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Healthy Individuals That Control a Latent Infection with *Mycobacterium tuberculosis* Express High Levels of Th1 Cytokines and the IL-4 Antagonist IL-4δ2[1]

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The majority of healthy individuals exposed to *Mycobacterium tuberculosis* will not develop disease and identifying what constitutes "protective immunity" is one of the holy grails of *M. tuberculosis* immunology. It is known that IFN-γ is essential for protection, but it is also apparent that IFN-γ levels alone do not explain the immunity/susceptibility dichotomy. The controversy regarding correlates of immunity persists because identifying infected but healthy individuals (those who are immune) has been problematic. We have therefore used recognition of the *M. tuberculosis* virulence factor early secretory antigenic target 6 to identify healthy, but infected individuals from tuberculosis (TB)-endemic and nonendemic regions (Ethiopia and Denmark) and have compared signals for cytokines expressed directly ex vivo with the pattern found in TB patients. We find that TB patients are characterized by decreased levels of Th1 cytokines and increased levels of IL-10 compared with the healthy infected and noninfected community controls. Interestingly, the healthy infected subjects exhibited a selective increase of message for the IL-4 antagonist, IL-4δ2, compared with both TB patients or noninfected individuals. These data suggest that long-term control of *M. tuberculosis* infection is associated not just with elevated Th1 responses but also with inhibition of the Th2 response. *The Journal of Immunology,* 2004, 172: 6938–6943.

With the introduction of drugs effective against *Mycobacterium tuberculosis* a half-century ago, it was assumed that the combination of chemotherapy and bacillus Calmette-Guérin (BCG)[3] vaccination would soon make tuberculosis (TB) a disease of the past. Even the most pessimistic would hardly have foreseen the present epidemic, with nearly 10 million new cases and more than 2 million deaths each year (1). Unfortunately, the BCG vaccine appears to give protection for only a limited number of years (2) and while TB chemotherapy is cheap and effective, identifying and treating infected individuals before they transmit infection to others has been problematic. The reason behind both of these failings is the complex etiology and immunology of TB. Not all infected individuals develop disease, and in those that do, the period between infection and onset can be years or decades. Unfortunately, the factors that determine these outcomes remain largely unknown.

It is known that immunity to mycobacterial infection requires a Th1 response: defects in either IFN-γ or IL-12 pathways render the host susceptible to infection (3–5). However, it is also clear that IFN-γ production alone is not sufficient for protection against *M. tuberculosis*. Most, if not all, individuals respond to mycobacterial infection by producing IFN-γ and animal studies show that there is no direct correlation between protection and the levels of this cytokine (6).

Thus, to identify protective immune responses in humans, we need first identify individuals who have been infected with *M. tuberculosis* so that those who successfully resist infection can be compared with those who have succumbed to clinical illness. Previously, the identification of latent or subclinical infection has been almost impossible. However, the characterization of Ags largely confined to the members of the TB complex now allows highly specific immunodiagnosis of infection (7). In particular, strong immune responses to the early secretory antigenic target 6 (ESAT-6) Ag appear to correlate with *M. tuberculosis* infection even in individuals without symptoms (8, 9). At the same time, the use of highly sensitive, quantitative RT-PCR allows analysis of gene activation directly ex vivo (10).

In this study, we compare immune responses in a cohort of healthy individuals (originally designated as community controls) from a TB-endemic region to immune responses from TB patients who have, by definition, failed to control their infection. We have divided the community controls into healthy latently infected, and uninfected based on ESAT-6 responses. Our data demonstrate that TB patients have a down-regulated expression of the Th1 cytokines (IFN-γ, IL-12) compared with both healthy infected and uninfected individuals. Interestingly, high levels of expression of the IL-4 antagonist IL-4δ2 (11, 12) are essentially limited to healthy

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; TB, tuberculosis; ESAT-6, early secretory antigenic target 6; PPD, purified protein derivative.

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infected individuals. To confirm these results, healthy individuals from a TB-nonendemic region were analyzed using the same approach and very similar results were obtained. This suggests that long-term control with TB in its latent stage is associated with not only increased expression of Th1 cytokines, but with suppression of the activity of IL-4, the prototypical Th2 cytokine.

Materials and Methods

Study design

The Ethiopian cohort analyzed was extracted from an ongoing, multicenter longitudinal study being conducted in Africa (VACSEL). Sputum-positive TB patients ($n = 30$) were recruited from TB clinics in the rural towns of Hossana and Butajira, Ethiopia. Community controls ($n = 17$) were randomly selected and recent TB contact excluded by questionnaire. Active TB was excluded in all healthy participants on entry to the study by radiological and clinical examination, sputum microscopy, and culture as previously described (9). Blood samples were obtained from all donors at entry to the study. All participants were screened for HIV and samples from HIV-positive individuals were excluded from the study. Pre- and posttest counseling was offered to all participants. The low incidence of TB and low prevalence of ESAT-6 reactivity in Denmark precluded the possibility of random sampling of community controls. Instead, the Danish cohort ($n = 13$) was recruited from laboratory workers and attendees at a local respiratory diseases clinic who had risk factors for TB, but no identified exposure to index cases. Since the Danish cohort was recruited as controls for the healthy Ethiopian subjects, no TB patients were recruited in Denmark. Only adults (18 years or older) who had given informed consent were included in the study and this work was performed under study protocols approved by the institutional and national ethical review boards.

IFN-γ ELISA

In vitro restimulation of PBMC with either purified protein derivative (PPD; 20 μg/ml) or ESAT-6 (2 μg/ml) was conducted as previously described (9). The supernatants were harvested at day 4 after stimulation and the levels of IFN-γ were assayed in duplicate culture supernatants using a commercial double sandwich ELISA kit in accordance with the manufacturer's instructions (Mabtech, Nacka, Sweden). The cutoff point (275 pg/ml) was based on the mean response of unstimulated well for the whole cohort plus 3 SDs, thus giving a very high probability that any response above this level was Ag specific. ROC analysis using a large PPD- and ESAT-6-negative Danish cohort as the negative population gave a very similar cutoff (292 pg/ml) and essentially identical results. We have therefore used the Ethiopian results for these analyses. The difference between the duplicate wells was consistently <10% of the mean.

Quantitative RT-PCR

Unstimulated PBMC were lysed immediately after drawing of blood with the Paxgene or RNEasy Blood RNA system (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The mRNA was transcribed into cDNA using the Omniscript reverse transcription kit (Qiagen) with oligo(dT) primers according to the manufacturer's instructions; the concentration was calculated from the OD using a GeneQuant spectrophotometer (Amersham Biosciences, Amersham, U.K.) and stored at −20°C until use. PCR was conducted in a total volume of 50 μl with 1 μg of cDNA using the HotStarTaq Master Mix kit (Qiagen) according to the manufacturer's instructions. Contamination from genomic DNA was tested for by comparing the results from PCR of RNA and the cDNA that was prepared from it. A negative (no template) control was also included in all PCR assays to test for contamination of reagents. PCR products were visualized by running on 1% agarose (Nusieve; FMC, Rockland, ME) gels containing SBYRgreen (Molecular Probes, Eugene, OR) at 1:10,000 (5 μl in a 50-ml gel) and normalized against the housekeeping gene β-actin, quantitated against standard curves using the same primers but based on standardized samples containing known copy numbers of cDNA, as previously described (10). The PCR conditions and primers used were β-actin forward, 5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3′; β-actin reverse, 5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′; IFN-γ forward, 5′-GGC TGT TAC TGC CAG GAC CCA TAT GT-3′; IFN-γ reverse, 5′-GAT GCT CTT CGA CCT CGA AAC AGC AT-3′; IL-4 forward, 5′-TGT GCT CCG GCA GTT CTA CAG-3′; IL-4 reverse, 5′-TGG CTT CCT TCA CAG GAC AGG-3′; IL-4δ2 forward, 5′-CAGAGCAGAAGAACACAACTG-3′; IL-4δ2 reverse, 5′-GTCTT TAGCCTTTCCAAGAAG-3′; IL-10 forward, 5′-ATG CCC CAA GCT GAG AAC CA-3′; IL-10 reverse, 5′-TCT CAA GGG GCT GGG TCA GC-3′; IL-12 forward, 5′-TCACAAAGG AGGCGAGGTT CTA-3′; and IL-12 reverse, 5′-CATGACCTCAATGGGCAGACTC-3′. The number of cycles was optimized for each cytokine (between 30 and 35), with an initial melting step of 15 min at 95°C followed by cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. The fluorescence of bands in the gel under UV transillumination was read using a 12-bit charge-coupled device camera (Sensicam; UVP, San Gabriel, CA) and the data were analyzed using the supplied Labworks software (UVP, San Gabriel, CA).

Statistical analysis

Data are presented as both means plus SDs and as medians and quartiles. Since ANOVA showed wide differences, comparisons between groups were assessed by the Kruskal-Wallis test. The Mann-Whitney *U* test was used for analyses within groups. In all instances, a $p < 0.05$ was considered to be significant.

Results

Strong ESAT-6 reactivity identifies latently infected healthy individuals

Previous studies from developing countries have shown the presence of strongly ESAT-6-positive individuals among community controls at percentages up to 30% (13). This confirms the theoretical assumption that a large proportion of the population in TB-endemic areas is latently infected with *M. tuberculosis* (13, 14). However, as the overall rate of breakdown with TB in individuals with no identified TB exposure in the Hossana region is <500/100,000 (0.05%) per year, it is likely that the vast majority of these ESAT-6-positive individuals control the infection, keeping it in the latent stage by the induction of a protective immune response. To identify these latently infected healthy individuals, we analyzed IFN-γ responses by ELISA of culture supernatants after restimulation of purified PBMC. PBMC were restimulated 4 days in vitro with either PPD, which contains many Ags shared among the mycobacteria, or with ESAT-6, which is largely restricted to members of the TB complex. A positive reaction was defined as >3 SDs above the mean of unstimulated cultures (275 pg/ml, indicated as a dotted line in Fig. 1). This allowed us to distinguish an Ag-specific reaction from background, in particular, the low level of IFN-γ secreted by cells from almost all individuals in developing countries without exogenous stimulation. Confirming earlier reports (9), a bimodal distribution of ESAT-6 responses was found in the Ethiopian cohort ($n = 17$, Fig. 1*A*) with a group of individuals ($n = 6$, ~30%) characterized by very high ESAT-6 responses (mean = 1052 pg/ml ± 714), whereas the rest showed low responses (mean = 210 pg/ml ± 10). Since ESAT-6 reactivity is the best marker of latent infection available (8, 14), we used this to identify latently infected individuals for subsequent cytokine analysis (medians and quartiles of the Ag responses are shown in Fig. 1*A*). In contrast, while PPD responses were elevated in the latently infected Ethiopian group (Fig. 1*A*), there remained significant overlap between the ESAT-6-positive and -negative groups with respect to PPD responses (for all individuals, mean = 775 pg/ml ± 1156). A similar pattern was seen with the Danish group ($n = 13$). Using the same approach, five individuals were identified as latently infected on the basis of their ESAT-6 response (mean for the positive group was 614 pg/ml ± 367 compared with 129 ± 87 for the negative group). As for the Ethiopian donors, their PPD responses showed a great degree of overlap between the ESAT-positive and -negative groups, with the mean PPD response for both groups being almost identical (6592 ± 872 vs 6344 ± 2410, respectively).

RT-PCR cytokine analysis differentiates uninfected individuals, latent TB, and active TB

Based on their ESAT-6 responses, the healthy individuals were segregated into noninfected (healthy, no record of TB exposure, ESAT-6 responses ≤274 pg/ml), and latently infected subjects

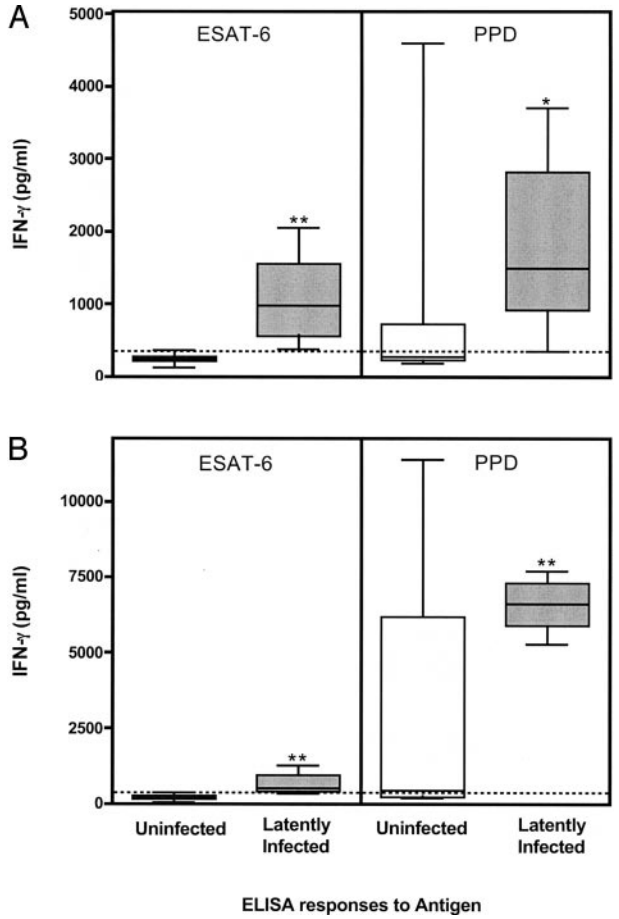

FIGURE 1. In vitro IFN-γ responses to restimulation with PPD or ESAT-6 of PBMC from uninfected ($n = 11$) or latently infected ($n = 6$) healthy individuals from Ethiopia (*A*) or Denmark ($n = 8$ and 5, respectively) (*B*) as assessed by ELISA. The cutoff point of the assay (based on mean ± 3 SDs of unstimulated responses) is indicated by the dotted line. Results are median responses plus 25th and 75th quartiles (boxes) with the range indicated by the single lines. Median levels of cytokine that were significantly different between groups are indicated (* = $p < 0.05$, ** = $p < 0.01$).

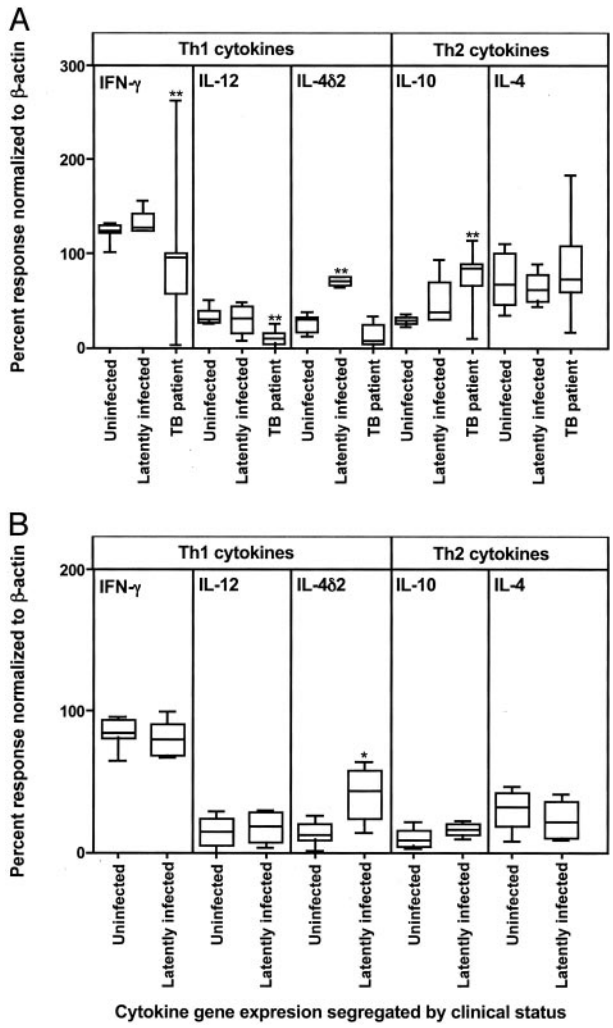

FIGURE 2. Comparison of cytokine message from unstimulated PBMC of uninfected ($n = 11$) or latently infected ($n = 6$) healthy individuals and TB patients ($n = 30$) from Ethiopia (*A*) or uninfected ($n = 8$) or latently infected ($n = 5$) healthy individuals from Denmark (*B*). Results are medians and quartiles (25th and 75th quartiles represented by boxes with the full range indicated by the single lines) of cytokine message normalized against β-actin and expressed as a percentage of the housekeeping gene. Results significantly different between the groups are indicated (* = $p < 0.05$, ** = $p < 0.01$).

(healthy, no record of TB exposure, but with ESAT-6 responses ≥276, or significantly above background) for cytokine analysis. Although ELISA has proven to be a reliable method for looking at IFN-γ responses, it is not sufficiently sensitive for analysis of all cytokines (Refs. 15 and 16 and T. M. Doherty, A. Demissier, D. Menzier, P. Andersen, G. Rook, A. Zumla, and the VACSEL Study Group, manuscript in preparation). We therefore used semiquantitative RT-PCR to analyze expression of genes for a panel of relevant cytokines. Cytokine levels were measured directly after lysis of PBMC to give a picture of the responses ex vivo. We also compared immune responses in TB patients to those of healthy latently infected or uninfected individuals. The Ethiopian TB patients had decreased levels of the Th1 cytokines IFN-γ and IL-12 compared with the healthy Ethiopian subjects, regardless of whether the latter were designated latently infected or uninfected (Fig. 2*A*). This tendency was significant for both IFN-γ ($p = 0.0015$) and IL-12 ($p < 0.001$). Consistent with this, the message for the immunosuppressive cytokine IL-10 was increased in the TB patient group ($p = 0.002$) relative to the healthy subjects (Fig. 2*A*). Baseline levels of IL-4 the message were not different between the groups, but interestingly, the healthy infected subjects exhibited a selective increase of message for the IL-4 antagonist, IL-4δ2, compared with TB patients or noninfected controls ($p < 0.001$).

It has been suggested that progress to clinical TB is associated with a Th1 to Th2 shift (16–18) and while this is consistent with the data presented for IFN-γ and IL-12, the similar level of IL-4 message in the different groups would argue against such an interpretation. However, the levels of IL-4 message seen in the Ethiopian participants were relatively high in all groups when compared with previous data from studies conducted in TB-nonendemic populations. We therefore analyzed cytokine mRNA levels in a cohort of healthy individuals from Denmark ($n = 13$, Fig. 2*B*). Since the results from Ethiopia were already known, this cohort was analyzed in a blinded fashion, with all samples number coded and only matched to clinical records at the study's conclusion. In general, relative levels of all cytokines tested in the Danish cohort were lower than in their Ethiopian counterparts, but this was only significant for IL-4 and IL-10 ($p < 0.001$), suggesting that these two cytokines may be elevated in

the Ethiopian population due to factors other than latent *M. tuberculosis* infection. Importantly, expression of IL-4δ2 was once again significantly elevated in the ESAT-6-positive or latently infected group ($n = 5$, $p < 0.05$).

Elevated levels of the IL-4 antagonist IL-4δ2 were only found when the healthy subjects were segregated on the basis of ESAT-6 reactivity in the ELISA and no differences were seen when the healthy individuals were reanalyzed using positivity to the common mycobacterial Ags PPD (data not shown) or Ag85A (Fig. 3). Thus, these data suggest that the alterations in IL-4δ2 message in this study are specifically associated with strong ESAT-6 reactivity in healthy individuals and therefore with latent *M. tuberculosis* infection.

Discussion

In the design of an effective TB vaccine, understanding what constitutes a protective immune response has long been one of the major stumbling blocks. Given the clear importance of IFN-γ in the control of *M. tuberculosis* infection, the hypothesis that there is a change in the Th1/Th2 balance during the development of disease or protective immunity has been an attractive one. Unfortunately, data to suggest such a change have been difficult to find. There are two reasons for this. The first is purely technical. Although the fact that IFN-γ levels decrease in TB patients as the disease progresses has been well documented (9, 13), measuring the quintessential Th2 cytokine IL-4 has proven more difficult. This is because IL-4 is labile, highly bioactive, and therefore generally present in relatively low quantities, making its measurement problematic (10, 15). This limitation has been overcome by two approaches: either the use of proxy markers for IL-4 such as IgE or soluble CD30 (17) or the use of more sensitive techniques (16). Both approaches support the notion that in TB patients the Th1/Th2 bias shifts toward a more marked Th2 response (16–18). However, the second obstacle that has blocked progress on defining the nature of the protective response has been the problem of identifying individuals who are protected; in short, those who are infected with *M. tuberculosis*, but control infection at the subclinical stage. This obstacle is now also yielding to improved technology as identification of Ags largely confined to the members of the TB complex allows highly specific immunodiagnosis of infection (7). The ESAT-6 protein is a mycobacterial virulence factor which is absent in all strains of BCG, and an immune response to this Ag correlates strongly with exposure to or infection with, *M. tuberculosis*, even in asymptomatic individuals (8, 9).

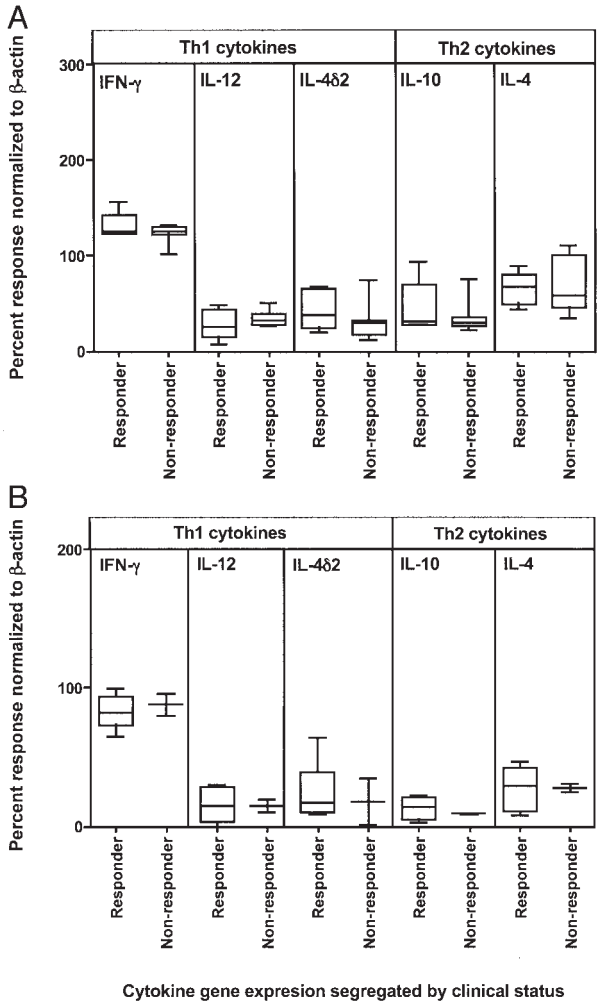

FIGURE 3. Comparison of cytokine message from unstimulated PBMC of healthy individuals ($n = 17$) from Ethiopia (*A*) or healthy individuals from Denmark ($n = 13$, *B*) segregated on the basis of reactivity to the common mycobacterial Ag Ag85A. Results are medians and quartiles (25th and 75th quartiles represented by boxes with the full range indicated by the single lines) of cytokine message normalized against β-actin and expressed as a percentage of the housekeeping gene.

It has long been suspected that many healthy individuals in TB-endemic regions are latently infected with *M. tuberculosis* based on epidemiology and the knowledge that only a small percentage of exposed (and presumably infected) individuals develop TB. That many such individuals harbor latent infections is attested to by the reactivation of disease years or decades after exposure (19), while the fact that latency is an active process, maintained by protective immunity, is shown by the high reactivation rate in HIV-infected individuals (20). ESAT-6 responses can persist years or decades after infection (21). We have thus used immune responses to ESAT-6 to identify healthy individuals who are, or have been, infected with *M. tuberculosis*. This analysis was paired with the highly sensitive quantitative RT-PCR to analyze immune responses, In this study, we have examined immune responses in latently infected subjects and compared them with individuals from the same communities who lack evidence of latent TB infection or with individuals who have developed TB and who, therefore, by definition have not made a protective immune response.

The results show clearly that when comparing overall immune responses by RT-PCR that healthy individuals are strongly biased toward the production of Th1 cytokines, compared with TB patients. This is consistent with data reported by many groups that in vitro Ag and mitogen-specific IFN-γ responses are decreased in African patients with advanced TB (9, 13, 22). The fact that immune responsiveness recovers in most patients during therapy suggests that this unresponsive state is directly related to TB (23–25). Interestingly, in this study, expression of the gene for the immunosuppressive cytokine IL-10 was significantly higher in TB patients than in community controls, consistent with earlier reports (26) and suggesting a possible mechanism for the decreased Th1 response.

However, although the median response is lower in healthy individuals, levels of message for IL-4 were not significantly different between the Ethiopian study groups. This contrasts with data from a smaller study performed in the U.K (18) where TB patients were found to have elevated levels of message for IL-4, but is consistent with reports of elevated IL-4 expression after helminth infection. Frequent, persistent helminth infection is a very common occurrence in this study population and has been shown to down-regulate immune responses to mycobacterial Ags (27, 28).

Consistent with this hypothesis, significantly lower levels of IL-4 mRNA were found in subjects from a region (Denmark) where helminth infections are rare ($p < 0.001$). It is therefore particularly interesting to see that message for the IL-4 antagonist IL-4δ2 is elevated in the latently infected group (regardless of country of residence), but not in TB patients or uninfected individuals. IL-4δ2 is a recently described splice variant of IL-4 that binds to the IL-4Rα chain of the IL-4R but has little if any activity and which can thus inhibit IL-4 activity (11, 12). Elevated levels of message for this cytokine are found in tissue from humans infected with pathogenic *Helicobacter pylori* (29), which induce a strong Th1 response but not in those infected with nonpathogenic strains where IL-4 is more dominant, suggesting that in *H. pylori* infection elevated message for IL-4δ2 is also associated with an elevated Th1 response.

These data suggest a new hypothesis: that maintenance of a prolonged Th1 response against *M. tuberculosis* requires not just elevation of IFN-γ, but also down-modulation of the Th2 response, specifically IL-4. IL-4 is not, of course, the only Th2 cytokine. In this regard, it is interesting to note a recent publication showing that modulation of the Th2 cytokine IL-13 by its natural antagonist IL-13Rα2 is also an important factor in the control of pathology during chronic helminth infections (30). Both the IL-4/IL-4δ2 and the IL-13/IL-13Rα2 interactions appear to modulate the development of fibrosis (11, 30), an important aspect of granulomatous pathology in both schistosomiasis and TB. This suggests that negative or inhibitory regulation of Th2-promoting cytokines may be as important as the positive feedback loops driven by IL-4 or IFN-γ/IL-12 in the immunopathology of chronic infections. Consistent with this hypothesis, IL-4, but not IL-4δ2, was elevated in atopic asthmatic patients whose immune dysfunction is associated with elevated Th2 responses, while early-stage TB patients who would be predicted to have a mixed Th1/Th2 response were found to be expressing both IL-4 and IL-4δ2 (31).

Most interesting of all, in this study, IL-4δ2 message in these subjects was only significantly elevated in healthy individuals who were strongly responsive to ESAT-6. This correlation appears to be specific for ESAT-6 recognition (and therefore, in this case, presumably latent TB infection), since separating healthy individuals on the basis of reactivity to the nonspecific Ag PPD or Ag85A or the mitogen PHA did not differentiate between the groups (whether for IL-4δ2 or any other cytokine).

Thus, the data demonstrate that presumptive protective responses against *M. tuberculosis* are associated with an elevated expression of an inhibitor for the Th2 cytokine IL-4, whereas disease is characterized by decreased expression of the Th1 cytokines IFN-γ and IL-12 and increased expression of the immunosuppressive cytokine IL-10. This is true of both the Ethiopian study population and the Danish cohort (which was performed blinded). Although both of these study groups were small, the conclusion is further strengthened by the fact that a parallel arm of the VACSEL Study in The Gambia using a slightly different methodology (ELISPOT and real-time quantitative PCR) and a slightly larger cohort has independently reached the same conclusion: that IL-4δ2 is specifically up-regulated in ESAT-6-positive healthy individuals.[4] Of course, these data do not prove that the increased Th2 response seen in TB patients is the cause of their TB. It is possible that their Th1 response is down-regulated in an attempt to limit pathology caused by rampant bacterial infection. However, these results also imply that long-term protection against TB requires inhibition of IL-4, arguing for a causal role of the Th1 to Th2 shift in TB. This study thus not only provides the first detailed comparison of cytokine responses between presumptively protected and nonprotected individuals, but points the way forward for further studies in this area.

[4] H. A. Fletcher, P. Owiafe, D. Jeffries, P. Hill, G. Rook, A. Zumla, T. M. Doherty, R. Brookes, and the VACSEL Study Group. Increased background expression of mRNA encoding IL-4 and its splice variant IL-4δ2 in contacts of *Mycobacterium tuberculosis*. *Submitted for publication*.

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