

Differential Expression of Interleukin-4 (IL-4) and IL-4 δ 2 mRNA, but Not Transforming Growth Factor Beta (TGF- β), TGF- β RII, Foxp3, Gamma Interferon, T-bet, or GATA-3 mRNA, in Patients with Fast and Slow Responses to Antituberculosis Treatment[∇]

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This study investigated interleukin-4 (IL-4), IL-4 δ 2, transforming growth factor beta (TGF- β), TGF- β RII, 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-4 δ 2 (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-4 δ 2 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- β RII, 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 discrim-

inate 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 adjunctive recombinant human interleukin-2 (IL-2) immunotherapy in multidrug-resistant TB subjects suggested that differential gene expression may provide an indication of antimicrobial response (16).

The present study investigated the transcription levels of selected genes that have been associated with immunity and immunopathology in TB patients at pretreatment and early after initiation of TB treatment. The objective was to identify candidate biomarkers for early segregation of patients with fast and slow responses to anti-TB therapy. The products of the targeted genes included IL-4, whose excess production during active TB has been associated with depressed Th1 response (15, 30) and disease severity (15, 25); IL-4 δ 2, an alternative spliced IL-4 variant and IL-4 antagonist which very effectively competes with IL-4 for their common binding sites (31) and has been shown to be associated with control of *Mycobacterium tuberculosis* infection (10); transforming growth factor beta (TGF- β) and TGF- β RII, 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

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TABLE 1. Sequences of primers used for amplification of target and housekeeping genes^a

Genes and gene product or primer name	Primer sequence		RefSeq identification no.
	Forward	Reverse	
Target genes			
IL-4* ^b			NM_000589, NM_172348
IL-4	5'-CGA GTT GAC CGT AAC AGA CAT-3'	5' CGT CTT TAG CCT TTC CAA GAA G-3'	
IL-4δ2	5'-CAG AGC AGA AGA ACA CAA CTG-3'	5'-GTC TTT AGC CTT TCC AAG AAG-3'	
TGF-β			NM_000660
TGF-βRII			NM_003242
Foxp3			NM_014009
GATA-3			NM_001002295, NM_002051
T-bet			NM_013351
IFN-γ			NM_000619
Housekeeping genes			
β-Actin			NM_001101
hGAPDH	5'-ACC CAC TCC TCC ACC TTT G-3'	5'-CTC TTG TGC TCT TGC TGG G-3'	
hG3PDH	5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3'	5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'	
hPRT1	5'-TGA CAC TGG CAA AAC AATGCA-3'	5'-GGT CCT TTT CAC CAG CAA GCT-3'	
huPO	5'-GCA ATG TTG CCA GTG TCT GT-3'	5'-GCC TTG ACC TTT TCA GCA AG-3'	

^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-4δ2.

Th2-type cytokines (22, 35, 36); T-bet (T-box 21), a Th1-specific transcription factor that plays a key function in Th1 development, polarization, gamma interferon (IFN-γ) gene transactivation, and promotion of delayed type 1 hypersensitivity 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-4δ2 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 observed 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 pyrazinamide (1,000 mg/day to 1,250 mg/day) during the 2-month intensive phase, followed by rifampin and isoniazid during the 4-month continuation phase.

Monitoring of bacteriology and classification of patients into treatment response groups. Mycobacterial sputum culture was done using the automated Bactec 12B liquid radiometric method (Becton-Dickinson, Bethesda, MD). Rigorous 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. Mycobacterial 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 negative) 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.

Quantitative real-time PCR. RNA was isolated from ex vivo blood samples by using mRNA isolation kits for blood and bone marrow (Roche kit). The isolation was done according to the manufacturer's instructions. The extracted mRNA was then reverse transcribed into cDNA (Qiagen kit). The transcripts of interest were relatively quantified using a light cycler (Roche) and a Quantitec Sybr green PCR kit (Qiagen). PCRs were done according to the manufacturer's instructions. The products of the genes targeted were IL-4, IL-4δ2, TGF-β, TGF-βRII, Foxp3, and GATA-3. The primers used for the amplification of IL-4* (both alternative IL-4 transcripts), TGF-β, TGF-βRII, Foxp3, GATA-3, T-bet, and IFN-γ were supplied by Qiagen. IL-4 and IL-4δ2 transcripts were amplified using specifically designed primers from the literature (24). The sequences of the primers used for amplification of the target and housekeeping genes are shown in Table 1. Qiagen did not disclose the sequences of their primers. Therefore, the reference sequence (RefSeq) identification numbers are shown for these genes instead of the primer sequences (Table 1). The products of the housekeeping genes used as reference genes for normalization of data included β-actin (Qiagen primers), human glyceraldehyde-3-phosphate dehydrogenase (using hGAPDH and hG3PDH primers), human PRT1, and human ribosomal protein (IDT, Inc.) (Table 1). Although validated primers were used, the amplification efficiency of each primer pair was assessed. This was done through a titration series of amplicons from a patient. The PCR mixtures consisted of a 20-μl reaction volume, including 10 μl of Sybr green master mix, 2 μl (0.1 μg) of cDNA, 2 μl (0.5 μM) of primers, and 6 μl of RNase-free water. Data were analyzed using the REST 2005 program from Corbett Life Science, which is a powerful randomization test that determines significant differences between two groups. The program takes into account reaction efficiency and normalization and performs 50,000 random reallocations of samples between two groups. It is based on the principle that if any perceived variation between two groups is due only to chance, then it will be possible to randomly swap values between the two groups

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

Analyte	Mean fold change in expression factor at wk 1	SE	<i>P</i>
IL-4 ^{*b}	2.77	0.5–17.95	0.038
IL-4	0.26	0.047–1.93	0.006
IL-4δ2	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

^a 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.

^b The primers used amplified both IL-4 and IL-4δ2.

and not see a greater difference than the one initially observed between the groups (adapted from the REST 2005 manual).

Immunoassay. The determination of IL-4 and IFN-γ protein levels in patient plasma samples was done with the Bio-Plex bead array system (Bio-Rad Laboratories). Assays were carried out with 96-well filter plates, using a LINCO-plex kit from Millipore. The reagent preparation and the assay protocol were in accordance with the manufacturer’s recommendation. The differences between the groups were analyzed using the Mann-Whitney U test.

RESULTS AND DISCUSSION

Changes in IL-4, IL-4δ2, 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-4δ2 (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-4δ2-specific primers showed that IL-4 mRNA expression was significantly down-regulated, whereas there was a marked and significant increase in IL-4δ2 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-4δ2 expression. Together, this resulted in a decrease of the IL-4/IL-4δ2 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 on-going 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-4δ2, TGF-β, TGF-βRII, Foxp3, and GATA-3 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-4δ2 mRNA 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-4δ2 mRNA expression determined using IL-4- and IL-4δ2-specific primers. IL-4 and IL-4δ2 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-4δ2 transcripts were observed between the responder groups (Table 3).

The analysis of IL-4 and IL-4δ2 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-4δ2 mRNA transcription was significantly upregulated by a

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^a

Analyte	Diagnosis time point			Wk 1		
	Mean FR/SR expression factor (fold)	SE	<i>P</i>	Mean FR/SR expression factor (fold)	SE	<i>P</i>
IL-4 ^{*b}	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-4δ2	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	0.75–28.92	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

^a 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.

^b The primers used amplified both IL-4 and IL-4δ2.

TABLE 4. mRNA expression changes from pretreatment to week 1 of therapy in slow and fast responders^a

Analyte	Fast responders			Slow responders		
	Mean FR/ SR expression factor (fold)	SE	<i>P</i>	Mean FR/ SR expression factor (fold)	SE	<i>P</i>
IL-4 ^{*b}	7.68	1.5–36.96	0.018	2	0.37–11.16	0.3
IL-4	0.71	0.11–7.13	0.69	0.06	0.26–0.98	0.002
IL-482	18.93	0.6–488.3	0.02	47.32	4.01–703.4	0.0001
TGF-β	2	0.091–22.5	0.4	0.94	0.12–6.61	0.74
TGF-βRII	2.25	0.17–43.41	0.39	1.1	0.087–14.4	0.71
Foxp3	5.03	0.51–43.98	0.11	1.95	0.25–21.1	0.34
GATA-3	3.61	0.34–29.18	0.13	1.5	0.18–13.4	0.42
T-bet	2.564	0.225–43.214	0.320	1.237	0.080–16.828	0.591
IFN-γ	1.875	0.375–10.708	0.472	2.163	0.293–12.832	0.257

^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-482.

factor of 47.3 (*P* = 0.0001) (Table 4). The simultaneous downregulation of IL-4 mRNA and upregulation of IL-482 mRNA 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-482 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-482 levels increased significantly (12).

The upregulation of IL-482 resulted in decreases of IL-4/IL-482 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 autocrine/paracrine growth factor and that the 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-482 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 together 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 ex-

pression 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.

(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-482 further complicates the interpretation of data on circulating IL-4 protein, as the currently available tests do not differentiate between IL-4 and IL-482 (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.

(vi) **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. Brahmhatt 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-4 δ 2 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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