Differential Expression of Interleukin-4 (IL-4) and IL-482 mRNA, but Not Transforming Growth Factor Beta (TGF-β), TGF-βRII, Foxp3, Gamma Interferon, T-bet, or GATA-3 mRNA, in Patients with Fast and Slow Responses to Antituberculosis Treatment\textsuperscript{7}

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This study investigated interleukin-4 (IL-4), IL-482, transforming growth factor beta (TGF-β), TGF-βRII, Foxp3, GATA-3, T-bet, and gamma interferon (IFN-γ) transcription in peripheral blood samples of adult pulmonary tuberculosis patients prior to and after 1 week of therapy. Twenty patients with positive results for sputum culture for Mycobacterium tuberculosis were enrolled and treated with directly observed short-course antituberculosis chemotherapy. Early treatment response was assessed. At the end of the intensive phase of treatment (month 2), 12 patients remained sputum culture positive (slow responders) and 8 converted to a negative culture (fast responders). Only the expression levels of IL-4 (4-fold decrease) and IL-482 (32-fold increase) changed significantly during the first week of therapy in the 20 patients. No baseline differences were present between the responder groups, but fast responders had significantly higher IL-4 transcripts than slow responders at week 1. Fast responders showed a 19-fold upregulation and slow responders a 47-fold upregulation of IL-482 at week 1. Only slow responders also showed a significant decrease in IL-4 expression at week 1. There were no significant differences in expression of TGF-β, TGF-βRII, Foxp3, IFN-γ, and GATA-3 between the groups. These data show that differential IL-4-related gene expression in the early stage of antituberculosis treatment accompanies differential treatment responses and may hold promise as a marker for treatment effect.

With more than 8 million people progressing to active tuberculosis (TB) every year and a death rate of about 25%, pulmonary TB is one of the most life-threatening human diseases (33). Although the majority of compliant patients are cured after conventional 6-month therapy, about 5% subsequently relapse, mostly within 2 years after completion of treatment. Currently, there is no reliable early predictor of relapse, although failure of sputum smear conversion after the 2-month intensive phase of anti-TB treatment (7, 34) and extent of pulmonary disease on chest radiography (1, 7, 18, 29) have been shown to be risk factors for relapse. Early identification of patients at risk for poor response to treatment may allow closer monitoring during and after treatment or intensified treatment regimens. Additionally, early markers of treatment effect may aid new TB drug discovery by shortening clinical trials.

Gene expression patterns in different clinical phenotypes may increase our understanding of disease processes. Mistry et al. (21) showed that host gene expression patterns can discriminate between active, latent, recurrent, and cured TB. Importantly, discrimination between recurrent and cured TB could be established at a time when both participant groups were not actively diseased. Furthermore, an investigation into adjunc-
tively transforming growth factor beta (TGF-β) and TGF-βRII, implicated in immune suppression and ineffective antimycobacterial responses (2, 9, 11); Foxp3, a transcription factor expressed by regulatory T cells (14, 23, 24); GATA-3, a Th2 cell-specific transcription factor that controls Th2-specific cytokine expression and functions as a negative regulator of Th1 cells independently of its ability to upregulate
samples were decontaminated using the Bactec 12B liquid radiometric method (Becton-Dickinson, Bethesda, MD). Rigs-
response groups. followed by rifampin and isoniazid during the 4-month continuation phase.

namide (1,000 mg/day to 1,250 mg/day) during the 2-month intensive phase, fol-
lowed by (480 mg/day to 600 mg/day), ethambutol (800 mg/day to 1,200 mg/day), and pyrazi-

weight-related, fixed combination of isoniazid (320 mg/day to 400 mg/day), rifampin 

served short-course anti-TB therapy as recommended by the South African National 

The patients received 6 months (26 weeks) of directly ob-

M. tuberculosis (20); and IFN-γ, a key 
ocytokine in immunity to TB, the nature of whose changes 
during treatment has been associated with disease activity and 
treatment outcome (19, 26). Our investigation showed that 

between patients with fast and slow responses to anti-TB treat-

Quantitative real-time PCR. RNA was isolated from ex vivo blood samples by 

was done according to the manufacturer's instructions. The extracted mRNA was 

and third days of treatment and at weeks 1, 2, 4, 8, 13, and 26 after the initiation 

microscopy was performed using the Ziehl-Neelsen method.

Sputum smear Ziehl-Neelsen stains and Bactec cultures were done on the first 

Sample handling. Five milliliters of freshly drawn blood (at pretreatment and 

week 1) was added to 10 volumes of guanidinium isothiocyanate and triton 
stabilization reagent (Roche kit) to achieve cell lysis and stabilization of the 
nucleic acids. Nucleic acid-stabilized blood samples were stored at –80°C until the 

extraction of mRNA.

Th2-type cytokines (22, 35, 36); T-bet (T-box 21), a Th1-spe-
icific transcription factor that plays a key function in Th1 de-
velopement, polarization, gamma interferon (IFN-γ) gene 
transactivation, and promotion of delayed type 1 hypersensi-
tivity responses needed for protection against intracellular pathogens, such as M. tuberculosis (20); and IFN-γ, a key 
cytokine in immunity to TB, the nature of whose changes 
during treatment has been associated with disease activity and 
treatment outcome (19, 26). Our investigation showed that 

there were differences in transcription of IL-4 and IL-4 
treatment has been associated with disease activity and 
treatment outcome (19, 26). Our investigation showed that 

between patients with fast and slow responses to anti-TB treatment.

MATERIALS AND METHODS

Patients. Twenty human immunodeficiency virus (HIV)-negative, first-episode, 
active TB patients infected with drug-sensitive M. tuberculosis strains (11 males and 
9 females, aged 18 to 51 years) and having positive results for sputum smear and 
culture at diagnosis were recruited and followed up for the duration of therapy. After 
the intensive phase of chemotherapy (2 months), eight fast responders and 12 slow 
responders were identified according to Bactec sputum culture results. 

Treatment protocol. The patients received 6 months (26 weeks) of directly ob-
served short-course anti-TB therapy as recommended by the South African National 
Tuberculosis Program based on WHO guidelines. The drug regimen consisted of a 
weight-related, fixed combination of isoniazid (320 mg/day to 400 mg/day), rifampin 
(480 mg/day to 600 mg/day), ethambutol (800 mg/day to 1,200 mg/day), and pyrazi-
namide (1,000 mg/day to 1,250 mg/day) during the 2-month intensive phase, fol-
lowed by rifampin and isoniazid during the 4-month continuation phase.

Monitoring of bacteriology and classification of patients into treatment re-
sponse groups. Mycobacterial sputum culture was done using the automated 
Bactec 12B liquid radiometric method (Becton-Dickinson, Bethesda, MD). Rig-
orous measures were used to prevent cross contamination (5, 6). The sputum 
samples were decontaminated using the N-acetyl-L-cysteine-sodium hydroxide 
(NALC-NaOH) method (4). Bactec 12B vials were incubated at 37°C, and the 
growth indexes were read daily at identical times to limit reading variability. 
Cultures were monitored for 60 days before being classified as negative. Myco-
bacterial drug susceptibility testing for resistance to first- and second-line drugs 
was done using the Bactec method (see Bactec 460TB systems product and 
procedure manual, 1996; Becton-Dickinson Diagnostic Instrument Systems, 
Sparks, MD) at diagnosis and at the end of therapy. Direct sputum smear 
microscopy was performed using the Ziehl-Neelsen method.

Sputum smear Ziehl-Neelsen stains and Bactec cultures were done on the first 
and third days of treatment and at weeks 1, 2, 4, 8, 13, and 26 after the initiation 
of chemotherapy. Patients were classified into groups with fast or slow responses 
to chemotherapy on the basis of their Bactec culture statuses (positive or neg-
ative) at week 8 (month 2) after being started on treatment.

Sample handling. Five milliliters of freshly drawn blood (at pretreatment and 
week 1) was added to 10 volumes of guanidinium isothiocyanate and triton 
stabilization reagent (Roche kit) to achieve cell lysis and stabilization of the 
nucleic acids. Nucleic acid-stabilized blood samples were stored at –80°C until the 

extraction of mRNA.

TABLE 1. Sequences of primers used for amplification of target and housekeeping genesa

| Genes and gene product or primer name | Primer sequence | RefSeq identification no. |
|--------------------------------------|----------------|--------------------------|
| **Forward**                          | **Reverse**   |                          |
| Target genes                         |               |                          |
| IL-4a                                | 5'-CGT CTT TAC CCA GAA GAG-3' | NM_000589, NM_172348     |
| IL-42                                | 5'-GTC TTT AGC CTT AAC AAG-3' | NM_000660                |
| TGF-β                                | 5'-MIT TGG TGC TGC TGG GAG-3' | NM_0003242               |
| TGF-βRII                             | 5'-CAT GTA GGC CAT GAG GTC CAC-3' | NM_014009               |
| FosP3                                | 5'-CTT CTT TAC CCA GAA GAG-3' | NM_001002295            |
| GATA-3                               | 5'-GTC TTT AGC CTT AAC AAG-3' | NM_002051                |
| T-bet                                | 5'-GTC TTT TAC CCA GAA GAG-3' | NM_013351                |
| IFN-γ                                | 5'-GTC TTT TAC CCA GAA GAG-3' | NM_000619                |
| **Housekeeping genes**               |               |                          |
| hGAPDH                               | 5'-ACC CAC TCC TCC ACC TTT GAG-3' | NM_001102               |
| hG3PDH                               | 5'-CAT TGG TGC TGC TGG GAG-3' | NM_0003242               |
| hPRT1                                | 5'-CTT CTT TAC CCA GAA GAG-3' | NM_0003242               |
| huPO                                 | 5'-GTC TTT TAC CCA GAA GAG-3' | NM_0003242               |
| **RefSeq**                           |               |                          |

a The RefSeq codes of the target genes are shown where the primer sequences were not disclosed by the manufacturers. huPO, human ribosomal protein.

b The primers used amplified both IL-4 and IL-42.
the groups were analyzed using the Mann-Whitney U test. According to the manufacturer’s recommendation, the differences between kit from Millipore. The reagent preparation and the assay protocol were in plasma samples was done with the Bio-Plex bead array system (Bio-Rad Laboratories). The primers used amplified both IL-4 and IL-4 regulated, whereas there was a marked and significant increase in IL-4 mRNA expression (P = 0.0001) after initiation of treatment (Table 2). Thus, the changes in IL-4 mRNA transcripts observed in patients between diagnosis and week 1 when nonspecific primers were used was driven by IL-4 mRNA expression. Together, this resulted in a decrease of the IL-4/IL-4 ratio from 802 at pretreatment to 1.6 after 1 week of therapy. Together, these changes may be due to a drop in therapy-induced bacterial load, with a decreased need for ongoing immune activation by the host, leading to immune regulation.

No significant changes were observed in the regulation of TGF-β, TGF-βRII, Foxp3, and GATA-3 mRNA expression from pretreatment to week 1 of therapy (Table 2). The week 1 time point might be too early for significant changes in TGF-β, TGF-βRII, Foxp3, and GATA-3 in response to anti-TB treatment to be appreciated, and future investigation of these parameters should include later time points.

Comparison of IL-4, IL-4, IL-4, IL-4, IL-4, and IL-4 mRNA expression levels in patients with fast and slow responses to anti-TB treatment. (i) IL-4 mRNA expression determined using primers that amplify both IL-4 and IL-4 reverse transcripts. Pretreatment and week 1 IL-4 mRNA expression levels showed no statistically significant differences between fast and slow responders (Table 3). However, the changes in IL-4 mRNA transcription in the fast responders between pretreatment and week 1 were significant and upregulated by a factor of 7.68, whereas no significant changes were observed in the slow responders between these time points (Table 4).

(ii) IL-4 mRNA and IL-4 mRNA expression determined using IL-4 and IL-4-specific primers. IL-4 and IL-4 mRNA expression levels were not significantly different for fast and slow responders at pretreatment. Direct comparison of the levels of IL-4 mRNA expression at week 1 showed significantly higher levels in fast responders (P = 0.038), whereas no significant differences in levels of IL-4 transcripts were observed between the responder groups (Table 3).

The analysis of IL-4 and IL-4 expression between pretreatment and week 1 for the two response groups showed that IL-4 mRNA transcription was significantly downregulated by a factor of 0.06 (P = 0.002) in the slow responders at pretreatment compared to the level at week 1 of therapy, whereas IL-4 mRNA transcription was significantly upregulated by a

### TABLE 2. Changes in mRNA expression from pretreatment to week 1 of therapy for all patients

| Analyte   | Mean fold change in expression factor at wk 1 | SE       | P   |
|-----------|---------------------------------------------|----------|-----|
| IL-4**  | 2.77                                        | 0.5–17.95| 0.038|
| IL-4      | 0.26                                        | 0.047–1.93| 0.006|
| IL-4      | 32.19                                       | 1.87–631.5| 0.0001|
| TGF-β     | 1.09                                        | 0.1–11.93| 0.7  |
| TGF-βRII  | 1.3                                         | 0.087–21.42| 0.54 |
| Foxp3     | 2.6                                         | 0.29–27.05| 0.093|
| GATA-3    | 1.94                                        | 0.24–16.58| 0.188|
| T-bet     | 2.047                                       | 0.109–40.55| 0.214|
| IFN-γ     | 2.48                                        | 0.304–17.49| 0.065|

*The expression factors represent the transcript expression level at week 1 compared to that at the pretreatment time point. Statistically significant P values are shown in bold.

The primers used amplified both IL-4 and IL-4.

RESULTS AND DISCUSSION

Changes in IL-4, IL-4, TGF-β, TGF-βRII, Foxp3, and GATA-3 expression in TB patients from pretreatment to week 1 after initiation of treatment. When primers that amplified both IL-4 (variant 1) and IL-4 (variant 2) mRNA reverse transcripts were used, IL-4 mRNA transcription was found to be moderately but statistically significantly upregulated (P = 0.038) after 1 week of treatment compared to pretreatment levels (Table 2). The use of IL-4 and IL-4-specific primers showed that IL-4 mRNA expression was significantly downregulated, whereas there was a marked and significant increase in IL-4 mRNA expression (P = 0.0001) after initiation of treatment (Table 2). Thus, the changes in IL-4 mRNA transcripts observed in patients between diagnosis and week 1

### TABLE 3. Differential mRNA expression levels for patients with fast and slow responses to TB treatment at pretreatment and 1 week after initiation of therapy

| Analyte   | Diagnosis time point | Wk 1 |
|-----------|----------------------|------|
|           | Mean FR/ SR expression factor (fold) | SE    | P     | Mean FR/ SR expression factor (fold) | SE    | P     |
| IL-4**  | 0.396                | 0.038–3.37 | 0.23 | 2.12 | 0.82–4.83 | 0.06 |
| IL-4     | 6.7                  | 0.05–4,400 | 0.24 | 1.75 | 0.9–2.94 | 0.038|
| IL-4      | 0.68                | 0.029–8.9 | 0.45 | 2.13 | 0.58–8.3 | 0.083|
| TGF-β     | 1                   | 0.12–6.83 | 0.793| 2.58 | 0.139–26 | 0.22 |
| TGF-βRII  | 1.350               | 0.13–13.48| 0.545| 3.4  | 0.21–71.42| 0.2  |
| Foxp3     | 0.892               | 0.088–8.17| 0.880| 2.7  | 0.5–15.17| 0.17 |
| GATA-3    | 1.387               | 0.178–12.3| 0.520| 3.84 | 0.75–29.92| 0.06 |
| T-bet     | 2.7                 | 0.222–45.334| 0.214| 5    | 0.307–82.680| 0.09 |
| IFN-γ     | 3.3                 | 0.241–46.181| 0.223| 2.16 | 0.430–11.724| 0.18 |

*Transcript expression factors for fast responders compared to those for slow responders are shown. For example, an expression factor of x indicates that the transcript level of the associated analyte in fast responders was x times higher or lower than that in slow responders. Values above 1 indicate increased expression, and values below 1 indicate decreased expression. FR, fast responder; SR, slow responder. Statistically significant P values are shown in bold.

The primers used amplified both IL-4 and IL-4.
factor of 47.3 (P = 0.0001) (Table 4). The simultaneous down-regulation of IL-4 mRNA and upregulation of IL-4/IL-42 transcripts in the slow responders may explain why primers that amplified both isoforms of IL-4 showed no significant changes over the two time points. In the fast responders, no significant changes in IL-4 transcription were observed between diagnosis and week 1. However, IL-4 transcription was significantly upregulated by a factor of 18.93 (P = 0.02) after initiation of treatment, possibly explaining the changes seen in the nonspecific IL-4 assay. Dheda et al. have also reported unchanged IL-4 expression during anti-TB treatment in HIV-infected and HIV-TB-coinfected patients, while IL-42 levels increased significantly (12).

The upregulation of IL-42 resulted in decreases of IL-4/IL-42 ratio from 176 to 1.1 in fast responders and from 212 to 2.96 in slow responders from pretreatment to week 1 of therapy, although these differences were not statistically significant.

It is not clear if the changes in expression among the different IL-4 isoforms observed here are responsible for or merely consequential to differences in rates of bacterial clearance. Increased intracellular IL-4 as measured by flow cytometry in fast responders was previously reported by our group for the same participants and was associated with increased apoptosis (32). In that paper, it was argued that IL-4 might be secreted by cells as an autocrine/paracrine growth factor and that secretion might be inhibited at the onset of apoptosis, leading to accumulation of intracellular IL-4. However, the present data show that there are indeed also increases in transcription of IL-42 and subsequently total IL-4 mRNA in the TB patients early during treatment.

Although IL-4 may play some role in the defense against mycobacterial infection (27), the dramatic upregulation of its inhibiting isoform during the first week of treatment in both responder groups and the drop in expression of active IL-4 in the TB group as a whole suggest that IL-4 inhibition is an important treatment effect. Th2-mediated responses might represent an inadequate immune response against the background of overwhelming bacterial infection, and subsequent chemotherapy-induced reduction of the bacterial burden might allow some restoration of the immune system. The reason for the more pronounced decrease in IL-4 mRNA expression in slow responders than in fast responders from pretreatment to week 1 does not support this reasoning. This might suggest that baseline differences in extent of disease and cytokine expression levels that might not be detectable in such a small group of participants contribute to the differences seen in responder phenotypes after the initiation of therapy.

Table 4: mRNA expression changes from pretreatment to week 1 of therapy in slow and fast respondersa

| Analyte  | Mean FR/ SR expression factor (fold) | SE   | P   | Mean FR/ SR expression factor (fold) | SE   | P   |
|---------|-------------------------------------|------|-----|-------------------------------------|------|-----|
| IL-4b   | 7.68                                | 1.5–36.96 | 0.018 | 7.68                                | 1.5–36.96 | 0.018 |
| IL-4    | 0.71                                | 0.11–7.13 | 0.69  | 0.71                                | 0.11–7.13 | 0.69  |
| IL-462  | 18.93                               | 0.6–488.3 | 0.02  | 18.93                               | 0.6–488.3 | 0.02  |
| TGF-β   | 2                                   | 0.091–22.5 | 0.4  | 2                                   | 0.091–22.5 | 0.4  |
| TGF-βRII| 2.25                                | 0.17–43.41 | 0.39  | 2.25                                | 0.17–43.41 | 0.39  |
| Foxp3   | 5.03                                | 0.51–43.98 | 0.11  | 5.03                                | 0.51–43.98 | 0.11  |
| GATA-3  | 3.61                                | 0.34–29.18 | 0.13  | 3.61                                | 0.34–29.18 | 0.13  |
| T-bet   | 2.564                               | 0.225–43.214 | 0.320  | 2.564                               | 0.225–43.214 | 0.320  |
| IFN-γ   | 1.875                               | 0.375–10.708 | 0.472  | 1.875                               | 0.375–10.708 | 0.472  |

a Changes in mRNA expression from pretreatment to week 1 of therapy are shown for fast and slow responders. The factors represent the level of transcript expression at week 1 compared to that at the pretreatment time point. Values above 1 indicate upregulation and values below 1 downregulation. FR, fast responder; SR, slow responder. Statistically significant P values are shown in bold.

b The primers used amplified both IL-4 and IL-42.

(iii) Plasma level of IL-4 protein. The analysis of circulating IL-4 protein in the plasma samples gave inconclusive results. IL-4 was undetected in 15 patients at pretreatment and in 14 (out of 20) at week 1 of treatment. The detection of IL-4 protein in clinical samples has always been difficult due to the lack of sensitivity among available tests (8), making it difficult to investigate whether the change that occurs at the transcriptional stage is reflected at the translational stage (13). Furthermore, the discovery of IL-42 further complicates the interpretation of data on circulating IL-4 protein, as the currently available tests do not differentiate between IL-4 and IL-42 (31).

(iv) TGF-β and TGF-βRII mRNA expression. In the present study, no differences in expression level of TGF-β or TGF-βRII were found between the patients with fast and slow responses to treatment at pretreatment or at week 1 of therapy (Table 3). The expression levels also did not change significantly over time within the two groups. Further studies including larger patient numbers and additional time points should be conducted in the future to investigate these findings further.

(v) Foxp3 mRNA expression. To investigate the association of a prominent regulatory T-cell marker with month 2 sputum culture conversion, we measured the expression levels of Foxp3 in the fast and slow responders at diagnosis and at week 1 after initiation of therapy. No differences in expression level of Foxp3 were found between the patients with fast and slow responses to treatment at either time point (Table 3).

When each group was looked at individually, the fast responders showed an increase in Foxp3 by a factor of 5.03 (P = 0.11) after a week of treatment, and the slow responders showed only a 2-factor increase (P = 0.3) (Table 4). However, the changes in Foxp3 expression recorded in the fast and slow responders were not statistically significant, most probably due to the large standard error in Foxp3 expression. Nevertheless, this does not rule out Foxp3 as a potential predictor of week 8
sputum culture conversion. Further studies including larger patient numbers and additional time points should be conducted.

(ii) GATA-3 mRNA expression. As Th2 responses have been implicated in susceptibility to TB, GATA-3 expression was investigated. No significant differences between fast and slow responders at pretreatment or week 1 (Table 3) were seen. Although the differences between fast and slow responders were not statistically significant, GATA-3 mRNA expression was 3.84-fold higher in the fast responders than in the slow responders at week 1 (P = 0.06). No significant changes in expression were found in either responder group.

(vii) T-bet and IFN-γ mRNA expression. T-bet and IFN-γ are central players in type 1 immunity against pathogens. Lack of T-bet has been shown to lead to increased susceptibility to intracellular pathogens and decreased IFN-γ production (28). Furthermore, upregulation of T-bet and IFN-γ transcripts has been shown by Kawashima and Miossec (17) to be associated with treatment-related improvement of patients with rheumatoid arthritis. In the present study, no differences between treatment response phenotypes and no changes from pretreatment to week 1 were observed in T-bet or IFN-γ mRNA transcription.

(viii) Plasma level of IFN-γ protein. The analysis of plasma IFN-γ levels also showed no significant differences between fast and slow responders. Brahmbhatt et al. (3) in a similarly designed study also found no significant differences in IFN-γ secretion between fast and slow responders in the early stage of treatment (week 4).

Conclusion. This study shows differential IL-4 and IL-4β gene expression profiles in patients with fast and slow responses to TB treatment early after initiation of treatment. This observation suggests that treatment-related changes in IL-4 biology may hold promise as early markers for treatment effect and outcome.

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