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Immunodiagnosis of latent tuberculosis : new answers to an old question?

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QUALITATIVE DIFFERENCES IN IMMUNE RESPONSE BETWEEN CURED TB PATIENTS AND LATENTLY INFECTED INDIVIDUALS

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MANUSCRIPT UNDER PREPARATION

ABSTRACT

Background

Peripheral blood mononuclear cells (PBMC) of individuals cured from manifest infection by *Mycobacterium tuberculosis* (MTB) often produce IFN- γ after 24 hrs *in vitro* stimulation with MTB antigens. Some latently infected individuals (LTBI) only respond by IFN- γ production after a period of 6 day stimulation (Leyten et al. 2007). We hypothesized that there may be qualitative differences between responding PBMC population between patients and those latently infected. We therefore assessed qualitative and quantitative differences in IFN- γ producing cell populations after short and long term stimulations with MTB antigens.

Methods

PBMC were isolated from healthy controls, patients cured from active TB and individuals with LTBI who tested negative in QuantiFERON-TB Gold, and were stimulated with TB specific antigens. Cytokine production was analyzed by intracellular staining and in supernatants

Results

PPD stimulation induced IFN- γ producing cells both in TB and in LTBI subjects, but TB patients had a significantly higher mean percentage of IFN- γ^+ cells compared to LTBI individuals. ESAT-6 and CFP-10 induced IFN- γ production in TB patients after 24 hours and 6 days stimulation. However, in the LTBI group, CFP-10 did induce a strong response of IP-10 in the absence of IFN- γ similar to that in TB patients. Multifunctional CD4 $^+$ cells, producing IFN- γ , IL-2 and TNF- α simultaneously, were detectable in TB patients after combined stimulation with ESAT-6 and CFP-10. After PPD or ESAT-6 stimulation, IFN- γ was predominantly produced by CD45RA $^-$ CD27 $^-$ effector-memory cells in TB patients. By contrast, in LTBI individuals IFN- γ was produced predominantly by CD45RA $^+$ CD27 $^+$ cells, i.e., of the naïve phenotype.

Conclusion

PBMC of cured TB patients and those with latent TB infection can differ both in quantity and quality with respect to MTB antigen-elicited cytokine producing cells. Cells of LTBI individuals responded poorly to ESAT-6 and CFP-10. Our findings suggest that the observed lack of response of some LTBI individuals in short term culture IGRA is related to these differences in circulating MTB responsive cells.

INTRODUCTION

Tuberculosis is still a major public health problem with more than one third of the world population estimated to be latently infected with TB. In the past few years new tools have become available to detect TB infection; the interferon- γ release assays (IGRA), QuantiFERON-TB® Gold in-tube and T-SPOT.TB®, which have been studied extensively (1). These assays can more accurately detect infection with *Mycobacterium tuberculosis* compared to the well known tuberculin skin test (TST), since they show no cross-reactivity after Bacille Calmette-Guérin (BCG) vaccination or infection with most non-tuberculous mycobacteria (2-5). From previous studies it appeared that not all latently infected individuals can be detected by short term culture IGRA. Although this can in part be explained by immune suppression (6;7), a study performed in our laboratory showed that some latently infected individuals with a normal immune system were also negative in the IGRA; interestingly, cells of most of these subjects were able to produce interferon- γ (IFN- γ) after 6 days of stimulation (8). Several hypotheses might explain this observation: firstly, it may be that these individuals responded through a different cell subset to TB infection, which would not be activated within the short term incubation period of the IGRA but only following prolonged culture. Secondly, it may be that IFN- γ , the readout cytokine for the IGRA, is not the most sensitive cytokine. Perhaps other cytokines or chemokines are more sensitive to detect immune responses to MTB. Ruhwald *et al.* showed that responses of some latently infected individuals were below the IFN- γ cut-off in the QFT-GIT, but that these individuals specifically produced high levels of IP-10 (also known as CXCL-10) in response to ESAT-6/CFP-10 stimulation (9-11).

It has been postulated that IGRA, since they have a short incubation period, can only detect effector cells (12). The presence of short-lived effector cells indicates the presence of a recent or persistent infection. Previous studies found that past infections could be associated with positive IGRA results (13)(and submitted data) and even cured TB patients often remain positive in the IGRA (unpublished data and (14-17). Furthermore, one study showed that long lived memory cells are not capable of producing IFN- γ within 20h stimulation period in ELISPOT (18).

At present it is unknown if sole production of IFN- γ after ESAT-6 or CFP-10 stimulation indicates protection from disease or indicates risk of progression to disease. There are reports of animal studies, as well as two studies in humans, suggesting that high levels of IFN- γ after ESAT-6 stimulation are predictive of progression to active TB (7;19-22). This seemingly contrasts to vaccination studies in animals in which IFN- γ response to ESAT-6 was associated with protection (23;24). Other studies have reported that instead of mono-functional cells producing IFN- γ only, multi-functional cells producing multiple cytokines (IFN- γ , IL-2 and TNF- α simultaneously) are associated with protection (25-27). Thus, latently infected individuals could have “protective” multi-functional cells, even though total IFN- γ production might be lower compared to individuals who progress to TB.

The aim of this study was to compare the qualitative and quantitative cellular subsets that produce IFN- γ in response to TB specific antigens in patients cured from active TB and selected late-responding latently infected individuals (8).

SUBJECTS AND METHODS

Reagents

Reagents were as follows: PPD (Statum Serum Institute, Copenhagen, Denmark), ESAT-6 (LUMC peptide facility as previously described (28)), CFP-10 (LUMC peptide facility as previously described (28)) and anti-CD3 (OKT3, Pharmacy LUMC).

Study subjects

Peripheral blood was obtained from healthy donors (n=4), cured TB patients known to be strong ESAT-6 responders (n=4) and from individuals of the previous study with discordant QFT-GIT and LST results (n=8), characteristics are summarized in Table 1 (8). All subjects volunteered to participate in this study. The protocol was approved by the ethics committee of the LUMC (Protocol number P07.048).

Table 1. Characteristics of the study population

Group	Male (%) (N/N)	Age mean (range)	QFT-GIT	TST mm (range)	Time since positive TST mean (years)
Healthy controls	25.0 (1/4)	29.9 (25-39)	Negative (4/4)	n.d.	-
Cured TB patients	50.0 (2/4)	60.3 (42-77)	Positive (4/4)	n.d.	19.5 (4-44)
Latently infected	62.5 (5/8)	44.1 (26-56)	Negative (8/8)	17 (10-27)	5.4 (3.3-8.9)

Stimulation experiments

Peripheral blood mononuclear cells (PBMC's) were isolated by density centrifugation. PBMC's were stimulated with antigen in Iscove's modified Dulbecco's medium (Invitrogen, Breda, The Netherlands) supplemented with 10% pooled human serum. Antigens used were PPD (10 µg/ml), ESAT-6 and CFP-10 peptides, tested in pools containing 1 µg/ml of each peptide, plastic coated OKT3 was included as positive control and unstimulated cells were used as negative controls. All FACS experiments and the analysis of cytokine production (described below) were performed with freshly isolated cells. The LSR-II experiments (see below) were performed with frozen and thawed cells, originally collected at the same moment as the cells for FACS analysis. The cells were frozen at minus 152°C.

FACS analysis

Cells were stained with combinations of the following antibodies: CD4-PercP, CD8-allophycocyanin (APC), CD45RA-fluorescein isothiocyanate (FITC), CD27-phycoerythrin (PE), (all BD Biosciences); CCR7-PE (R&D Systems, Abingdon, UK). Intracellular staining, following Golgiplug for the last 18 h (BD Biosciences), was performed using Intrastain reagents (DakoCytomation) and the following antibodies were used: IFN γ -FITC/APC/PE, IL-2-FITC and TNF- α -PE (BD Biosciences). Samples were measured on a FACSCalibur and analyzed using CellQuestPro software (BD Biosciences).

Multiparameter flowcytometric analysis

Cells were stained with combinations of the following antibodies: CD3-APC-Cy7/Pacific Blue, CD4-Pacific Blue/Alexa 700/PeCy7, CD8-AmCyan, CD25-APC-Cy7, CD14-FITC (all from BD Biosciences). Intracellular staining, after adding Brefeldin A for the last 18 h (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands), was performed using Intrastain reagents (DakoCytomation) and the

following antibodies: IFN γ -Alexa 700, IL2-PE, TNF α -APC, IP10-PE (all from BD Biosciences). Samples were measured on a LSR-II and analyzed using FACSDiva software (BD Biosciences).

Cytokine analysis

Cytokine production in supernatants was measured using a custom-made 7-plex suspension array system (Luminex; Bio-Rad, Veenendaal, The Netherlands). The following cytokines were measured: IFN- γ , IL-2, TNF- α , IP-10, IL-17, IL-15 and MIP-1 β . Data of the cytokine analysis are shown for 48 hours and 6 days of stimulation, since after 24 hours the number of responses was too small.

QuantiFERON-TB Gold in-tube

The QFT-GIT was performed according to the manufacturers' instructions (<http://www.cellestis.com>).

Statistics

Differences between groups were calculated with Mann-Whitney. Cytokine analyses were performed with the Bon Feroni test where all measurements of the same stimulation were pooled and analyzed for group differences. For all tests, a two-sided p-value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 16.0 for Windows (Chicago, IL, USA) and Prism (Graphpad version 4.02, La Jolla, CA, USA).

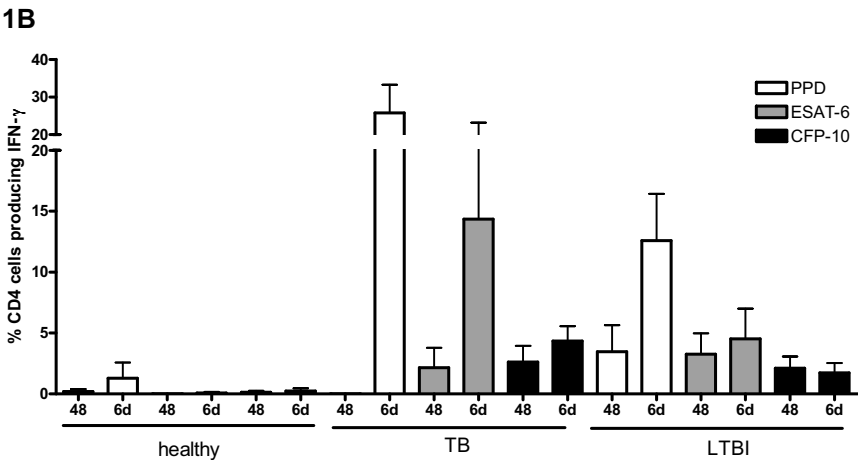
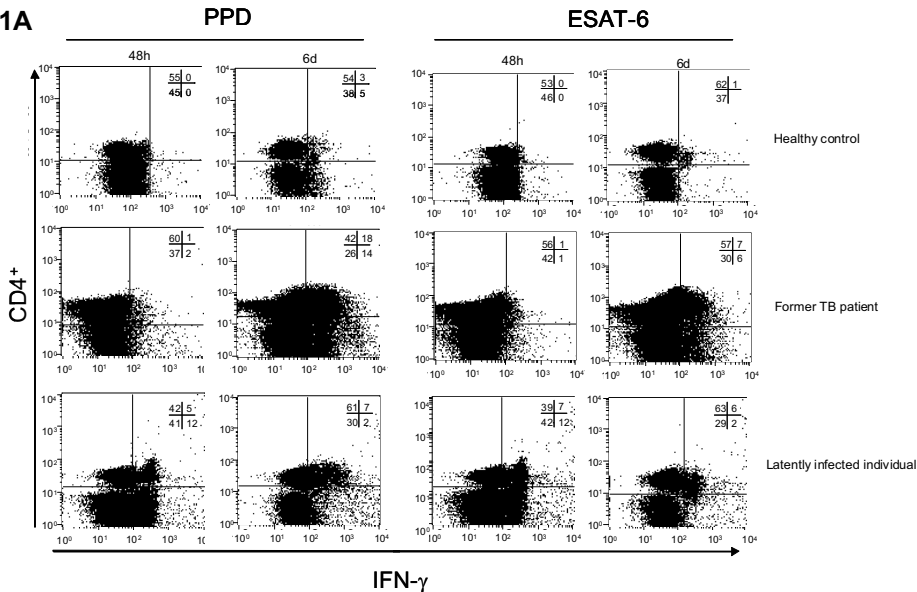
RESULTS

Study population

All healthy controls, and the latently infected individuals selected from the previous study (8), were negative in the QFT-GIT performed at the moment of blood collection, in contrast to the cured TB patients who all had a positive result. Table 1 shows the characteristics of the study population. The time elapsed since TST conversion for the LTBI individuals mounted to an average of 5.4 years, 2 years more than in the previous study. The cured TB patients were diagnosed on average 19.5 years ago.

IFN- γ

In Figure 1A an example of IFN- γ producing cells after PPD and ESAT-6 stimulation of a TB patient and latently infected individual is shown. In Figure 1B and 1C the average percentages of the complete groups are shown. Cured TB patients as well as latently infected individuals had increased numbers of IFN- γ producing cells compared to the healthy control group after MTB specific stimulation. Furthermore, stimulation with PPD resulted in a higher number of IFN- γ producing cells than did separate stimulation with ESAT-6 or CFP-10. The number of IFN- γ producing cells induced was higher in cured TB patients than in the latently infected individuals.



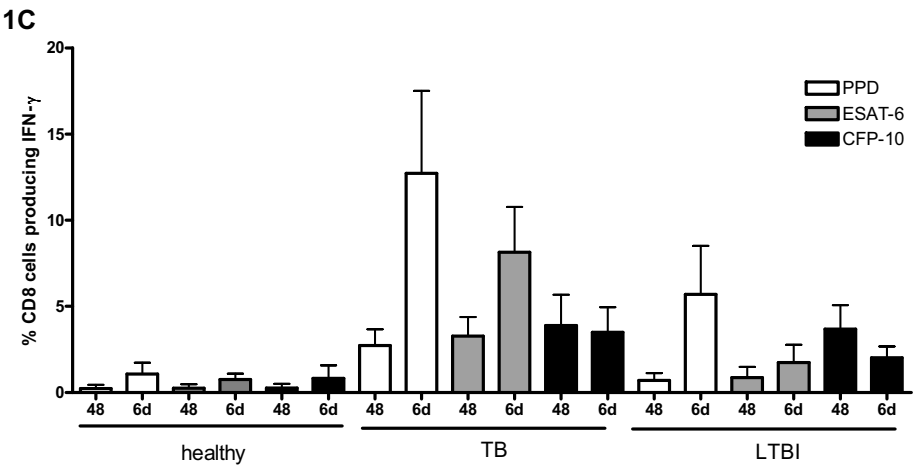


Figure 1. Cured TB patients have more IFN- γ producing cells PBMCs from healthy controls, cured TB patients and LTBI were stimulated with PPD, ESAT-6 or CFP-10. At 48 hours and 6 days cells were harvested and stained intracellularly for IFN- γ production.

1A. CD4 versus IFN- γ , gated on lymphocytes for both time points in a representative donor from each group. Percentages within the quadrants represent the percentage of cells from the total lymphocyte gate.

1B. Percentage of IFN- γ producing CD4⁺ cells upon stimulation with PPD, ESAT-6 or CFP-10, mean + SD of 4 healthy controls, 4 cured TB patients and 8 LTBI measured on 2 different time points. Values were corrected for IFN- γ production in unstimulated samples.

1C. Percentage of IFN- γ producing CD8⁺ T cells, corrected for IFN- γ production in unstimulated samples.

The concentration of IFN- γ in culture supernatants is depicted in Figure 2A. Both TB patients and latently infected individuals produced and secreted large amounts of IFN- γ after PPD stimulation. Surprisingly, only TB patients produced IFN- γ after ESAT-6 or CFP-10 stimulation. Cells from latently infected individuals did produce small amounts of IFN- γ after 6 days of CFP-10 stimulation, but this was just above the cut-off value. This was in contrast with the previous study two years ago where they produced higher concentrations of IFN- γ in response to prolonged stimulation with TB antigens. This shows that significant waning of circulating effector immunity can take place following infection in LTBI.

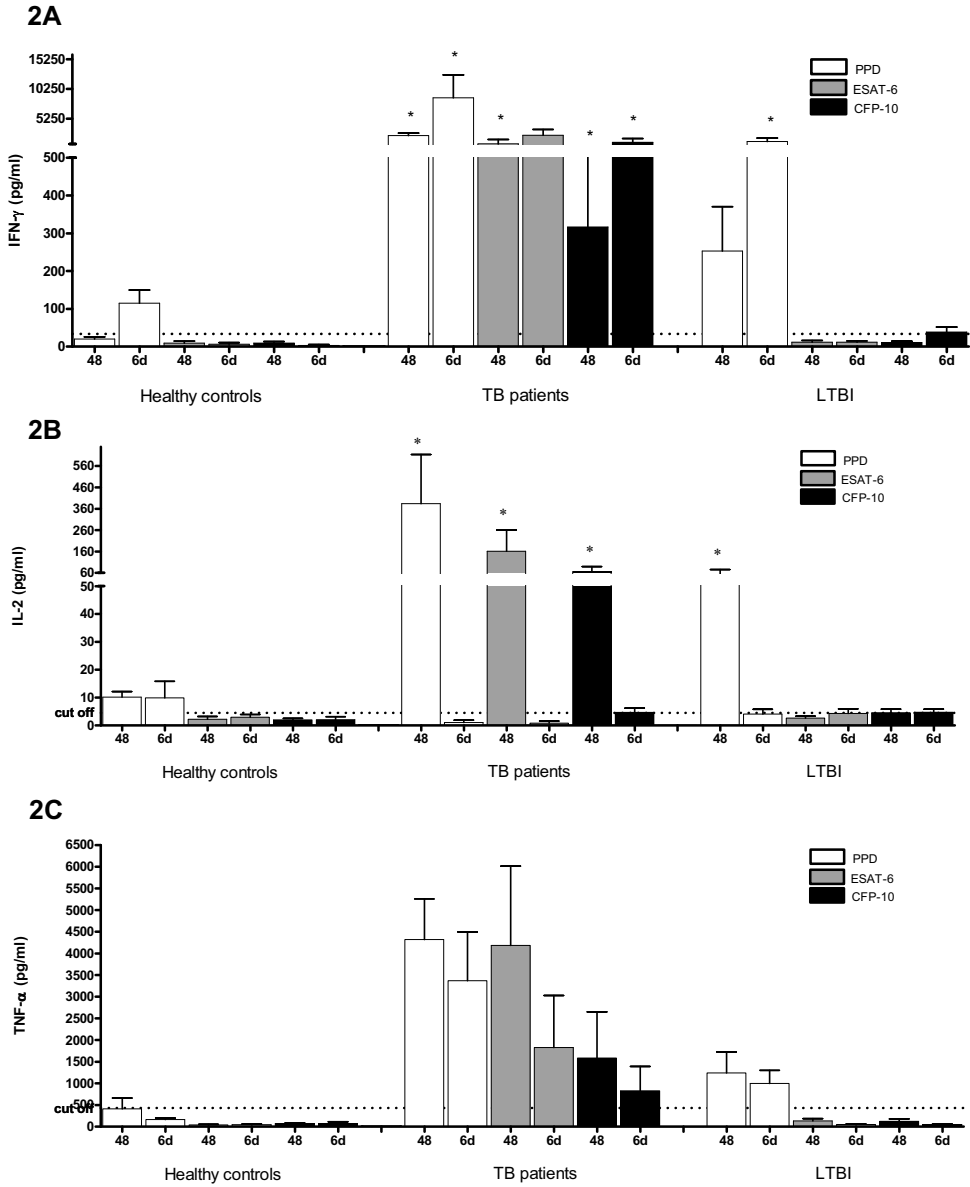


Figure 2. Cured TB patients produce more IFN- γ , IL-2 and TNF- α PBMCs from healthy controls, cured TB patients and LTBI were stimulated with PPD, ESAT-6 or CFP-10. At 48 hours and 6 days supernatant was harvested and the concentration of cytokines determined.

2A. Concentration of IFN- γ (pg/ml) produced after PPD, ESAT-6 or CFP-10 stimulation (mean + SD of 4 healthy controls, 4 cured TB patients and 8 LTBI). The * indicates a significant difference compared to the healthy control group. For IFN- γ all TB and LTBI measurement are also significantly different from each other ($p < 0.05$). Dotted line represents cut-off value, average mean + 2x SD.

2B. Concentration of IL-2 (pg/ml) produced after PPD, ESAT-6 or CFP-10 stimulation (mean + SD of 4 healthy controls, 4 cured TB patients and 8 LTBI). The * indicates a significant difference compared to the healthy control group. Dotted line represents cut-off value, average mean + 2x SD.

2C. Concentration of TNF- α (pg/ml) produced after PPD, ESAT-6 or CFP-10 stimulation (mean + SD of 4 healthy controls, 4 cured TB patients and 8 LTBI). The * indicates a significant difference compared to the healthy control group. For TNF- α all TB and LTBI measurement are also significantly different from each other ($p < 0.05$). Dotted line represents cut-off value, average mean + 2x SD.

IP-10

IP-10 or CXCL-10 has been suggested to be a more sensitive marker to detect those LTBI individuals who are below the cut-off in the QFT-GIT, where IFN- γ production is measured. In Figure 3, the concentration of IP-10 in supernatants of stimulated PBMC cultures at 48 hours and 6 days is shown. Healthy controls did not produce IP-10 after TB specific stimulations; cured TB patients produced IP-10 after PPD, ESAT-6 and CFP-10 stimulation. Surprisingly, the latently infected group produced IP-10 only after CFP-10 stimulation, but not upon PPD stimulation. Interestingly, IFN- γ and IP-10 did not always show similar patterns as one might expect based on the known link between IFN- γ and IP-10, suggesting an antigen specific induction of IP-10. Therefore PBMCs were analyzed to detect possible IP-10 production by T-cells. In Figure 3B it is shown that both CD3⁺CD4⁺ as well as CD3⁺CD8⁺ cells produced IP-10. The plots suggest that cells first produced IP-10 before starting to produce IFN- γ , as most IFN- γ producing cells were also positive for IP-10. These observations would fit into the current concept that IP-10 can be used for more sensitive detection of anti-mycobacterial immunity since IP-10 is expressed earlier than IFN- γ .

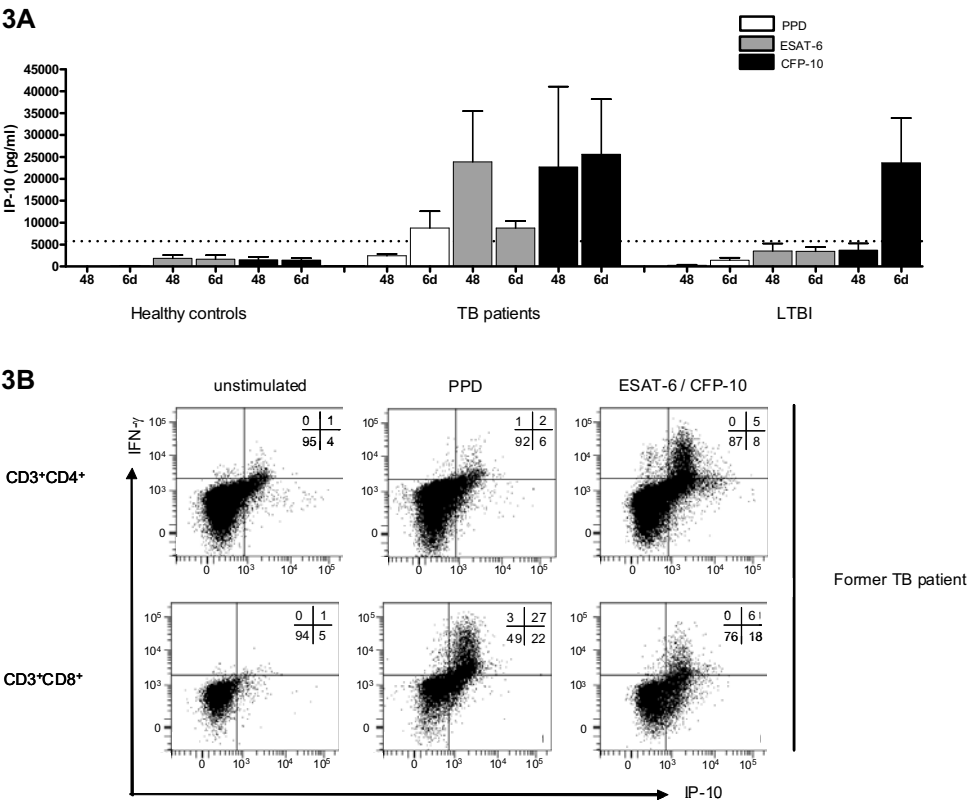


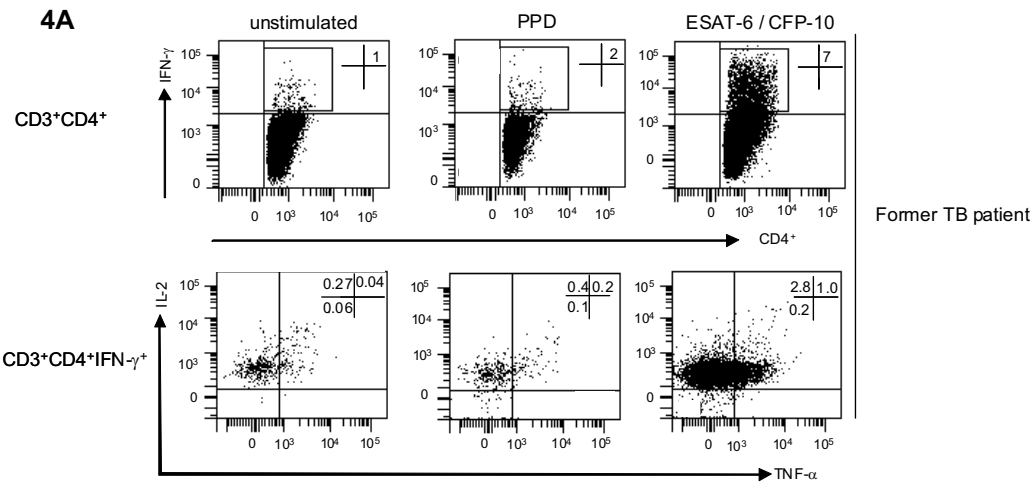
Figure 3. IP-10 is specifically produced after CFP-10 stimulation in the latently infected group, as well as by cured TB patients.

3A. Concentration IP-10 (pg/ml) produced after 48 hours and 6 days stimulation with PPD, ESAT-6 or CFP-10 (mean + SD of 4 healthy controls, 4 cured TB patients and 8 LTBI). The dotted line represents the cut-off value, average mean + 2x SD.

3B. IP-10 versus IFN- γ , gated on CD3+CD4+ or CD3+CD8+ T cells in a representative cured TB patient after 6 days stimulation with PPD or a combination of ESAT-6 and CFP-10.

Multifunctional T cells

Since multifunctional T cells, producing IFN- γ , IL-2 and TNF- α simultaneously, are associated with protection from disease in several models of infectious diseases, it was postulated that they might also have a protective role in human TB (26;29). In Figure 4A an example is shown of cells of a cured TB patient stimulated for 6 days with ESAT-6 and CFP-10, the upper panels show the CD3⁺CD4⁺IFN- γ ⁺ cells, in the lower panels CD3⁺CD4⁺IFN- γ ⁺ cells were analyzed for concomitant IL-2 and TNF- α production. The percentages shown are those of the total CD3⁺CD4⁺ population. In Figure 4B and 4C the induction of single, double and multiple cytokine producing cells after combined stimulation with ESAT-6 and CFP-10 is shown for the CD3⁺CD4⁺ and the CD3⁺CD8⁺ population, respectively. Cured TB patients displayed a strong induction of multifunctional T cells after 6 days of MTB specific antigen stimulation, whereas PBMCs from healthy controls as well as from the latently infected individuals did not display such cells. In the CD3⁺CD4⁺ population TB patients also had IFN- γ ⁺TNF- α ⁺ cells. All three groups induced IFN- γ ⁺IL-2⁺ cells, although healthy controls had lower levels of these cells.



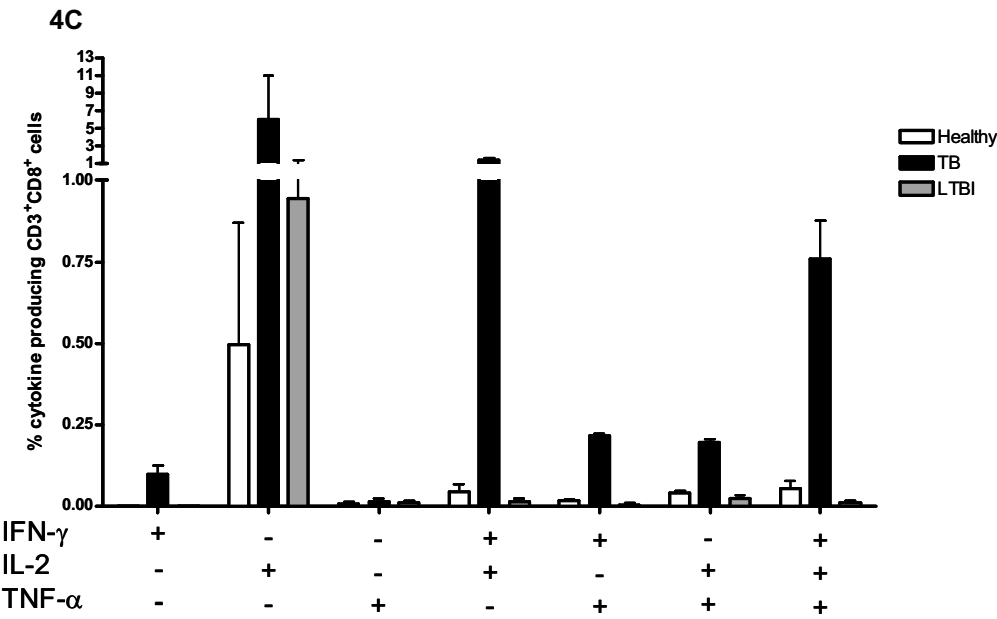
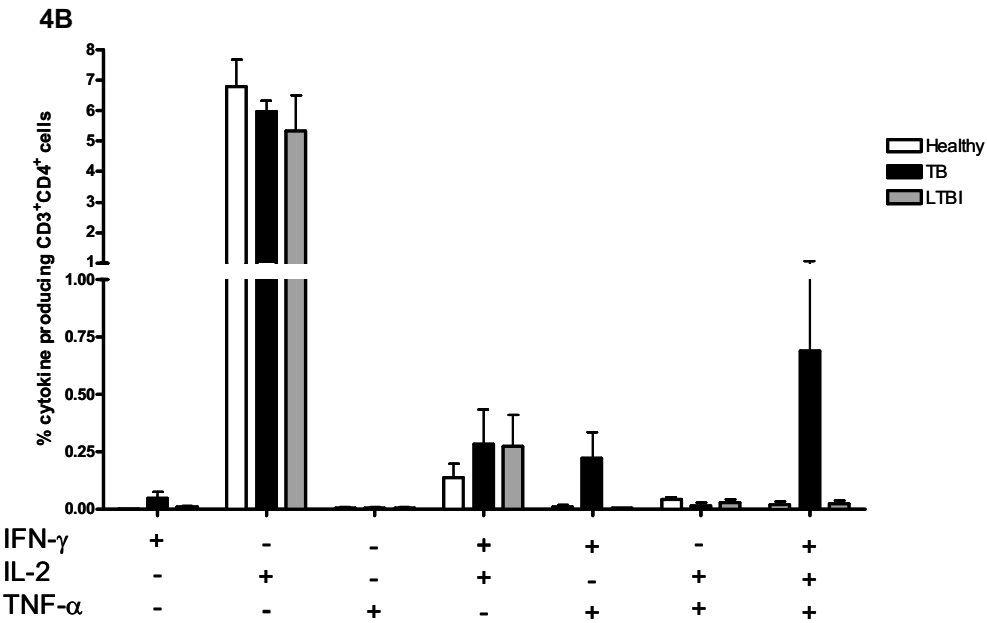


Figure 4. Multifunctional CD4⁺ and CD8⁺ T cells producing IFN- γ , IL-2 and TNF- α simultaneously are induced in cured TB patients

4A. Percentage of CD3⁺CD4⁺ and CD3⁺CD8⁺ multifunctional, double functional and single functional T cells of a representative cured TB patient after combined ESAT-6 CFP-10 stimulation. The percentages in the quadrants are those of the CD4⁺ or CD8⁺ population.

4B. Average percentage + SD of multifunctional, double functional and single functional CD3⁺CD4⁺ cells after combined ESAT-6/CFP-10 stimulation for 6 days of 3 healthy controls, 3 cured TB patients and 8 latently infected individuals (corrected for unstimulated cells).

4C. Average percentage + SD of multifunctional, double functional and single functional CD3⁺CD8⁺ cells after combined ESAT-6/CFP-10 stimulation for 6 days of 3 healthy controls, 3 cured TB patients and 8 latently infected individuals (corrected for unstimulated cells).

Phenotype of IFN- γ producing cells

To investigate the effector or memory phenotypes of the IFN- γ producing cells, the latter were stained for CD45RA and CD27. Cells that are CD45RA⁺CD27⁺ are regarded as naïve T cells, CD45RA⁺CD27⁺ cells are central memory cells, CD45RA⁺CD27⁻ are known as effector-memory cells and CD45RA⁺CD27⁻ are regarded terminally differentiated effector cells (30-32). From the analysis it appeared that IFN- γ production by cells from cured TB patients could be ascribed almost completely to effector cells (Figure 5A). In contrast, IFN- γ production by cells from LTBI individuals was much more diverse, and most cells had a central memory or naïve phenotype. The average percentages of IFN- γ producing cells with the phenotype of naïve and effector-memory cells after PPD stimulation are shown in Figure 5B. Thus different cell populations are responsible for the IFN- γ production in cured TB patients compared to LTBI individuals.

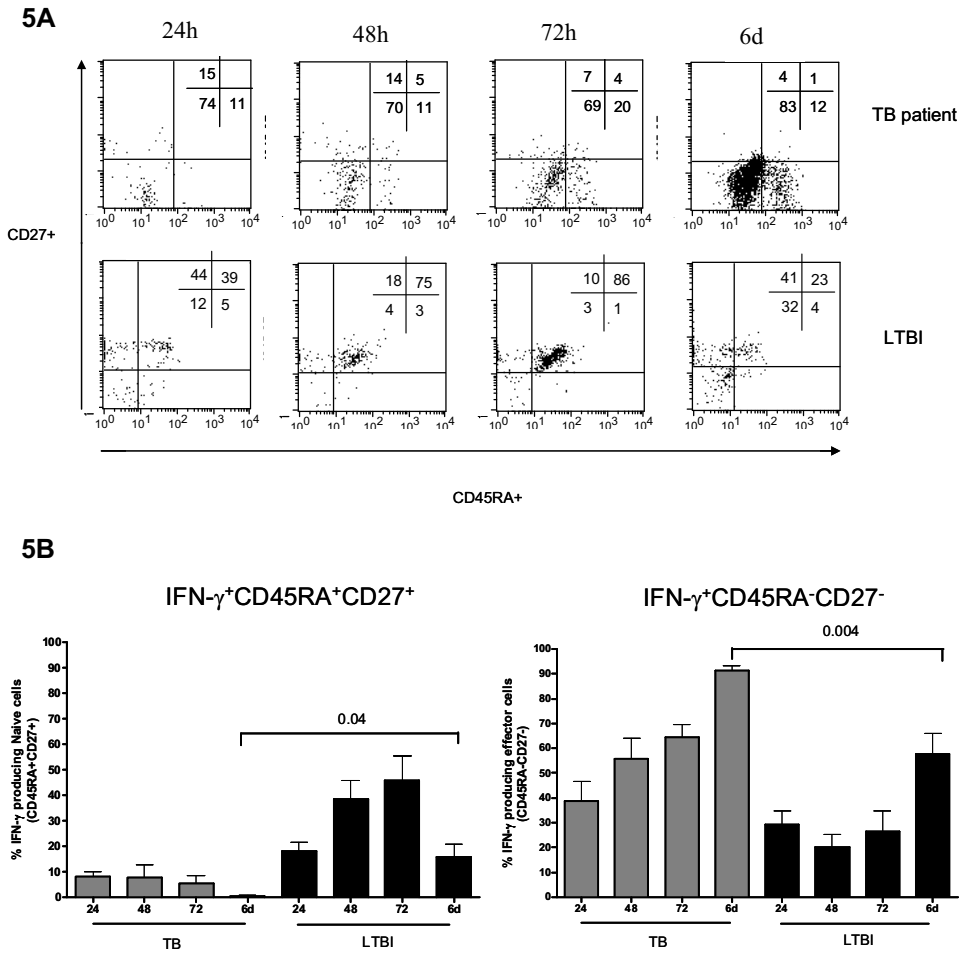


Figure 5. IFN- γ producing T cells of cured TB patients and those of latently infected individuals have a different phenotype.

5A. CD45RA versus CD27 expression, gated on the CD4⁺ IFN- γ producing cells for a representative cured TB patient and latently infected individual after 24, 48 and 72 hours and 6d stimulation with PPD.

5B. In the left panel the average percentage + SD of IFN- γ producing CD4⁺ cells with an CD45RA⁺CD27⁺ (naïve) phenotype are shown after 24, 48 and 72 hours and 6 days PPD stimulation for 4 cured TB patients and 8 latently infected individuals. In the right panel the average percentage + SD of IFN- γ producing CD4⁺ cells with a CD45RA⁻CD27⁻ (effector) phenotype is depicted.

DISCUSSION

The main finding of the present study is that multifunctional T cells producing IFN- γ , IL-2 and TNF- α simultaneously, can be detected among PBMC's from patients cured from a manifest TB infection, but not in a subgroup of latent TB infected individuals who are TST and 6-day LST positive but have an negative QFT-GIT. Secondly, IP-10 was strongly induced upon stimulation with TB specific antigens in cells of both cured TB patients and latently infected individuals; cells responsible for IP-10 production included CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in both groups. Thirdly, the phenotype of the PBMC's producing IFN- γ appears to be different in cured TB patients as compared to those latently infected individuals with TB not responding in short term culture assays.

We like to mention several limitations concerning the findings presented in this study. Firstly, we have selected a special latently infected subpopulation with no detectable IFN- γ response in the short term IGRA and cured TB patients included in this study have been selected on the basis of their strong responses to ESAT-6 stimulation. We have chosen for these two extremes because we anticipated this would make it possible to detect pronounced differences, which might have been less obvious in a random sample of the LTBI population. Of note, most of the experiments were performed with freshly isolated PBMCs and some with frozen PBMC's. This freezing process may have affected PBMC function. Secondly, the LTBI were diagnosed as latently infected on the basis of a TST conversion ≥ 10 mm after documented contact to an active TB patient and by production of IFN- γ by their PBMC's after 6 days of stimulation with ESAT-6 & CFP-10 in a previous study (8). In our study these individuals produced no or only small amounts of IFN- γ , lower than that obtained in a previous study. There can be several explanations for this observation. In this study, IFN- γ was measured by Luminex, whilst IFN- γ in the previous study was measured by ELISA. Most likely the Luminex was less sensitive than the IFN- γ ELISA; therefore we analyzed our supernatants also by ELISA. From our small dataset it appeared that the ELISA was a factor 5 more sensitive than the Luminex. A second explanation might be waning of the response over time, as the previous measurement was performed two years earlier and individuals might have lost their responsiveness towards the TB specific antigens. Thirdly, the number of participants included in this study was small and they were preselected as based on their immunological response. The findings therefore cannot be generalized to the total TB infected population. In any case, the present findings should now be confirmed in a random sample of the total latently TB infected.

IP-10 has been proposed to be a more sensitive marker than IFN- γ after stimulation with TB specific antigens in order to detect active and latently infected individuals (9-11;33;34). The data in our study showed that although the selected latently infected individuals did not produce detectable levels of IFN- γ after stimulation with ESAT-6 or CFP-10, they did produce IP-10 after 6-days of CFP-10 stimulation. This observation seems to be in line with findings of others, that IP-10 might be a more sensitive marker than IFN- γ (9-11;33). Interestingly, monocytes and macrophage are reportedly the main source of IP-10 (10;35;36). Monocytes and macrophages respond after paracrine stimulation from T cells by interferons and other proinflammatory cytokines with the expression of IP-10. IP-10 is involved in trafficking monocytes and activated T helper cells to sites of inflammation (11). Given the striking antigen specificity of the IP-10 production that we observed, we decided to evaluate whether IP-10 could also be produced by T cells. The data presented in Figure 3B clearly show that CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were capable of producing IP-10 after TB specific stimulation. This observation might be the explanation for the specific induction of IP-10 in plasma of the QFT-GIT assay described by Ruhwald et al (9-11). Furthermore, our data seem to suggest that IP-10 is produced prior to IFN- γ , but future research should address the pathway of IP-10 induction in T cells as well as the kinetics of IP-10 versus IFN- γ .

The presence or induction of multifunctional T cells producing IFN- γ , IL-2 and TNF- α simultaneously, has been associated with protection from disease (25). This protective role is also demonstrated in mouse models of *Mycobacterium tuberculosis* infection (27). Only one study in children analyzed the presence of multifunctional T cells in active TB, showing that these cells can indeed be induced in humans (26). It is interesting to see that in our study multifunctional T cells could be induced in cured TB patients, which seems to contradict the hypothesis that multifunctional T cells protect from TB disease. However, a recent study published by Tchilian showed that in a murine model the presence of systemic multifunctional T cells was not correlated with protection from TB disease (37), which is in line with our data. The question is whether the presence of multifunctional T cells prevents progression to active TB or whether these cells are only induced after massive presentation of antigen, as is the case during active TB? More definite information in this regard could be provided by a prospective study among individuals with recent latent infection who are not treated for LTBI, and patients with active TB during treatment and assess the presence of multifunctional T cells over time in order to relate the presence of multifunctional T cells to clinical status and clinical status in particular.

The last observation of this study was the different phenotype of the cells producing IFN- γ in cured TB patients and latently infected individuals. IFN- γ producing cells from cured TB patients predominantly had the effector phenotype, being CD27⁻ and CD45RA⁻. In contrast, IFN- γ producing T cells of latently infected individuals were more distributed over the 4 possible different subsets, with an unexpected high percentage of cells with the naive phenotype producing IFN- γ . From other reports it is believed that effector cells are needed for IFN- γ production in the short term culture IGRA and that the absence of these effector cells might be an explanation for a negative IGRA result in association with a positive response in a long term assay (12;18). Therefore, it is interesting that presumably naive cells produced IFN- γ after TB specific stimulation, which led to the hypothesis that those supposedly naive cells producing IFN- γ are not truly naive cells. Further research is needed to define the precise memory-effector phenotype of cells producing IFN- γ in cured TB patients, latently infected individuals who respond only after 6 days of stimulation, as well as in latently infected individuals who respond in a short term culture assay.

In conclusion, the data collected in this pilot study suggest that the immunological response of cured TB patients and those latently infected with TB may differ both with regard to the phenotype of responding cells as well as the magnitude of response. These findings confirm earlier reports that short term culture IGRA are not fully sensitive for detection of all forms of past latent TB infection, which can be relevant in patients who are screened before or during immunosuppressive treatment. In particular the finding of multi-functional T cells only among cured TB patients prompts further study addressing the role of such cells in relation to protection.

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