staphylococcal enterotoxin B (SEB; Sigma; 200 ng ml^{-1}) was used as a positive control on 50,000 cells. Results are expressed as the mean number of SFU per 10^6 cells from quadruplicate assays. Only cell samples with >80% viability after thawing were analyzed, and only assays with <50 SFU per 10^6 cells for the negative control and >500 SFU per 10^6 cells after SEB stimulation were considered as valid. An ELISPOT result was defined as positive if the number of SFUs was ≥55 SFU per 10^6 cells and more than fourfold higher than the negative control.

Flow cytometry analysis. For ICS, cryopreserved blood mononuclear cells ($1–2 \times 10^6$) were rested for 6–8 h and then stimulated overnight in 1 ml of complete medium containing Golgiplug (1 μl ml^{-1}, Becton Dickinson) and CD28-specific antibodies (0.5 μg ml^{-1}, Becton Dickinson) as previously described[19]. For stimulation of blood mononuclear cells, peptide pools were used at 1 μl ml^{-1} for each peptide. SEB stimulation (200 ng ml^{-1}) served as positive control. At the end of the stimulation period, cells were stained for dead cells (LIVE/DEAD kit, Invitrogen), permeabilized (Cytofix/Cytoperm, Becton Dickinson) and then stained with antibodies specific for CD3, CD4, CD8, IFN-γ, TNF-α and IL-2. All antibodies but those specific for CD3 (Invitrogen) and CD4 and CD19 (VWR International) were purchased from Becton Dickinson. Cells were then fixed, acquired on an LSRII SORP (four lasers) and analyzed with FlowJo 8.8.2 and SPICE 4.2.3 (developed by M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, US National Institutes of Health) as previously described[18]. The number of lymphocyte-gated events ranged between 10^5 and 10^6 in the flow cytometry experiments shown.

Statistical analyses. Comparisons of categorical variables were made with Fisher's exact test. Statistical significance (P values) of the magnitude of ELISPOT responses was calculated by unpaired two-tailed Student's t test using GraphPad Prism 5. Bonferroni's correction for multiples analyses was applied. The selection of the optimal parameters to discriminate between cases of latent infection and cases of active disease was performed using a logistic regression analysis followed by a receiver operating characteristic (ROC) curve analysis[20–22] to evaluate the diagnostic performance of each parameter. Results for the optimal parameter (single-positive TNF-α) are summarized as a contingency table giving sensitivity, specificity and positive and negative predictive values (PPV and NPV). Analyses provided include a ROC-curve graph and a sensitivity and specificity graph as a function of the probability cutoff.

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