



Universiteit
Leiden
The Netherlands

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

Franken, W.P.J.

Citation

Franken, W. P. J. (2009, June 10). *Immunodiagnosis of latent tuberculosis : new answers to an old question?*. Retrieved from <https://hdl.handle.net/1887/13840>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/13840>

Note: To cite this publication please use the final published version (if applicable).

12

SUMMARY AND GENERAL DISCUSSION

The results of the studies described in Chapters 2 through 11 will be briefly summarized and discussed in the following sections.

- 9.1.) Detection of LTBI in the native Dutch population
- 9.2.) Detection of LTBI in the immigrant Dutch population
- 9.3.) Technical aspects of IGRA
- 9.4.) Differences in immune responses between cured TB patients and subjects with LTBI
- 9.5.) The future of TB diagnostics

Two new commercial assays for the diagnosis of LTBI have been evaluated in this thesis. Both of these assays rely on the presence of MTB specific T cells who will respond with IFN- γ production after recognition of the TB specific antigens. The difference between the two assays is the method with which they visualize this reaction, the QFT-GIT measures the concentration of the IFN- γ produced while the T-SPOT.TB visualizes the number of cells responding. Although both of these assays have proven useful in the diagnosis of LTBI, they are not ideal. But what are then the demands of an ideal TB detection method? It has to be simple, easy to perform, stable at room temperature as well as the temperatures in Africa, with a minimum of discomfort for the patient, cheap, 100% sensitive and 100% specific for the detection of LTBI. Furthermore, ideally the assay would discriminate recent TB infection from infection acquired longer ago, since only recent infections are usually considered for preventive treatment. So far such an assay is not yet available simply because we do not have the knowledge to construct such an assay. Therefore, we have to work with the best possible alternatives and in the mean while try to optimize the available tools in order to come close to the ideal TB detection assay.

9.1. LTBI in the native Dutch population

In the Netherlands we have two different situations regarding the control of latent TB infection in the population. On the one hand, there are individuals who are born in the Netherlands and who have the most risk of encountering tuberculosis when they visit a high endemic country, since the incidence of TB among native Dutch individuals is less than 2 per 100,000 per year (1). On the other hand we have immigrants coming to the Netherlands from high endemic countries, who are predominantly BCG vaccinated and most of whom are likely to be latently infected when entering the Netherlands (2). Furthermore the latter group is at higher risk than native Dutchmen of acquiring TB infection in the Netherlands by re-exposure to an immigrant with active TB (incidence of TB among the immigrant population is

40.4 per 100,000) (1). Thus, we can conclude that these are two different groups, which asks for different approaches regarding tracing and treatment of latent TB infections. Therefore these two populations are discussed separately.

9.1.1 IGRA and influence of NTM infections

In the study described in **chapter 2** QFT-GIT was evaluated in a BCG-naïve population of employees of the Royal Dutch Armed Forces. After deployment they were monitored for possible exposure to TB as well as exposure to NTM during a military mission in high endemic areas. The results showed that QFT-GIT was positive only in 11% of TST positive participants after mission abroad, while QFT-GIT was positive in 44% of TST positive new recruits that had not yet been send on a mission. Bruins et al (3) already demonstrated in 1995 that more than half of all skin test conversions in the Royal Dutch Armed Forces were due to infections with NTM. Therefore we think that QFT-GIT detected only those individuals with a true TST conversion due to TB infection while the positive TST in the remaining 89% of subjects were related to NTM exposure. So far few studies have been published evaluating in humans the influence of NTM on IGRA results (4-6). One study showed that T cell responses against ESAT-6 and/or CFP-10 can occur also after infection with *M. marinum* or *M. kansasii* (4). These latter findings have been confirmed in a study in cattle, showing cross-reactivity with ESAT-6 with *M. kansasii* infection (7). Another obstacle for interpretation of the TST is prior BCG vaccination, which leads to high false-positive TST rates. Since the antigens used in QFT-GIT are absent from all BCG strains this is expected not to affect QFT-GIT results, as was shown in several studies (8-12). To definitely conclude that the TST positive QFT-GIT negative individuals indeed were not infected with TB, long term, untreated follow-up of these individuals would be required. However, untreated follow-up of military personnel with positive TST is at present not feasible and would require additional scientific evidence regarding the risk among QFT-GIT negative subjects of developing active TB. In conclusion it appeared that QFT-GIT is more specific than the TST for detection of LTBI infections in a group of immuno-competent individuals who are at high risk of false positive TST results due to NTM exposure (13;14).

9.1.2 IGRA during follow-up of TST positive individuals

The study described in **Chapter 3** was initiated to analyze the commercially available IGRA, QFT-GIT and T-SPOT.TB, during a large-scale contact investigation. This contact investigation was carried out since a supermarket employee in Zeist, the Netherlands, appeared to have had highly contagious open lung TB during at least 8 months. By consequence, all supermarket customers

were offered TB screening. The results of the study showed that in contrast to the TST both IGRA were associated with exposure to TB, expressed as cumulative shopping time in the supermarket. Agreement between both IGRA was strong, although the T-SPOT.TB was more often positive in the low range TST indurations as compared with the QFT-GIT. Analysis of cut-off values revealed that optimal agreement between both IGRA was obtained when the cut-off of the QFT-GIT was adjusted to 0.20 IU/ml and the cut-off of the T-SPOT.TB to 13 spots. It has to be investigated whether or not the individuals with a TST of more than 15 mm but with a negative IGRA are at risk of developing active TB or not. Other studies have been published showing that the IGRA are quantitatively correlated to exposure in contrast to the TST, which implies that they indeed are better markers to identify those latently infected with TB (15).

One of the hypotheses was that IGRA, in contrast to the TST, will turn negative after a certain period of time after TB exposure and that they can be used to detect re-infections or even might be used as a correlate of treatment success (16). The study described in **Chapter 4** followed individuals with a positive TST after being exposed to a contagious supermarket employee. It appeared that individuals with a negative IGRA at the beginning of the study, even though they had a positive TST, remained IGRA negative during the two year follow-up period. Responses of individuals with a quantitatively high positive IGRA result remained strongly positive and limited or no fluctuations were observed in follow-up. However, if the initial IGRA result was moderately to strongly positive a dynamic pattern of follow-up results was observed. A possible explanation could be that the immune response of those individuals had not yet established equilibrium with the bacteria and the results represent an 'ongoing immunological battle'. Alternatively, it could represent inherent variability of the assay, with a cut-off at high positive levels. As of the time of this writing few data are available about the reproducibility of IGRA in short and long term periods. The studies reported so far have analyzed the inter-assay variability of QFT-GIT within a short period of time, i.e., with repeated measurements over a maximum interval of 3 months in a population not recently exposed to TB (17;18). These studies indicate that variability between measurements (i.e., positive or negative) of about 16% can be expected. Another recent study showed that in a 12 month interval most reversions and conversions occurred in samples with values that were close to the cut-off (19). None of our participants developed TB despite persistently positive results indicating that a positive IGRA result not necessarily implicates that active TB will develop.

Several studies have been published suggesting that the effectiveness of treatment in active TB patients can be monitored using IGRA, since responses decreased during treatment (20-25). However, other studies reported that even though there

is a decrease in response test reversions are rarely observed (16;26;27). Thus, IGRA do not appear to have clinical value during follow-up.

The evaluation of the effect of preventive treatment on IGRA result in individuals with latent infection has not yet resulted in a generally accepted conclusion. In agreement with our findings there are studies showing that some individuals with a positive IGRA will remain positive (15;28;29). A possible explanation of persistent positive IGRA results in the absence of development of active TB could be that some individuals mount a very effective immune response during which many cells acquire an intermediate effector-memory phenotype that can become activated in the short term culture of IGRA (30-32). Another hypothesis, that does not exclude the previous one, might be that these individuals have mounted a strong immune response, but have failed to clear the infection completely, thus antigens are persistently presented to the immune system.

Another observation of this study was that some individuals can have discordant IGRA results, most often a negative QFT-GIT combined with a positive T-SPOT. *TB* result, the significance of which was not understood. It has been described that T-SPOT. *TB* is more sensitive than QFT-GIT (33), especially in immuno-compromised patients like those with HIV/AIDS (34;35). The most likely explanation for this observation is that the number of PBMC's is diminished in immuno-compromised patients and since a defined number of isolated PBMC's is used in T-SPOT. *TB*, while a defined volume of whole blood is used for QFT-GIT, this could underly the enhanced sensitivity of T-SPOT. *TB* (36-38). On the other hand it has been reported that more positive T-SPOT. *TB* results can be found in association with a negative TST (**Chapter 3**)(39), which might indicate lower specificity.

The study described in **Chapter 5** followed individuals from the study described in **Chapter 3** and who had a negative TST combined with a positive IGRA, predominantly positive T-SPOT. *TB* results, at the time of the contact investigation. The reverse combination of a positive TST and negative IGRA could be explained by false positive TST results due to prior BCG-vaccination or NTM infection or alternatively by lower sensitivity of IGRA for past infection as was first hypothesized in the study in **Chapter 3**. It is more difficult to contrive an explanation for a negative TST in association with a positive IGRA. In the century of experience with the TST it has not been reported that individuals with a TST <5mm are at risk of developing active TB and the association of TST negative and IGRA positive is thus highly unlikely to reflect an increased risk of active TB. One hypothesis could be that individuals with such discordant results have been infected with a low inoculum (low dose of bacteria) and have mounted an adequate immune

response which truly eradicated all bacteria. It has been described in other studies that ESAT-6 and CFP-10 have a different significance with regard to the state of latent infection. For instance, responses to ESAT-6 will remain as an immunological scar whereas those to CFP-10 might be more related to recent infection (40). In our study, all individuals with an initial positive T-SPOT.TB response reverting to negative one year later were CFP-10 positive, indicating that this might have been a recent infection. However, unpublished data of the study described in **Chapter 3** indicate that responses to ESAT-6 were more specific for recent infection while those to CFP-10 were more frequent among subjects with negative TST and only positive T-SPOT.TB result thus possibly reflecting such transient infection (S. Arend, personal communication). It is overall surprising to find these differences since ESAT-6 and CFP-10 are secreted as a 1:1 complex by the MTB bacteria (41). Further research is clearly required to analyze the observed differences in antigen-specific response patterns.

9.2 LTBI in the immigrant Dutch population

The incidence of TB is highest in underdeveloped countries where resources, drugs and diagnostics are scarce. Already at a young age individuals are exposed to TB, and many will be re-infected repeatedly during their lifetime. In order to protect children from the most severe and lethal forms of TB, they are vaccinated with BCG, the only registered TB vaccine today. One drawback of BCG vaccination is that the tuberculin skin test can become false positive due to crossreactive immune responses to the antigens in PPD shared by MTB and BCG, so that a TST cannot distinguish between infection and vaccination (42). Furthermore, once positive the TST will remain positive for a life-time precluding the possibility to detect re-infections (42-44). This is a problem when already infected individuals from high endemic countries immigrate to low endemic countries like the Netherlands and are exposed to smear-positive TB. Due to the lack of specificity of the TST in this population, it was not possible to assess latent infection. In the Netherlands, immigrants are therefore followed-up for two years by radiographic imaging of the lungs in order to detect active TB at an early stage.

9.2.1 Influence of prior TB infection on IGRA

One of the great bottlenecks in the eradication of tuberculosis is the large pool of latently infected individuals from which new active TB cases arise every day. Since one third of the world population is thought to be infected, it is impossible to provide all those individuals with preventive treatment. Not just because of the magnitude of drug distribution but also because large groups will have relative contra-indications for taking the medications such as chronic viral hepatitis or other

liver diseases which increases the risk of severe side-effects like liver failure (45). Due to these potentially serious side-effects elderly people are usually not advised to take preventive treatment. Therefore a different approach needs to be taken into consideration for eradication of TB. At the moment, only those individuals with a recent infection are taken into consideration for preventive isoniazid (INH) treatment, since most TB cases arise among these within two years after infection. Most convenient would be an assay that will single out individuals who will actually develop active TB, allowing targeted treatment. As yet, no such assay exists. It has been suggested that IGRA could be more suitable in this regard, since these are short term incubation assays that will only detect activated effector cells present in the blood thus reflecting ongoing bacterial replication (46), in contrast to the TST which will induce memory responses as well since it depends on a delayed type hypersensitivity reaction. It has been postulated that during older latent infections, when bacilli are hiding from the immune system, some individuals may have no, or a relative small number of circulating effector cells, while most effector cells have progressed to become true memory cells that are supposedly not capable of becoming activated within the short term culture of IGRA (47). In **Chapter 6** the baseline analysis of a prospective cohort study among immigrant, BCG-vaccinated, close contacts of Ziehl-Neelsen sputum positive TB patients are presented. As described above, the predominantly BCG-vaccinated immigrants are not screened for LTBI in the Netherlands; therefore it was considered ethically justified to withhold treatment from participants and follow them up for active TB and analyze risk factors associated with development of active TB. At enrollment in the study, all participants received a TST and if the induration was at least 5 mm they were tested with IGRA and questionnaire about their past exposure to TB and demographic data. In chapter 6 we analyzed risk factors associated with a positive TST or IGRA result. We showed that both IGRA are strongly correlated to factors associated with past exposure indicating that a positive IGRA result can reflect past infection as well as recent infection. QFT-GIT and T-SPOT.TB were more often positive in close contacts of an active TB patient when they were born in a high TB endemic country, which indicates prior exposure to TB. Furthermore individuals with known previous exposure to TB, like previous participation in contact investigations or being a former TB patient, were more likely to have a positive IGRA than individuals who were not. A study in Norway showed that 29% of recent immigrants were positive in QFT-GIT at entry (2), most likely reflecting prior TB exposure, although it cannot be excluded with certainty that these individuals were recently infected during travel or shortly after entering Norway. Few other studies have addressed the issue of the effect of previous exposure to TB on IGRA results. However, when analyzing results obtained in high endemic

countries it is clear that a considerable proportion of the population can be positive in IGRA or in-house versions of these assays (28;48) suggesting that repeated exposure could increase the chance of persistent positive IGRA results compared to brief exposure from one smear-positive case as is the common situation in a low-endemic setting nowadays. In conclusion, based on the data reported in **Chapter 6** it appeared that IGRA results are influenced by remote and likely repeated exposure to TB which may limit the usefulness of IGRA to detect recent TB infections in immigrants.

9.2.2 Predictive value of IGRA and TST for developing active TB

Chapter 7 presents the follow-up data of the individuals untreated despite TB exposure, as described in chapter 6. The main goal of this study was to analyze the predictive value of IGRA and TST for identification of individuals who will progress to active TB. After the analysis of the data in chapter 4 were completed it was clear that the positive predictive value (PPV) of IGRA for active TB would be very low, as almost 60% of participants was positive in one or both IGRA. After follow-up of two years eight participants had developed active TB. Of these 8 TB cases, only 6 were positive in T-SPOT.TB and 5 in QFT-GIT at the time of inclusion. Both manufacturers of the assays do not claim that the assay is 100% sensitive and it is therefore possible that results of some of the infected individuals were false negative. However, several studies have reported high responses to ESAT-6 and CFP-10 stimulation in individuals who later progressed to TB (35;49-52). A plausible explanation for the negative IGRA in the progressors might be that the IGRA were performed too soon after exposure. It has been suggested that a cellular immune response can be measured as soon as two weeks after infection with TB (53), making the time window more narrow than that of the TST which is 6 to 8 weeks, but it has not been proven if this also applies to IGRA. On evaluation of the interval between diagnosis of the TB index case and phlebotomy for IGRA of the 8 participants who developed active TB it appeared that this was quite short for some individuals (5, 19 and 34 days after diagnosis of the index patient while the median of total group is 37 days with an inter-quartile range of 15-177 days), although not significantly different from the interval in all individuals with a positive IGRA response. It is known that TB patients are most infectious in the later stages of their illness, so it is possible that the progressors with negative IGRA had been infected shortly before the TB index case was identified and that they therefore had not yet mounted a detectable immune response to the antigens. In practice this could implicate that IGRA must be performed at least 6 weeks after diagnosis of the index patient for optimal yield, since otherwise individuals who are at risk of progressing to active TB may be missed. Unfortunately, we were not able to repeat

IGRA at the second round of the contact investigation and further studies should address the minimal time to conversion. Apart from the interval between exposure and blood test, there are other potential explanations for why some progressors were negative in IGRA. The assays may not be completely sensitive and might miss some infected individuals. Since none of the participants had a recognized immune disorder or immunosuppressive treatment, it was highly unlikely that impaired immune status had affected the results. Interestingly, all TB progressors had a positive TST result, in contrast to IGRA response. This would indicate enhanced sensitivity of the TST for detecting those infected individuals. However, since the study population was predominantly BCG vaccinated and participants eligible for IGRA were selected based on a TST result of at least 5 mm, it is also possible that the positive TST responses reflected the non-specificity of the TST, although that would not hold true for TST responses exceeding 15 mm which are indicative of TB infection even in BCG vaccinated individuals (54). However, the negative predictive value (NPV) of IGRA was very high, almost 100%, which was also found in other studies (55-57). There have been only a few prospective studies evaluating the predictive value of responses to ESAT-6 and CFP-10 in untreated subjects, but none included an immigrant population. One study in Germany showed that QFT-GIT had a significantly higher PPV than the TST at a cut-off value of 5 mm, but not at a cut-off of 10 mm (12). In conclusion, the TST appeared to be more sensitive than both IGRA formats for prediction of disease in this immigrant population, but the specificity could not be compared directly as it is not possible to discriminate between true and false positive TST results. Thus, further studies are needed to evaluate the use of these tests with regard to the value of prophylactic treatment in a population with a high rate of BCG vaccination as well as of preexisting latent TB infection.

9.3 Technical aspects of IGRA

Chapter 8 and 9 discussed some technical issues of IGRA. In **Chapter 8**, T-SPOT.*TB* results of the PREDICT study (Chapter 6) were used for comparison of inter-rater interpretation of the assay. All 27 T-SPOT.*TB* plates were read and interpreted by four individual human raters of two different laboratories who did not have knowledge of each other results, and by two automated spot raters. From the analysis it appeared that there was considerable variation between the six raters. The highest rater interpreted 17% more samples as positive than the lowest rater even though five out of six raters were within a 6% difference of each other. So far no studies have been published that addressed T-SPOT.*TB* reading. It should be noted that the population on which this study was based included an extraordinarily high rate of latently infected individuals. Since the number of positive tests will in

general be smaller in daily practice it is expected that the absolute differences will be smaller while the relative difference will remain, indicating that there is a subjective component in the T-SPOT.*TB* result that may affect the decision whether or not to treat. A possible explanation for the observed inter-rater differences could be that each rater has his or her own internal standard about the definition of what signifies a true spot. The manufacturer has developed a web based tool to practice rating spots in order to learn the definition of a spot. However, all human raters in this study had had personal training in rating the spots and still there was considerable discordance. Future research should focus on this discrepancy in interpretation of spots by independent raters of multiple laboratories. Other populations like hospital patients or participants of contact investigations among non-immigrants should also be included since our study included an unusually high rate of positive results. Since this study only analyzed the agreement in spot rating, future research should also include distributing samples among different laboratories to analyze between-laboratory variation in overall performance of the assay.

There have been suggestions to use quantitative as well as qualitative results of IGRA. However, the significance of quantitative test results is unclear at present, especially whether very high test results could indicate an increased risk of progression. The standard curve used to calculate the concentration of IFN- γ in QFT-GIT ends at the high end at 4 IU/ml. The cut off value is 0.35 IU/ml and all higher measurements are considered positive. Due to the limited range of the standard curve, values in the high range may therefore not be precise. In **Chapter 9** the results were shown of a concise study in which ELISA results of QFT-GIT samples with a high test result were compared to their diluted equivalent. The data in Chapter 7 show that for values above 10 IU/ml it is no longer justified to report them as such as they can be a factor 10 higher than the ELISA result suggests. This could be a problem when researchers try to analyze the effect of treatment on test outcome and report values initially exceeding 10 or 50 IU/ml which appeared to have decreased on follow-up. The newest version of the software provided by the manufacturer to analyze results now contains an upper limitation and values above 10 are reported as >10 IU/ml.

9.4 Differences in immune responses between cured TB patients and subjects with LTBI

The study which is described in **Chapter 10** of this thesis was initiated to explain an observation made by Leyten et al (58). Among latently infected individuals, defined by TST conversion after reported contact with a TB patient, they detected a subgroup which had negative results in QFT-GIT and ELISPOT, but after

prolonged stimulation their PBMC's did respond with IFN- γ production in response to ESAT-6 and/or CFP-10 stimulation indicating that they were truly latently infected with TB. Since this subgroup comprised almost 50% of the participants this prompted further study to analyze the discordance between the negative QFT-GIT and positive long term culture result. The results in **Chapter 10** showed that these latently infected individuals did have IFN- γ producing cells after stimulation with ESAT-6 or CFP-10, even after a short stimulation, but in the supernatants no IFN- γ was detected suggesting that the cells had not (yet) secreted their IFN- γ . Further analysis showed that the phenotype of the IFN- γ producing cells of these LTBI was different from that of the IFN- γ producing cells of cured TB patients, who did have high IFN- γ levels in their supernatants. The cells of the cured TB patients producing IFN- γ had an effector-memory phenotype; in contrast the phenotype of the IFN- γ producing lymphocytes of the subjects with LTBI was much more diverse and was dominated by supposedly naïve and central memory cells. Together these data suggest that cells producing IFN- γ in the short-term culture assay indeed are of the effector-memory phenotype, as was suggested previously (46;47). An explanation for the negative IGRA result could be a lack of antigen-specific cells of the effector-memory phenotype in part of the subjects with LTBI, as could be associated with absence of bacterial replication and subsequent lack of antigen presentation that is required to drive persistence of a cell type with the capacity of immediate responsiveness. It is not known whether such state indicates the complete absence of any live bacilli or just a deeply dormant state from which periods of limited replication may still occur, which would be relevant in case treatment of persistent latent infection is indicated as e.g. before immunosuppressive treatment.

It has also been described in other studies that some latently infected individuals are negative in QFT-GIT (59;60). Questions have been raised about whether or not IFN- γ is the most sensitive readout cytokine, or whether other cytokines or a combination of cytokines should be used. Ruhwald et al have demonstrated that interferon- γ inducible protein (IP-10) might be a more sensitive marker than IFN- γ , since patients with active TB and latently infected individuals who were negative in QFT-GIT did mount a strong IP-10 response (61-63), findings that have been confirmed by others (64;65). When we analyzed IP-10 production of PBMC's of cured TB patients and latently infected individuals, it appeared that latently infected individuals indeed produced IP-10 at the same level as cured TB patients after prolonged stimulation with the TB specific antigens. Unfortunately, we were unable to analyze QFT-GIT plasmas of the individuals to confirm that they also produce IP-10 after short-term stimulation. Future research should therefore include the analysis of IP-10 in QFT-GIT supernatants of these individuals.

The study also showed that PBMC's of cured TB patients can be induced to become multifunctional T cells, a cell type that has been associated with protection from disease (66;67). However, a recent study in a murine model showed that the presence of systemic multifunctional T cells was not correlated to protection from TB disease (68). More definite information about the role and induction of multifunctional T cells should be provided with a longitudinal study among recently TB infected individuals who are not treated and assess the presence of multifunctional T cells over time in relation to clinical status and protected or non-progressor state in particular.

9.5 Future of TB diagnostics

Although IGRA have proven to be useful in the diagnosis of active as well as latent TB in several populations, there are potential limitations. As discussed above, the usefulness of IGRA in individuals with a history of prior TB infection seems to be limited (**Chapter 6**). Furthermore, the assays are quite expensive and require good laboratory equipment. IGRA may therefore not be very suitable for use in poorly developed countries, where most LTBI and TB cases can be found. Therefore other initiatives have been employed in order to better separate those infected with TB from those who are not.

The study described in **Chapter 11** evaluated the use of the TB specific antigen ESAT-6 as a skin test reagent, as compared with PPD. This study was a Phase-I clinical trial and the first to evaluate the safety in healthy volunteers and treated TB patients. From the study it appeared that uninfected volunteers did not experience significant adverse effects. Furthermore, the healthy TB naïve volunteers did not respond to ESAT-6 stimulation demonstrating the specificity of the antigen. Secondly, it was evaluated if skin reactions could be induced in previously treated TB patients. This was indeed the case; at 0.1 µg ESAT both redness and induration were induced to the same extent as the reactions to PPD. From this data it appeared that ESAT-6 can be safely used as skin test reagent and further research should be performed to analyze the sensitivity and predictive value in latently infected individuals. A skin test based on ESAT-6 could be useful in developing countries, as there is already extensive experience with the tuberculin skin test which makes implication of the assay quite straightforward and costs will likely be lower compared to IGRA. However, although a new skin test may solve the specificity problem of the TST, other drawbacks remain like the need to return for reading and the fact that it cannot differentiate between past or recent infection or between latent and active TB infection.

With regard to patients suspected of active TB, others have tried to enhance the sensitivity of IGRA by using cells obtained from biopsies, pleural fluid or bronchial lavage fluid (69-74). The idea behind this is that most specific T cells could be present at the site of infection and not in the blood compartment (75), which might explain the high rate of false negative IGRA results in patients with active TB. Using pleural effusion mononuclear cells for T-SPOT.TB resulted in enhanced sensitivity compared to using PBMC (72). Another study showed that directly detecting IFN- γ in pleural fluid was more accurate in identifying active TB patients (all patients were detected) than the QFT-GIT or the QFT-GIT using pleural fluid as cell source (73).

Apart from immunodiagnosis as was studied in this thesis, future research should be extended to other possible methods for detecting TB infection. With the PCR technique it is already possible to detect TB bacteria in sputum, other excreta or in biopsies of patients (76;77), but obtaining biopsies can be very difficult and uncomfortable for the patient (78;79). A limitation of latent TB infection is that no material can be readily obtained to perform assays to directly detect bacilli or their components. New initiatives focus on detecting excretion products of the TB bacilli, like latency proteins and specific fatty lipids, directly in urine or breath condensates (unpublished data). This can be accomplished by different techniques like e-nose, nuclear magnetic resonance spectroscopy or mass spectrometry.

During the latent phase of TB infection certain proteins, named latency proteins, are strongly upregulated due to exposure to adverse intracellular conditions such as hypoxia (80). The best characterized latency antigen is the 16kDa- α -crystallin protein encoded by Rv2031c. Latency antigens are encoded by 48 genes named the dormancy regulon (DosR) (81). During latent infection it might thus be possible to directly detect those proteins. However, it was demonstrated that T cell responses to latency antigens were found in patients with past active TB as well, indicating that those proteins may not be truly phase-specific (82).

Another topic of intensive research is vaccine development. Vaccination of individuals at risk of encountering TB has so far been with limited success. The only vaccine registered today is the BCG vaccine. This will protect small children from the most severe forms of TB, but does not protect adults from the contagious lung TB. A lot of effort is put in designing new TB vaccines which will protect everyone from acquiring TB disease on the one hand and preventing reactivation in those who are already infected with TB (83;84). Some have altered the existing BCG-vaccine so that an over expression of strongly recognized epitopes, like Ag85B, is accomplished (85)(86). Others have prepared a recombinant fusion vaccine of several MTB antigens like combinations of Ag85B and TB10.4 or Ag85B and

ESAT-6 (86). Some of these vaccines are being evaluated in clinical trials for safety and efficacy. At the department of Infectious Diseases in Leiden, a phase-I clinical trial has been performed using a fusion protein of Ag85B with ESAT-6. Results of this study are not yet published. Other studies used latency antigens in a post-exposure vaccination strategy (87),

In conclusion, the studies described in this thesis have shown that IGRA have added value for the specific diagnosis of LTBI, but as is the case for any other assay, they have limitations as well. The results of IGRA should therefore be interpreted carefully in the light of the setting and the complete clinical data. Future research should not be limited to enhance the performance of IGRA but also include new opportunities, like an improved skin test or the detection of bacterial products in clinical specimens.

REFERENCE LIST

- (1) Erkens C.G.M, Kalisvaart N.A., Slump E., Sebek M., van Soolingen D. Tuberculose in Nederland 2006. KNCV Tuberculosefonds; 2008 Mar.
- (2) Winje BA, Oftung F, Korsvold GE, Mannsaker T, Jeppesen AS, Harstad I, et al. Screening for tuberculosis infection among newly arrived asylum seekers: comparison of QuantiFERON(R)TB Gold with tuberculin skin test. BMC Infect Dis 2008 May 14;8(1):65.
- (3) Bruins J, Gribnau JH, Bwire R. Investigation into typical and atypical tuberculin sensitivity in the Royal Netherlands Army, resulting in a more rational indication for isoniazid prophylaxis. Tuber Lung Dis 1995 Dec;76(6):540-4.
- (4) Arend SM, van Meijgaarden KE, de Boer K, de Palou EC, van Soolingen D, Ottenhoff TH, et al. Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with *Mycobacterium marinum* or *M. kansasii*. J Infect Dis 2002 Dec 15;186(12):1797-807.
- (5) Kobashi Y, Obase Y, Fukuda M, Yoshida K, Miyashita N, Oka M. Clinical reevaluation of the QuantiFERON TB-2G test as a diagnostic method for differentiating active tuberculosis from nontuberculous mycobacteriosis. Clin Infect Dis 2006 Dec 15;43(12):1540-6.
- (6) Arend SM, de Haas P, Leyten E, Rosenkrands I, Rigouts L, Andersen P, et al. ESAT-6 and CFP-10 in clinical versus environmental isolates of *Mycobacterium kansasii*. J Infect Dis 2005 Apr 15;191(8):1301-10.

- (7) Vordermeier HM, Brown J, Cockle PJ, Franken WP, Arend SM, Ottenhoff TH, et al. Assessment of cross-reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP-10 at the T cell epitope level. *Clin Vaccine Immunol* 2007 Aug 1.
- (8) Porsa E, Cheng L, Seale MM, Delclos GL, Ma X, Reich R, et al. Comparison of a new ESAT-6/CFP-10 peptide-based gamma interferon assay and a tuberculin skin test for tuberculosis screening in a moderate-risk population. *Clin Vaccine Immunol* 2006 Jan;13(1):53-8.
- (9) Diel R, Nienhaus A, Lange C, Meywald-Walter K, Forssbohm M, Schaberg T. Tuberculosis contact investigation with a new, specific blood test in a low-incidence population containing a high proportion of BCG-vaccinated persons. *Respir Res* 2006 May 17;7(1):77.
- (10) Codecasa L, Mantegani P, Galli L, Lazzarin A, Scarpellini P, Fortis C. An In-House RD1-Based Enzyme-Linked Immunospot-Gamma Interferon Assay Instead of the Tuberculin Skin Test for Diagnosis of Latent *Mycobacterium tuberculosis* Infection. *J Clin Microbiol* 2006 Jun;44(6):1944-50.
- (11) Brock I, Weldingh K, Lillebaek T, Follmann F, Andersen P. Comparison of tuberculin skin test and new specific blood test in tuberculosis contacts. *Am J Respir Crit Care Med* 2004 Jul 1;170(1):65-9.
- (12) Diel R, Loddenkemper R, Meywald-Walter K, Niemann S, Nienhaus A. Predictive Value of a Whole-blood IFN- γ Assay for the Development of Active TB Disease. *Am J Respir Crit Care Med* 2008 Feb 14.
- (13) Franken WP, Timmermans JF, Prins C, Slootman EJ, Dreverman J, Bruins H, et al. Comparison of Mantoux and QuantiFERON TB Gold tests for diagnosis of latent tuberculosis infection in Army personnel. *Clin Vaccine Immunol* 2007 Apr;14(4):477-80.
- (14) Mazurek GH, Zajdowicz MJ, Hankinson AL, Costigan DJ, Toney SR, Rothel JS, et al. Detection of *Mycobacterium tuberculosis* infection in United States Navy recruits using the tuberculin skin test or whole-blood interferon-gamma release assays. *Clin Infect Dis* 2007 Oct 1;45(7):826-36.
- (15) Ewer K, Millington KA, Deeks JJ, Alvarez L, Bryant G, Lalvani A. Dynamic antigen-specific T-cell responses after point-source exposure to *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med* 2006 Oct 1;174(7):831-9.
- (16) Dominguez J, Souza-Galvao MD, Ruiz-Manzano J, Latorre I, Prat C, Lacom A, et al. T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment. *Diagn Microbiol Infect Dis* 2008 Nov 20.

- (17) Veerapathran A, Joshi R, Goswami K, Dogra S, Moodie EE, Reddy MV, et al. T-cell assays for tuberculosis infection: deriving cut-offs for conversions using reproducibility data. *PLoS ONE* 2008;3(3):e1850.
- (18) Perry S, Sanchez L, Yang S, Agarwal Z, Hurst P, Parsonnet J. Reproducibility of QuantiFERON-TB gold in-tube assay. *Clin Vaccine Immunol* 2008 Mar;15(3):425-32.
- (19) Pai M, Joshi R, Dogra S, Zwerling AA, Gajalakshmi D, Goswami K, et al. T-cell assay conversions and reversions among household contacts of tuberculosis patients in rural India. *Int J Tuberc Lung Dis* 2009;13(1):84-92.
- (20) Katiyar SK, Sampath A, Bihari S, Mamtani M, Kulkarni H. Use of the QuantiFERON-TB Gold In-Tube test to monitor treatment efficacy in active pulmonary tuberculosis. *Int J Tuberc Lung Dis* 2008 Oct;12(10):1146-52.
- (21) Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Oka M. Transitional changes in T-cell responses to *Mycobacterium tuberculosis*-specific antigens during treatment. *J Infect* 2008 Oct 9.
- (22) Carrara S, Vincenti D, Petrosillo N, Amicosante M, Girardi E, Goletti D. Use of a T cell-based assay for monitoring efficacy of antituberculosis therapy. *Clin Infect Dis* 2004 Mar 1;38(5):754-6.
- (23) Goletti D, Butera O, Bizzoni F, Casetti R, Girardi E, Poccia F. Region of Difference 1 Antigen-Specific CD4+ Memory T Cells Correlate with a Favorable Outcome of Tuberculosis. *J Infect Dis* 2006 Oct 1;194(7):984-92.
- (24) Lalvani A, Nagvenkar P, Udwadia Z, Pathan AA, Wilkinson KA, Shastri JS, et al. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. *J Infect Dis* 2001 Feb 1;183(3):469-77.
- (25) Pathan AA, Wilkinson KA, Klenerman P, McShane H, Davidson RN, Pasvol G, et al. Direct ex vivo analysis of antigen-specific IFN-gamma-secreting CD4 T cells in *Mycobacterium tuberculosis*-infected individuals: associations with clinical disease state and effect of treatment. *J Immunol* 2001 Nov 1;167(9):5217-25.
- (26) Bosshard V, Roux-Lombard P, Perneger T, Metzger M, Vivien R, Rochat T, et al. Do results of the T-SPOT.TB interferon-gamma release assay change after treatment of tuberculosis? *Respir Med* 2008 Oct 31.
- (27) Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Okimoto N, et al. Clinical evaluation for diagnosing active TB disease and transitional change of two commercial blood tests. *Scand J Infect Dis* 2008;40(8):629-34.

- (28) Pai M, Joshi R, Dogra S, Mendiratta DK, Narang P, Dheda K, et al. Persistently elevated T cell interferon-gamma responses after treatment for latent tuberculosis infection among health care workers in India: a preliminary report. *J Occup Med Toxicol* 2006 May 23;1(1):7.
- (29) Higuchi K, Harada N, Mori T. Interferon-gamma responses after isoniazid chemotherapy for latent tuberculosis. *Respirology* 2008 May;13(3):468-72.
- (30) Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 2008 Nov;73(11):975-83.
- (31) Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, et al. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med* 1997 Nov 3;186(9):1407-18.
- (32) Caccamo N, Dieli F, Wesch D, Jomaa H, Eberl M. Sex-specific phenotypical and functional differences in peripheral human Vgamma9/Vdelta2 T cells. *J Leukoc Biol* 2006 Apr;79(4):663-6.
- (33) Menzies D, Pai M, Comstock G. Meta-analysis: New Tests for the Diagnosis of Latent Tuberculosis Infection: Areas of Uncertainty and Recommendations for Research. *Ann Intern Med* 2007 Mar 6;146(5):340-54.
- (34) Rangaka MX, Wilkinson KA, Seldon R, Van Cutsem G, Meintjes GA, Morroni C, et al. Effect of HIV-1 infection on T-Cell-based and skin test detection of tuberculosis infection. *Am J Respir Crit Care Med* 2007 Mar 1;175(5):514-20.
- (35) Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, et al. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 2006 Apr 22;367(9519):1328-34.
- (36) Vincenti D, Carrara S, Butera O, Bizzoni F, Casetti R, Girardi E, et al. Response to region of difference 1 (RD1) epitopes in human immunodeficiency virus (HIV)-infected individuals enrolled with suspected active tuberculosis: a pilot study. *Clin Exp Immunol* 2007 Oct;150(1):91-8.
- (37) Kobashi Y, Mouri K, Obase Y, Fukuda M, Miyashita N, Oka M. Clinical evaluation of QuantiFERON TB-2G test for immunocompromised patients. *Eur Respir J* 2007 Nov;30(5):945-50.
- (38) Brock I, Ruhwald M, Lundgren B, Westh H, Mathiesen LR, Ravn P. Latent tuberculosis in HIV positive, diagnosed by the *M. tuberculosis* specific interferon-gamma test. *Respir Res* 2006;7:56.
- (39) Arend SM, Thijsen SF, Leyten EM, Bouwman JJ, Franken WP, Koster BF, et al. Comparison of two interferon-gamma assays and tuberculin skin test for tracing tuberculosis contacts. *Am J Respir Crit Care Med* 2007 Mar 15;175(6):618-27.

- (40) Chee CB, Khinmar KW, Gan SH, Barkham TM, Pushparani M, Wang YT. Latent tuberculosis infection treatment and T-cell responses to Mycobacterium tuberculosis-specific antigens. *Am J Respir Crit Care Med* 2007 Feb 1;175(3):282-7.
- (41) Renshaw PS, Panagiotidou P, Whelan A, Gordon SV, Hewinson RG, Williamson RA, et al. Conclusive evidence that the major T-cell antigens of the Mycobacterium tuberculosis complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6*CFP-10 complex. Implications for pathogenesis and virulence. *J Biol Chem* 2002 Jun 14;277(24):21598-603.
- (42) Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. *Clin Infect Dis* 1993 Dec;17(6):968-75.
- (43) Menzies RI. Tuberculin Skin Testing. Tuberculosis, a comprehensive international approach. 2nd ed. New York: Marcel Dekker Inc; 2000. p. 279-322.
- (44) Menzies D. Interpretation of repeated tuberculin tests. Boosting, conversion, and reversion. *Am J Respir Crit Care Med* 1999 Jan;159(1):15-21.
- (45) Kopanoff DE, Snider DE, Jr., Caras GJ. Isoniazid-related hepatitis: a U.S. Public Health Service cooperative surveillance study. *Am Rev Respir Dis* 1978 Jun;117(6):991-1001.
- (46) T SPOT Technology. www.oxfordimmunotec.com/eu/products_services/background.html. 12-1-2008. Ref Type: Internet Communication
- (47) Wilkinson KA, Kon OM, Newton SM, Meintjes G, Davidson RN, Pasvol G, et al. Effect of treatment of latent tuberculosis infection on the T cell response to Mycobacterium tuberculosis antigens. *J Infect Dis* 2006 Feb 1;193(3):354-9.
- (48) Hill PC, Brookes RH, Fox A, Fielding K, Jeffries DJ, Jackson-Sillah D, et al. Large-scale evaluation of enzyme-linked immunospot assay and skin test for diagnosis of Mycobacterium tuberculosis infection against a gradient of exposure in The Gambia. *Clin Infect Dis* 2004 Apr 1;38(7):966-73.
- (49) Doherty TM, Demissie A, Olobo J, Wolday D, Britton S, Eguale T, et al. Immune responses to the Mycobacterium tuberculosis-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. *J Clin Microbiol* 2002 Feb;40(2):704-6.
- (50) Dietrich J, Aagaard C, Leah R, Olsen AW, Stryhn A, Doherty TM, et al. Exchanging ESAT6 with TB10.4 in an Ag85B fusion molecule-based tuberculosis subunit vaccine: efficient protection and ESAT6-based sensitive monitoring of vaccine efficacy. *J Immunol* 2005 May 15;174(10):6332-9.

- (51) Buddle BM, Parlane NA, Keen DL, Aldwell FE, Pollock JM, Lightbody K, et al. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. *Clin Diagn Lab Immunol* 1999 Jan;6(1):1-5.
- (52) Vordermeier HM, Chambers MA, Cockle PJ, Whelan AO, Simmons J, Hewinson RG. Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infect Immun* 2002 Jun;70(6):3026-32.
- (53) Andersen P, Doherty TM, Pai M, Weldingh K. The prognosis of latent tuberculosis: can disease be predicted? *Trends Mol Med* 2007 May;13(5):175-82.
- (54) Wang L, Turner MO, Elwood RK, Schulzer M, FitzGerald JM. A meta-analysis of the effect of Bacille Calmette Guerin vaccination on tuberculin skin test measurements. *Thorax* 2002 Sep;57(9):804-9.
- (55) Higuchi K, Harada N, Mori T, Sekiya Y. Use of QuantiFERON-TB Gold to investigate tuberculosis contacts in a high school. *Respirology* 2007 Jan;12(1):88-92.
- (56) Nsutebu E, Moffitt SJ, Mullarkey C, Schweiger MS, Collyns T, Watson JP. Use of QuantiFERON-TB Gold test in the investigation of unexplained positive tuberculin skin tests. *Public Health* 2008 Nov;122(11):1284-7.
- (57) Franken WP, Arend SM, Thijsen SF, Bouwman JJ, Koster BF, van Dissel JT, et al. Interferon-gamma release assays during follow-up of tuberculin skin test-positive contacts. *Int J Tuberc Lung Dis* 2008 Nov;12(11):1286-94.
- (58) Leyten EM, Arend SM, Prins C, Cobelens FG, Ottenhoff TH, van Dissel JT. Discrepancy between *Mycobacterium tuberculosis*-specific gamma interferon release assays using short and prolonged in vitro incubation. *Clin Vaccine Immunol* 2007 Jul;14(7):880-5.
- (59) Codeluppi M, Cocchi S, Guaraldi G, Di Benedetto F, De Ruvo N, Meacci M, et al. Posttransplant *Mycobacterium Tuberculosis* Disease Following Liver Transplantation and the Need for Cautious Evaluation of Quantiferon TB GOLD Results in the Transplant Setting: A Case Report. *Transplant Proc* 2006 May;38(4):1083-5.
- (60) Franken WP, Koster BF, Bossink AW, Thijsen SF, Bouwman JJ, van Dissel JT, et al. Follow-up study of tuberculosis-exposed supermarket customers with negative tuberculin skin test results in association with positive gamma interferon release assay results. *Clin Vaccine Immunol* 2007 Sep;14(9):1239-41.

- (61) Ruhwald M, Bjerregaard-Andersen M, Rabna P, Kofoed K, Eugen-Olsen J, Ravn P. CXCL10/IP-10 release is induced by incubation of whole blood from tuberculosis patients with ESAT-6, CFP10 and TB7.7. *Microbes Infect* 2007 Jun;9(7):806-12.
- (62) Ruhwald M, Petersen J, Kofoed K, Nakaoka H, Cuevas LE, Lawson L, et al. Improving T-cell assays for the diagnosis of latent TB infection: potential of a diagnostic test based on IP-10. *PLoS ONE* 2008;3(8):e2858.
- (63) Ruhwald M, Bodmer T, Maier C, Jepsen M, Andersen MB, Eugen-Olsen J, et al. Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of TB. *Eur Respir J* 2008 Aug 6.
- (64) Whittaker E, Gordon A, Kampmann B. Is IP-10 a Better Biomarker for Active and Latent Tuberculosis in Children than IFN γ ? *PLoS ONE* 2008;3(12):e3901.
- (65) Petrucci R, Abu AN, Gurgel RQ, Sherchand JB, Doria L, Lama C, et al. Interferon gamma, interferon-gamma-induced-protein 10, and tuberculin responses of children at high risk of tuberculosis infection. *Pediatr Infect Dis J* 2008 Dec;27(12):1073-7.
- (66) Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* 2007 Jul;13(7):843-50.
- (67) Forbes EK, Sander C, Ronan EO, McShane H, Hill AV, Beverley PC, et al. Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against *Mycobacterium tuberculosis* aerosol challenge in mice. *J Immunol* 2008 Oct 1;181(7):4955-64.
- (68) Tchilian EZ, Desel C, Forbes EK, Bandermann S, Sander C, Hill AV, et al. Immunogenicity and protective efficacy against murine tuberculosis of prime-boost regimes with Δ ureC hly+ rBCG and MVA85A. *Infect Immun* 2008 Dec 8.
- (69) Jafari C, Ernst M, Strassburg A, Greinert U, Kalsdorf B, Kirsten D, et al. Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot. *Eur Respir J* 2007 Nov 7.
- (70) Jafari C, Lange C. Suttons's Law: Local Immunodiagnosis of Tuberculosis. *Infection* 2008 Oct 18.
- (71) Kusters K, Nau R, Bossink A, Greiffendorf I, Jentsch M, Ernst M, et al. Rapid Diagnosis of CNS Tuberculosis by a T-Cell Interferon-gamma Release Assay on Cerebrospinal Fluid Mononuclear Cells. *Infection* 2008 Jan 12.
- (72) Losi M, Bossink A, Codecasa L, Jafari C, Ernst M, Thijsen S, et al. Use of a T-cell interferon-gamma release assay for the diagnosis of tuberculous pleurisy. *Eur Respir J* 2007 Dec;30(6):1173-9.

- (73) Chegou NN, Walzl G, Bolliger CT, Diacon AH, van den Heuvel MM. Evaluation of Adapted Whole-Blood Interferon-gamma Release Assays for the Diagnosis of Pleural Tuberculosis. *Respiration* 2008 Apr 24.
- (74) Luca MC, Petrovici CM, Vata A, Dorobat C, Nastase E, Luca V, et al. [Gamma interferon testing in blood and cerebrospinal fluid--rapid method for the diagnosis of tuberculous meningitis]. *Rev Med Chir Soc Med Nat Iasi* 2008 Jan;112(1):108-10.
- (75) Moonka D, Milkovich KA, Rodriguez B, Abouljoud M, Lederman MM, Anthony DD. Hepatitis C specific T cell IFN- γ and proliferative responses are more common in perihepatic lymph nodes than in peripheral blood or liver. *J Virol* 2008 Aug 20.
- (76) Chakravorty S, Sen MK, Tyagi JS. Diagnosis of extrapulmonary tuberculosis by smear, culture, and PCR using universal sample processing technology. *J Clin Microbiol* 2005 Sep;43(9):4357-62.
- (77) Chakravorty S, Tyagi JS. Novel use of guanidinium isothiocyanate in the isolation of *Mycobacterium tuberculosis* DNA from clinical material. *FEMS Microbiol Lett* 2001 Nov 27;205(1):113-7.
- (78) Migliori GB, Hopewell PC, Blasi F, Spanevello A, Raviglione MC. Improving the TB case management: The International Standards for Tuberculosis Care. *Eur Respir J* 2006 Oct;28(4):687-90.
- (79) Valdes L, Alvarez D, San JE, Penela P, Valle JM, Garcia-Pazos JM, et al. Tuberculous pleurisy: a study of 254 patients. *Arch Intern Med* 1998 Oct 12;158(18):2017-21.
- (80) Yuan Y, Crane DD, Simpson RM, Zhu YQ, Hickey MJ, Sherman DR, et al. The 16-kDa alpha-crystallin (Acr) protein of *Mycobacterium tuberculosis* is required for growth in macrophages. *Proc Natl Acad Sci U S A* 1998 Aug 4;95(16):9578-83.
- (81) Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, et al. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med* 2003 Sep 1;198(5):705-13.
- (82) Leyten EM, Lin MY, Franken KL, Friggen AH, Prins C, van Meijgaarden KE, et al. Human T-cell responses to 25 novel antigens encoded by genes of the dormancy regulon of *Mycobacterium tuberculosis*. *Microbes Infect* 2006 Jul;8(8):2052-60.
- (83) Kaufmann SH, Baumann S, Nasser EA. Exploiting immunology and molecular genetics for rational vaccine design against tuberculosis. *Int J Tuberc Lung Dis* 2006 Oct;10(10):1068-79.

- (84) Williams A, Hatch GJ, Clark SO, Gooch KE, Hatch KA, Hall GA, et al. Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis. *Tuberculosis (Edinb)* 2005 Jan;85(1-2):29-38.
- (85) Horwitz MA, Harth G. A new vaccine against tuberculosis affords greater survival after challenge than the current vaccine in the guinea pig model of pulmonary tuberculosis. *Infect Immun* 2003 Apr;71(4):1672-9.
- (86) Andersen P. Vaccine strategies against latent tuberculosis infection. *Trends Microbiol* 2007 Jan;15(1):7-13.
- (87) Lin MY, Ottenhoff TH. Not to wake a sleeping giant: new insights into host-pathogen interactions identify new targets for vaccination against latent *Mycobacterium tuberculosis* infection. *Biol Chem* 2008 May;389(5):497-511.

