Dominant TNF-α *Mycobacterium Tuberculosis*-Specific CD4 T-Cell Responses Discriminate Between Latent Infection and Active Disease

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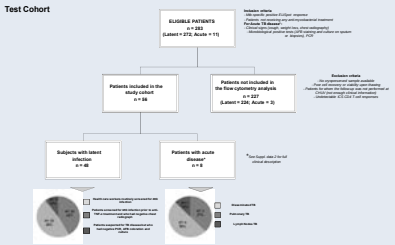
Abstract

Background: Diagnosis of *Mtb* infection remains complex and requires several clinical, radiological, histo-pathological, bacteriological and molecular parameters. IFN-γ-release assays (IGRAs), i.e. Quantiferon and ELISpot, measure responses to antigens (e.g. ESAT-6 or CFP-10) that are mainly limited to *Mtb*, and discriminate infection from immunity induced by vaccination with Bacille Calmette-Guérin. IGRA however do not discriminate between active TB disease and latent *Mtb* infection.

Methods: *Mtb*-specific T-cell responses were investigated in a test cohort of 283 subjects with known diagnosis of latent Mtb infection and active TB disease and subsequently in a validation cohort of 114 subjects with blinded clinical status. *Mtb*-specific T-cell responses were analyzed by polychromatic flow cytometry using peptide pools (15-mers overlapping by 11 aa) encompassing proteins ESAT-6 and CFP-10 but also PPD antigens. In particular, *Mtb*-specific CD4 T-cell responses were analyzed for the simultaneous expression of IFNg, TNFa and IL-2.

Results: *Mtb*-specific IFNg ELISpot responses were not different between patients with active TB disease or latent *Mtb* infection. In contrast, the functional profile of *Mtb*-specific CD4 T-cell responses was significantly different between active TB disease and latent *Mtb* infection (ESAT-6, CFP-10 or PPD, all $P<0.0001$) in the test cohort. Overall, *Mtb*-specific CD4 T-cell responses from patients with latent *Mtb* infection were polyfunctional (i.e. mostly composed of cells producing simultaneously TNFa+IFNg+IL-2) while single TNFa-producing Mtb-specific CD4 T-cell responses were dominant in patients with active TB disease. We then investigated the possibility to use this parameter (i.e. % of single TNFa-producing cells) as a diagnostic tool in an independent cohort of 101 patients with blinded TB diagnosis. The concordance between the clinical and the cytokines profile in predicting active TB disease and latent *Mtb* infection diagnosis was confirmed in >90% of cases thus validating the use of the profile of single TNFa-producing CD4 T-cell response in the timely diagnosis of acute TB disease.

Conclusions: These results indicate that analysis of cytokines profiles in *Mtb*-specific CD4 T-cells by polychromatic flow cytometry is a strong immunological measure discriminating between active and latent *Mtb* infection. Therefore, polychromatic flow cytometry is a novel and reliable laboratory tool for the timely diagnosis of active *Mtb* infection.



Flow chart description of subjects included in the Test Cohort

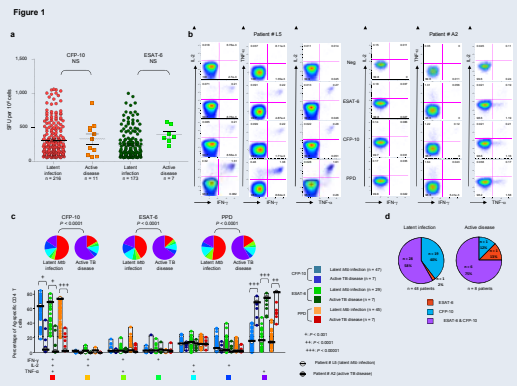
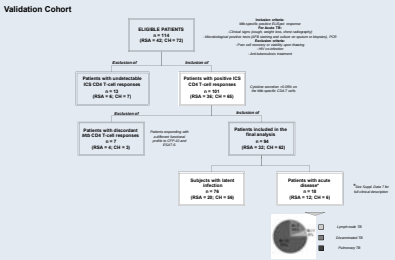


Figure 1. Quantitative and qualitative analysis of *Mtb*-specific T-cell responses in the Test Cohort. **a)** IFN-γ ELISpot responses following stimulation with ESAT-6 or CFP-10 peptide pools in a cohort of 283 participants with latent *Mtb* infection (n = 272) or active TB disease (n = 11). Shown are the numbers of spot-forming units (SFU) per 10^6 mononuclear cells. Statistical significance (P values) of the results was calculated by unpaired two-tailed student t test using GraphPad Prism 5. Bonferroni correction for multiples analyses was applied. **b)** Qualitative analysis of *Mtb*-specific CD4 T-cell responses by polychromatic flow cytometry. Shown are representative flow cytometry analysis of the functional profile of *Mtb*-specific CD4 T-cell responses in participants with either latent *Mtb* infection (Pt#L5, left panels) or active TB disease (Pt#A2, right panels). Profiles are gated on live $CD3^+CD4^+$ T cells and the various combinations of IFN-γ, IL-2 and TNF-α are shown following stimulation with ESAT-6 and CFP-10 peptide pools or PPD. **c)** Simultaneous analysis of the functional profile of *Mtb*-specific CD4 T-cells on the basis of IFN-γ, IL-2 or TNF-α production. ESAT-6-, CFP-10- and PPD-specific CD4 T-cell responses are shown from 48 and 8 participants with latent *Mtb* infection or active TB disease, respectively. Representative examples from Pt#L5 and A#2 shown in figure 1b are also identified. All the possible combinations of the different functions are shown on the x axis whereas the percentages of the distinct cytokine-producing cell subsets within *Mtb*-specific CD4 T-cells are shown on the y axis. The pie charts summarize the data, and each slice corresponds to the proportion of *Mtb*-specific CD4 T-cells positive for a certain combination of functions. **d)** Distribution of CFP-10- and/or ESAT-specific CD4 T-cell responses among subjects with latent *Mtb* infection or active TB disease.



Flow chart description of subjects included in the Validation Cohort

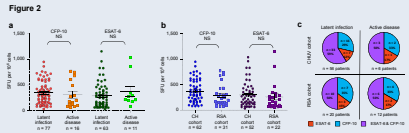


Figure 2. Analysis of *Mtb*-specific T-cell responses in the Validation Cohort following unblinding of the clinical status. **a)** IFN-γ ELISpot responses following stimulation with ESAT-6 or CFP-10 peptide pools. Shown are the numbers of SFU per 10^6 mononuclear cells. Statistical significance (P values) of the results was calculated by unpaired two-tailed student t test using GraphPad Prism 5. Bonferroni correction for multiples analyses was applied. **b**) Analysis of *Mtb*-specific IFN-γ ELISpot T-cell responses in patients enrolled in Switzerland (CH) and Republic of South Africa (RSA). **c**) Distribution of CFP-10- and/or ESAT-specific CD4 T-cell responses among patients from the Validation Cohort with positive and concordant *Mtb*-specific CD4 T-cell responses.

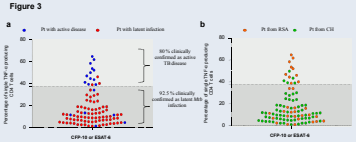


Figure 3. Percentages of CFP-10- or ESAT-6-specific single TNF-α-producing CD4 T-cells of the 94 subjects from the Validation Cohort with concordant responses against CFP-10 and ESAT-6. Dashed line represents the cutoff of 37.4% of single TNF-α. **a**) Subjects with active disease or latent infection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

Conclusions

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

These results indicate that the analysis of cytokines profiles in *Mtb*-specific CD4 T-cells by polychromatic flow cytometry is an important immunological measure discriminating between active and latent *Mtb* infection. Therefore, polychromatic flow cytometry is a novel and reliable laboratory tool for the timely diagnosis of active *Mtb* infection.

Reference

Harari et al, Nature Medicine 2011