

## Effect of Chemotherapy on Whole-Blood Cytokine Responses to *Mycobacterium tuberculosis* Antigens in a Small Cohort of Patients with Pulmonary Tuberculosis<sup>∇†</sup>

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Received 17 March 2011/Returned for modification 18 April 2011/Accepted 15 June 2011

**The development of genomic and proteomic tools has enabled studies that begin to characterize the molecular targets of an effective host immune response to *Mycobacterium tuberculosis*, including understanding the specific immune responses associated with tuberculosis (TB) disease progression, disease resolution, and the development of latency. One application of such tools is the development of diagnostic reagents and assays useful as a test of cure. Such a test could be of considerable importance for the evaluation of new therapeutics. We and others have previously described immunodominant proteins of *M. tuberculosis*, including both vaccine and diagnostic candidates. In the present study, we describe the changes in immune responses to a panel of 71 *M. tuberculosis* antigens in six patients during the course of therapy. The levels of six cytokines were measured in 24-h whole-blood assays with these antigens, revealing that gamma interferon (IFN- $\gamma$ ), tumor necrosis factor (TNF), and interleukin-10 (IL-10) were differentially regulated in response to a subset of antigens. Therefore, measuring the production of these three cytokines in response to a panel of carefully selected *M. tuberculosis* proteins during the course of TB therapy might be a promising path toward the development of a test of cure and warrants further validation in larger cohorts of pulmonary TB patients.**

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex bacilli is one of the leading causes of death worldwide (42). Upon exposure to *M. tuberculosis*, 30 to 40% of close contacts will develop TB infection, of whom 5% would be expected to develop active disease within a 24-month period while the other 95% enter a state of controlled latent TB infection (LTBI), which can reactivate later in life following decreased immunocompetence of the host. T cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, and the cytokines gamma interferon (IFN- $\gamma$ ) and tumor necrosis factor (TNF) play important roles in the prevention of active disease and the control of LTBI, as demonstrated by gene-knockout animal models and human subjects with mutations affecting the expression of these two cytokines (17).

The traditional diagnosis of active TB disease relies on positive identification of acid-fast bacilli (AFB) in a sputum smear or *M. tuberculosis* identified in culture and is supported by delayed-type hypersensitivity reactions to intradermal injection of *M. tuberculosis*-specific and nonspecific purified protein derivative (PPD). The tuberculin skin test (TST) utilizes PPD and has a number of drawbacks, notably that TST cross-reacts with the *Mycobacterium bovis* vaccine strain bacillus Calmette-

Guérin (BCG) and other environmental mycobacteria, increasing the number of false positives (37). As IFN- $\gamma$  is required for a T helper 1 (Th1) response to *M. tuberculosis*, this cytokine has been measured *ex vivo* in serum (35, 39), sputum (35), bronchoalveolar lavage fluid (4), pleural effusions of TB patients (21, 25), or culture supernatants of peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with *M. tuberculosis* antigens (2, 18, 40). IFN- $\gamma$  responses to antigen stimulation are easily induced in PBMC or whole blood and can be detected using simple technologies, such as enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunosorbent spot assay (ELISPOT), since IFN- $\gamma$  is not labile and is usually produced in measurable quantities. Differences in the levels of IFN- $\gamma$  measured in culture supernatants of stimulated lymphocytes from TB patients and controls varied considerably depending on the study and were inconclusive as a diagnostic tool (40), probably due to nonstandardized sample handling and variable duration of stimulation with *M. tuberculosis* antigens (13).

More recently, standardized short (<24 h) peripheral blood T-cell IFN- $\gamma$  responses to three *M. tuberculosis*-specific antigens, early secreted antigenic target 6 (ESAT-6), culture filtrate protein 10 (CFP-10), and antigen TB7.7 (Rv2654), have been investigated for the management of TB. Two assays, the ELISPOT assay T-SPOT.TB (Oxford Immunotec, Oxford, United Kingdom) that uses PBMC and the ELISA QuantiFERON-TB gold (Cellestis, Victoria, Australia) that uses whole blood, measure IFN- $\gamma$  responses to overlapping ESAT-6/CFP-10 peptides and ESAT-6/CFP-10/TB7.7, respectively, in less than 24 h. It was reported that the number of ESAT-6/CFP-10-specific IFN- $\gamma$ -producing cells in circulating

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† Supplemental material for this article may be found at <http://cvi.asm.org/>.

∇ Published ahead of print on 29 June 2011.

PBMC reflects bacterial load and the relative risk for pathology and illness. While both assays have advanced the diagnosis of latent TB infection, their utility in monitoring immune responses during the course of chemotherapy for active TB disease and in correlating IFN- $\gamma$  levels after antigen stimulation with the decrease in bacterial load has yet to be confirmed (12). The results obtained to date from a small number of studies using these two assays are controversial for both active TB and LTBI. Some of these studies report decreased IFN- $\gamma$  responses to specific mycobacterial antigens (1, 8, 10, 20, 26, 28, 33, 34, 36) during and after chemotherapy, while others have shown persistently positive or even stronger responses with chemotherapy (3, 16, 31, 38, 41, 43). Decreases in IFN- $\gamma$  responses to CFP-10 during treatment appeared to correlate better with reduction in bacterial loads than analogous responses to ESAT-6 or ESAT-6 and CFP-10 combined (9, 14, 27). Host immune responses to *M. tuberculosis* antigens during therapy are complex, dynamic events that will likely require the measure of more than one parameter—cytokine and/or antigen. It has been suggested that the IFN- $\gamma$ /IL-10 ratio might be used to successfully predict the development of active disease after exposure to TB (24). In addition, Eum et al. recently showed that the regulation of TNF during therapy might be better than IFN- $\gamma$  in predicting sputum conversion at 6 months (15).

The identification of surrogate markers of bacterial clearance in TB patients receiving chemotherapy is important for a number of reasons, including early diagnosis of treatment efficacy, prevention of relapse due to incomplete cure, and as endpoints in clinical trials evaluating new tuberculosis medications (22). The objective of this study was to identify in pulmonary TB patients surrogate markers for successful response to therapy by characterizing the dynamic of cytokine responses to a large array of *M. tuberculosis* antigens before, during, and after completion of treatment. For that purpose, cytokines associated with Th1, Th2, and Th17 were analyzed. The levels of IFN- $\gamma$ , TNF, interleukin-10 (IL-10), IL-5, IL-2, and IL-17 were characterized in whole blood after <24 h of stimulation with PPD, *M. tuberculosis* lysate (MtbL), ESAT-6, and 70 additional recombinant *M. tuberculosis* proteins, including both secreted and latency antigens.

**MATERIALS AND METHODS**

**Study subjects.** TB patients ( $n = 6$ ), recruited at the TB Control Program, Public Health—Seattle & King County (Seattle, WA), were assessed for cytokine profiles during therapy using *M. tuberculosis* antigen-stimulated whole-blood cultures. The study was approved by the Human Subjects Review Committee of the University of Washington, and written informed consent was obtained from each study subject. All six patients were diagnosed with active pulmonary TB by sputum culture, were PPD positive (indurations of >10 mm), and were negative for human immunodeficiency virus (HIV) and hepatitis B and hepatitis C viruses. Four patients had a history of BCG vaccination, one did not, and the status of one patient was unknown. The mean age was 32.8 years (range, 19 to 49), and the male/female ratio was 5:1. Each patient was given the standard 6 months of directly observed therapy (DOT) for TB, which consisted of a combination of at least two of the following drugs: isoniazid, rifampin, ethambutol, pyrazinamide or rifamate (a combination of isoniazid and rifampin). Each patient was initially treated with standard short-course therapy according to the guidelines of the American Thoracic Society and Centers for Disease Control and Prevention (7), consisting of a 2-month intensive phase with four drugs (isoniazid, rifampin, ethambutol, and pyrazinamide) and 4 months of isoniazid and rifampin. One patient (TBC-04) harbored a multidrug-resistant *M. tuberculosis* strain that was

TABLE 1. Categories of selected *M. tuberculosis* antigens

Category	Antigens
Secreted/membrane .....	Rv0125, Rv0153c, Rv0164, Rv0390, Rv0455c, Rv0496, Rv0577, Rv0733, Rv0909, Rv1411, Rv1511, Rv1626, Rv1827, Rv1860, Rv1926c, Rv1980c, Rv1984c, Rv2031, Rv2220, Rv2873, Rv2875, Rv3029c, Rv3881, Ag85B
PE/PPE protein family .....	Rv0915, Rv1789, Rv1818c, Rv2608, Rv3478
EsX protein family .....	Rv0287, Rv1793, Rv3619, Rv3620c, ESAT-6
Database .....	Rv0952, Rv1009, Rv1174, Rv1211, Rv1253, Rv1270c, Rv1288, Rv1884c, Rv2389c, Rv2450, Rv3204, Rv3407
Latency	
Growth in	
macrophage .....	Rv0467, Rv0523c, Rv0655, Rv0716, Rv1099, Rv1397c, Rv1410c, Rv1569, Rv1589, Rv1590, Rv3541c, Rv3587
Hypoxia .....	Rv1240, Rv1738, Rv1813c, Rv2032, Rv2428, Rv2558, Rv2624c, Rv2626c, Rv2801c, Rv3044, Rv3129c, Rv3130, Rv3810

resistant to both isoniazid and rifampin and was treated with an alternative regimen for 18 months. All patients had converted to culture negative at the end of the treatment and were considered adequately treated. In addition, healthy PPD-negative subjects ( $n = 7$ ) with no history of BCG vaccination were included as controls.

**Reagents.** *M. tuberculosis* PPD (lot P A0814-1) was obtained from Mycos Research LLC (Loveland, CO). Phytohemagglutinin (PHA) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Target *M. tuberculosis* recombinant His-tagged proteins were prepared in Tris buffer as previously described (5), and all showed residual endotoxin levels of <100 endotoxin units (EU)/mg of protein.

**Whole-blood assay.** At the time of enrollment in the study, patients had been started on chemotherapy for <2 weeks. Blood samples were drawn into heparinized Vacutainers (BD Biosciences, San Jose, CA) between the hours of 10 a.m. and 2 p.m. at the initial visit (time zero [T0]) and at 2 weeks (T0.5) and 1, 3, 6, 9, and 12 months (T1, T3, T6, T9, and T12) thereafter. Blood (20 ml) was diluted 1:1 with sterile RPMI 1640 (Invitrogen) tissue culture medium. Diluted blood (450  $\mu$ l/well) was plated in 48-well tissue culture plates within 2 h of collection. Blood cultures were stimulated with 50  $\mu$ l/well of Tris buffer, 10  $\mu$ g/ml PPD, 10  $\mu$ g/ml PHA, 10  $\mu$ g/ml MtbL, or 10  $\mu$ g/ml of each recombinant protein of *M. tuberculosis* (Table 1) for 20 to 22 h at 37°C in 5% CO<sub>2</sub>. Culture supernatants were harvested and stored for later use in separate polypropylene plates as three 100- $\mu$ l aliquots at -80°C.

**Cytokine determination by Luminex.** Supernatants were filtered through 1.2- $\mu$ m and 0.2- $\mu$ m filters to remove possible *M. tuberculosis* bacilli. The filtrates were analyzed for IL-2, IL-5, IL-10, IL-17, IFN- $\gamma$ , and TNF cytokine production using a customized human 6-plex Procarta cytokine assay kit (Affymetrix, Santa Clara, CA), following the directions of the manufacturer. Samples were read on a Luminex 200 (Luminex Corporation, Austin, TX) machine powered by the MasterPlex Software Suite program and analyzed using the MasterPlex QT 3.0 (MiraiBio, Inc., Alameda, CA) quantification software in order to obtain cytokine concentration values. The sensitivities and ranges of the assays were determined by running a dose-response curve of a reference standard for each cytokine from the manufacturer on each plate.

**Data analyses.** All data were analyzed by using MasterPlex QT 3.0 and Microsoft Excel. Antigen-specific cytokine responses are shown after subtraction of the spontaneous cytokine secretion that occurs in the absence of a stimulant. A macro was written with Microsoft Excel to convert cytokine levels from pg/ml or fold change to colored heat maps. Comparison of the levels of IFN- $\gamma$  and IL-10 or those of TNF and IL-10 were provided as differences rather than ratios to account for zero values where present. Intra-assay variation was initially calculated for each cytokine at the T0 and T6 time points on duplicate wells of cells stimulated with PHA, PPD, ESAT-6, or antigen 85B (Ag85B). The intra-assay cytokine variation averaged 1.1  $\pm$  0.3 (mean  $\pm$  standard deviation [SD]; range, 1.0 to 1.5). There were no statistically significant differences observed between stimulants or cytokines, as determined using a 1-way analysis of variance (ANOVA) test. A cutoff for fold-change cytokine expression compared to the

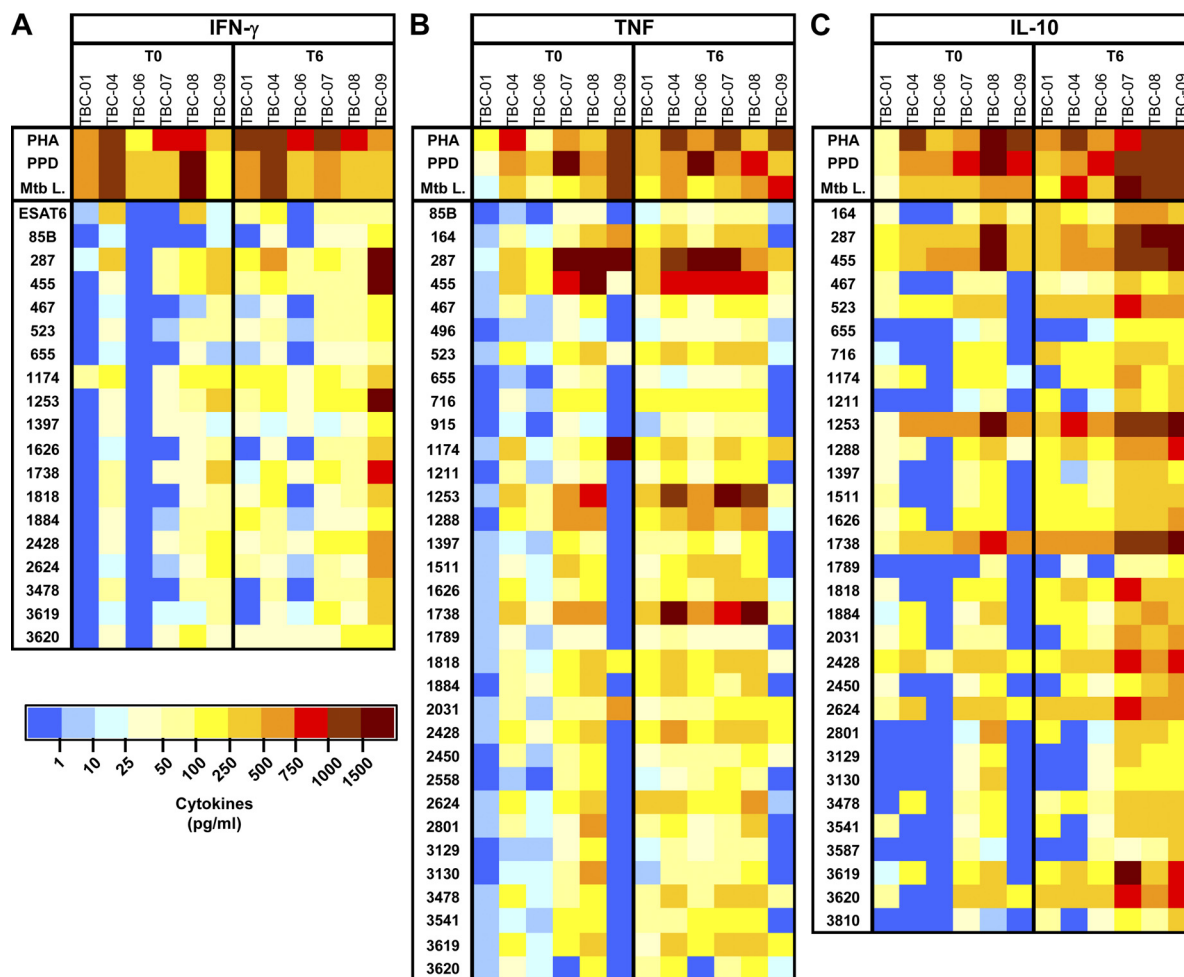


FIG. 1. Heat map of whole-blood cytokine responses to a subset of *M. tuberculosis* antigens recognized by two-thirds of the subjects. Whole blood was obtained from six TB patients at time 0 (T0) and 6 months (T6) of antibiotic treatment and stimulated with Tris buffer, PHA, PPD, MtbL, or an individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured using multiplexing ELISA. Antigen-specific cytokine concentrations are represented in a heat map from blue (>1 pg/ml) to dark brown (>1,500 pg/ml).

expression at T0 was considered different when greater than the absolute value of  $2 (\text{average}_{\text{variation}} + 3 \times \text{SD}_{\text{variation}})$ .

## RESULTS

To explore whether changes in cytokine responses to defined *M. tuberculosis* antigens would provide a surrogate marker for sputum smear and culture conversion, whole blood was obtained from six pulmonary TB subjects during the course of chemotherapy. The blood samples were stimulated for 24 h with an extensive panel of 71 single *M. tuberculosis* proteins, including ESAT-6 and Ag85B, and PPD. The levels of cytokines associated with Th1/effector (IFN- $\gamma$  and TNF), memory (IL-2), regulatory (IL-10), Th2 (IL-5), and Th17 (IL-17) cells were measured in culture supernatant with the Luminex technology.

**Profile of cytokine responses to *M. tuberculosis* antigens at initiation and end of therapy.** Whole-blood cells' cytokine responses to *M. tuberculosis* antigen stimulation were initially compared at the beginning (T0) and end (T6) of therapy (see Fig. S1 in the supplemental material). While the six cytokines

were upregulated in all patients in response to PHA stimulation, indicating that active disease did not inhibit cytokine responses, only IFN- $\gamma$ , TNF, IL-10, and IL-2 were increased in response to PPD and MtbL. Furthermore, cytokine responses to single *M. tuberculosis* antigens were mostly restricted to IFN- $\gamma$ , TNF, and IL-10. Based on these observations, we focused our analyses on a subset of *M. tuberculosis* antigens that induced antigen-specific cytokine responses of >25 pg/ml in four of six patients (67%) at T6 for one or more of the latter three cytokines (Fig. 1). Nineteen, 33, and 31 *M. tuberculosis* antigens met these criteria for IFN- $\gamma$  (Fig. 1A), TNF (Fig. 1B), and IL-10 (Fig. 1C), respectively. Interestingly, among these *M. tuberculosis* antigens, 17 upregulated all three cytokines, 12 more were associated with increases in TNF and IL-10 responses, and one was associated with IFN- $\gamma$  and TNF responses. When concentrations of effector (IFN- $\gamma$  and TNF) versus regulatory (IL-10) cytokines were compared, the IL-10 levels were generally higher than the IFN- $\gamma$  and TNF levels for most *M. tuberculosis* antigens and TB subjects at both T0 and T6, resulting in net negative values (Table 2). In addition, there

TABLE 2. Differences between plasma levels of IFN- $\gamma$  effector and IL-10 regulatory cytokines with stimulation by various *M. tuberculosis* antigens

Cytokine pair measured and <i>M. tuberculosis</i> antigen used	Amt of IFN- $\gamma$ or TFN – amt of IL-10 (pg/ml) in patient at indicated time point <sup>a</sup>											
	TBC-01		TBC-04		TBC-06		TBC-07		TBC-08		TBC-09	
	T0	T6	T0	T6	T0	T6	T0	T6	T0	T6	T0	T6
<b>IFN-<math>\gamma</math> – IL-10</b>												
PHA	580	1182	-761	-556	-223	456	-261	4380	-542	-565	-2933	-3350
PPD	600	110	3714	1895	-331	-462	-1166	-1046	49	-1781	-641	-1412
MtbL	620	315	4402	1726	-25	-187	-612	-915	551	-1327	-428	-1380
ESAT-6	7	69	324	179	0	0	-2	-19	250	8	16	36
Ag85B	0	0	24	25	0	0	5	-71	-58	-91	23	15
Rv0287	-139	-387	-76	-46	-488	-249	-498	-1400	-853	-1209	58	82
Rv0455	-114	-406	-243	-491	-594	-445	-993	-1703	-1030	-1608	-107	-267
Rv0467	-62	-83	20	-15	0	-86	-116	-156	-76	-163	65	-132
Rv0523	-84	-356	-200	-367	-174	-317	-553	-779	-232	-574	86	-438
Rv0655	0	6	11	27	0	-19	3	-109	-24	-165	5	-69
Rv1174	-18	204	-44	83	0	-125	-267	-418	65	-147	105	-117
Rv1253	-35	-395	-549	-783	-647	-624	-801	-1417	-1136	-1668	-203	-189
Rv1397	-42	-90	32	5	0	-20	-64	-239	-83	-247	15	-54
Rv1626	-37	-175	-84	-193	0	-151	-215	-381	-127	-381	75	-245
Rv1738	-88	-519	-410	-570	-286	-630	-662	-1575	-831	-1981	-351	-473
Rv1818	-50	-178	85	-184	0	-115	-918	-823	-164	-297	50	-85
Rv1884	-24	-85	-62	-54	0	-23	-568	-241	-328	-621	80	-82
Rv2428	-130	-182	-250	-378	-72	-459	-289	-775	-217	-461	-75	-319
Rv2624	-57	-298	-226	-334	0	-256	-322	-789	-352	-671	13	-170
Rv3478	0	-80	-146	-44	0	-55	-215	-316	-30	-222	51	-42
Rv3619	-24	-173	-137	-264	0	-234	-254	-905	-393	-443	63	-312
Rv3620	-79	-302	28	-295	0	-246	-163	-869	-190	-583	-180	-749
<b>TNF – IL-10</b>												
PHA	33	-233	-3643	-6254	-358	111	1226	1274	-889	-1074	-376	-2192
PPD	-58	29	-50	130	-305	257	-729	-1007	-606	-1190	1199	-1373
MtbL	-18	-39	-126	-213	-250	-234	76	-1045	-295	-1064	2989	-883
Ag85B	0	20	10	59	1	30	0	-60	-24	-49	0	-161
Rv0164	-38	-248	64	30	11	0	-348	-332	-58	-201	564	-382
Rv0287	-132	-186	21	1114	-338	730	-799	-444	35	-539	862	-761
Rv0455	-106	-213	-21	343	-466	264	-1284	-879	-2	-696	-463	-1238
Rv0467	-59	-39	89	91	3	-41	-148	-32	20	-4	0	-249
Rv0496	1	11	5	35	1	39	0	31	-3	-49	0	-77
Rv0523	-78	-256	-55	-81	-151	-199	-713	-594	-45	-310	31	-639
Rv0655	1	37	3	18	0	17	0	-118	18	-115	0	-155
Rv0716	-18	-163	31	-5	1	14	-377	-150	-35	-73	0	-169
Rv0915	0	9	15	71	0	30	-31	14	-8	-22	0	-194
Rv1174	-72	102	21	122	12	-66	-423	-218	48	0	1463	29
Rv1211	1	-65	69	129	3	15	-123	-56	14	-55	0	-295
Rv1253	-34	-174	-173	625	-553	-133	-1130	-471	-236	-80	-528	-1280
Rv1288	-36	-84	52	197	53	439	-317	-201	98	3	-40	-869
Rv1397	-41	-76	12	54	5	126	-84	-202	-22	-140	0	-217
Rv1511	-58	-127	40	90	14	207	-140	-179	-24	-167	0	-312
Rv1626	-34	-125	26	-7	12	-80	-301	-156	-43	-139	0	-569
Rv1738	-82	-203	-164	404	-238	-108	-924	-779	-293	-922	-613	-1283
Rv1789	1	32	26	31	2	45	-148	-11	-24	-68	0	-140
Rv1818	-49	-74	75	39	15	12	-1119	-573	136	-25	103	-376
Rv1884	-24	-91	-54	220	36	116	-848	-179	-7	-227	0	-259
Rv2031	-38	31	-56	-28	9	-28	-382	-345	3	-81	597	-385
Rv2428	-122	-83	-72	51	-47	-282	-440	-482	32	-180	-226	-739
Rv2450	-41	32	65	-81	8	8	-262	-88	-3	-144	0	-520
Rv2558	0	21	4	25	0	59	0	-233	39	-87	0	-140
Rv2624	-54	-51	-99	48	15	-94	-462	-621	-68	-200	-132	-682
Rv2801	2	-134	69	113	24	20	-457	-234	-52	-159	0	-206
Rv3129	1	11	10	70	4	4	-186	-365	23	-67	0	-140
Rv3130	1	8	18	96	12	12	-676	-171	110	-26	0	-217
Rv3478	2	-23	-73	146	13	-2	-303	-36	11	-24	0	-315
Rv3541	-48	-110	15	64	9	104	-199	-244	2	-114	0	-304
Rv3619	-21	-128	-58	108	10	-131	-338	-733	27	-212	0	-667
Rv3620	-78	-271	26	-123	13	-276	-171	-825	-174	-583	-209	-856

<sup>a</sup> Whole blood was obtained from six TB patients at time zero (T0) and 6 months (T6) of antibiotic treatment and stimulated with individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$  or TNF and IL-10 were measured using multiplexing ELISA. A negative value indicates a higher level of IL-10 than of IFN- $\gamma$  or TNF.

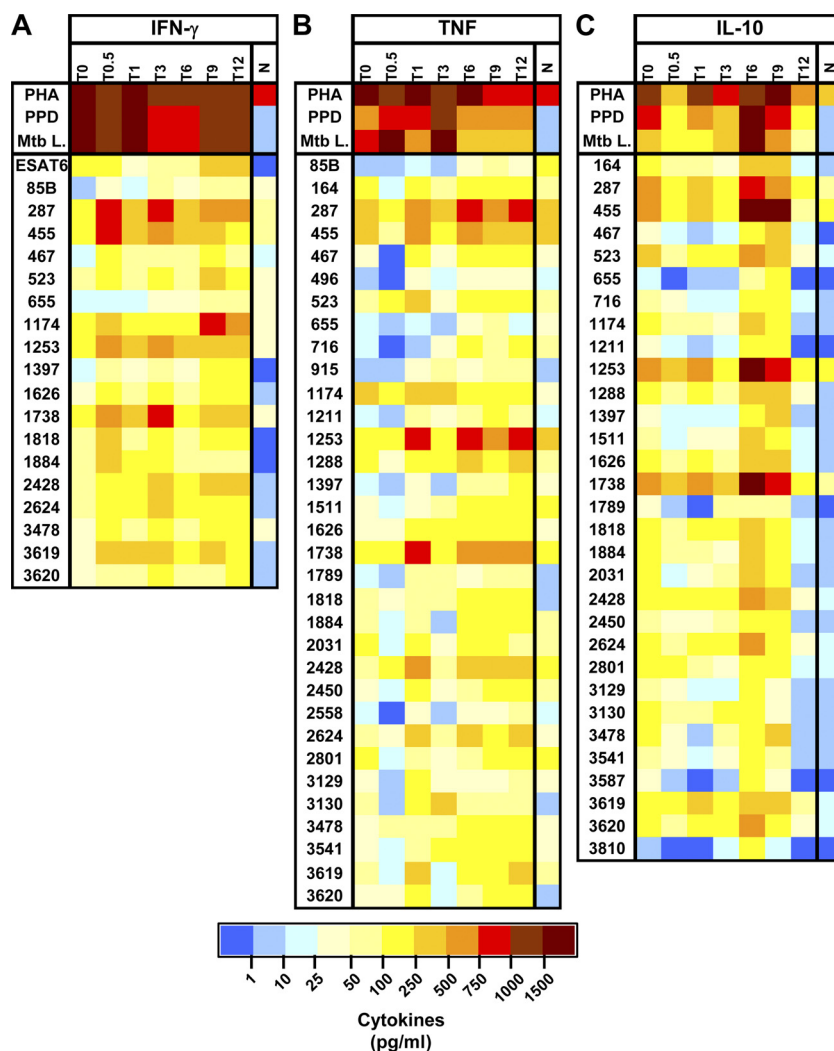


FIG. 2. Kinetics of whole-blood cytokine responses to a subset of *M. tuberculosis* antigens. Whole blood was obtained from six TB patients at time 0 (T0), 2 weeks (T0.5), and 1 (T1), 3 (T3), 6 (T6), 9 (T9), and 12 months (T12) of antibiotic treatment and from seven PPD-negative healthy controls (N) and stimulated with Tris buffer, PHA, PPD, MtbL, or an individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured using multiplexing ELISA. At each time point, data from the six subjects were averaged for a given *in vitro* stimulation and color coded depending on cytokine concentration.

was a trend for a greater IL-10 than IFN- $\gamma$  response detected at T6 compared to their levels at T0 (T6 value < T0 value).

**Kinetics of cytokine responses to *M. tuberculosis* antigens during chemotherapy.** Whole-blood samples were obtained at T0, T0.5 (2 weeks), and T1, T3, T6, T9, and T12 (months) after the initiation of therapy and stimulated for 24 h with the subset of stimulating *M. tuberculosis* antigens identified in the experiments discussed above (Fig. 1). Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured and averaged for the six patients at each time point. For IFN- $\gamma$ , the cytokine level associated with each single *M. tuberculosis* antigen was either maintained or increased at all the other time points except T0 (Fig. 2A). This observation is of particular interest as it suggests that blood cells from TB subjects already become more responsive to antigen-specific stimulation within 2 weeks after starting on antibiotics. In comparison, IFN- $\gamma$  levels measured from blood cells of PPD-negative healthy controls were low (<50 pg/ml, except for Rv0287 and Rv0455). The TNF levels in response to

*M. tuberculosis* antigen stimulation seemed generally higher at the later time points, T6 through T12 (Fig. 2B), with the exception of Rv0287, Rv0455, Rv1174, Rv1253, Rv1288, Rv1738, and Rv2428, which induced strong TNF responses at all time points, as well as in healthy controls. Responses to these *M. tuberculosis* antigens in healthy controls are probably due to homology with and exposure to environmental nontuberculous mycobacteria. Likewise, the highest IL-10 responses were generally observed at T6 and T9, with Rv0287, Rv0455, Rv1253, and Rv1738 inducing IL-10 levels of >500 pg/ml (Fig. 2C). At T12, however, the IL-10 responses resemble those of healthy controls. It appears that secretions of TNF and IL-10 have both more similar kinetics and more reactive *M. tuberculosis* antigen subsets than those of IFN- $\gamma$  and IL-10.

**Quantitative changes in cytokine responses to *M. tuberculosis* antigens during chemotherapy.** In the experiments discussed above, we identified a subset of 36 *M. tuberculosis* antigens inducing IFN- $\gamma$ , TNF, and/or IL-10 responses in the

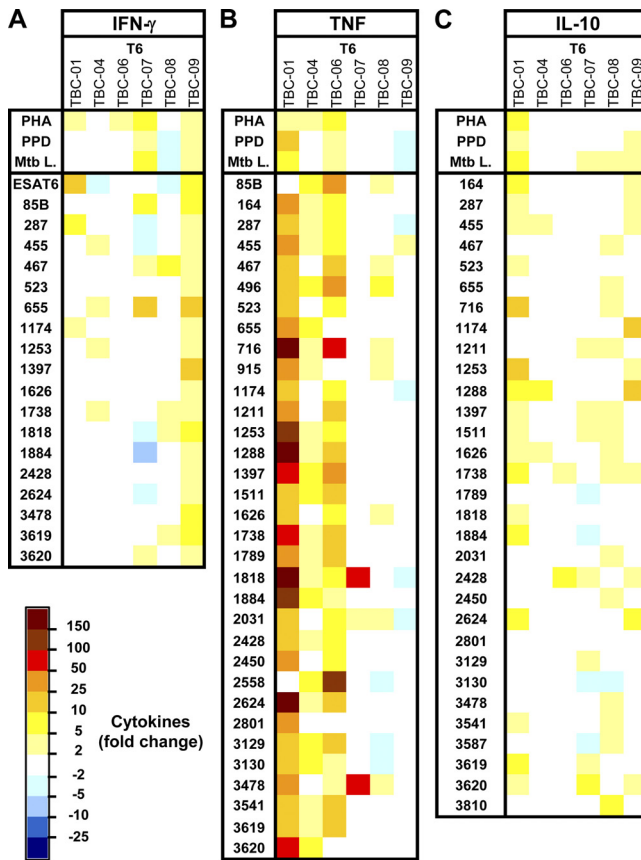


FIG. 3. Fold changes in cytokine responses to a subset of *M. tuberculosis* antigens 6 months into antibiotic therapy. Whole blood was obtained from six TB patients at time 0 (T0) and 6 months (T6) of antibiotic treatment and stimulated with Tris buffer, PHA, PPD, MtbL, or an individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured using multiplexing ELISA. Increases (positive fold change) or decreases (negative fold change) in cytokine responses at T6 compared to the data at T0 are shown and color coded based on intensity.

blood of two-thirds of the TB subjects. We further examined whether cytokine response(s) to some of these *M. tuberculosis* antigens changed during the course of therapy (Fig. 3 and Fig. S2).

The fold changes in IFN- $\gamma$ , TNF, and IL-10 responses to the subset of 36 *M. tuberculosis* antigens over the period from T0 to T6 were first calculated for each subject. Among the 19 *M. tuberculosis* antigens selected, IFN- $\gamma$  responses to latency antigens Rv467, Rv655, and Rv1738 were upregulated (2- to 25-fold) in 50% of the TB subjects (Fig. 3A). Similarly, at T6, 25 of the 33 *M. tuberculosis* antigens initially selected induced increased TNF levels (2- to 150-fold) (Fig. 3B), and 8 of 31 increased IL-10 responses (2- to 25-fold) (Fig. 3C) in 50% of the TB subjects. Finally, a subset of 8 *M. tuberculosis* antigens did induce positive changes at T6 for at least two cytokines in  $\geq 50\%$  of the patients (Table 3) and might be pursued further as a test of cure. While cytokine responses to some antigens were downregulated at T6 compared to their levels at T0, the occurrence was rare and never occurred in more than one-third of the subjects. Finally, fold changes in cytokine levels during the course of therapy were averaged for the six subjects

and presented as a group response at the different time points tested. IFN- $\gamma$  responses to Rv0467, Rv1738, and Rv3619 were already upregulated 5- to 25-fold at T0.5, while responses to ESAT-6, Ag85B, Rv0655, and Rv1174 increased at T3 or later (Fig. 4A). TNF responses to all but three antigens were upregulated at T6 and further increased at T9 and/or T12 (Fig. 4B). In addition, 2- to 10-fold changes in TNF responses to Rv0164, Rv1818, and Rv2425 were already occurring at T0.5. Finally, IL-10 responses to most antigens were downregulated, especially at T12 (Fig. 4C).

DISCUSSION

The identification of surrogate markers of bacterial clearance in pulmonary TB patients receiving chemotherapy is a critical step toward early assurance of treatment success and identification of patients at risk of relapse and subsequent transmission of TB to others following short-course TB therapy. These biomarkers may also serve as a surrogate endpoint in clinical trials evaluating new TB medications. In this longitudinal study, we demonstrated that whole-blood cytokine production patterns from pulmonary TB patients vary during standard TB treatment. Blood culture stimulation with a set of 71 recombinant *M. tuberculosis* proteins and complex antigen mixtures (PPD and MtbL) resulted predominantly in Th1 (IFN- $\gamma$  and TNF) and regulatory (IL-10) cytokine responses; the levels of Th2 (IL-5)-, Th17 (IL-17)-, and memory (IL-2)-associated cytokines were low to undetectable for most antigens in all subjects. IFN- $\gamma$ , TNF, and/or IL-10 changes in expression profiles during chemotherapy were associated with a subset of the *M. tuberculosis* antigens tested.

Diagnosis of active TB relies on clinical criteria, patient

TABLE 3. Increase in IFN- $\gamma$ , TNF, and IL-10 responses to a subset of *M. tuberculosis* antigens

Antigen	% of patients with positive cytokine response <sup>a</sup>		
	IFN- $\gamma$	TNF	IL-10
Rv287	33	50	33
<b>Rv455</b>	<b>33</b>	<b>67</b>	<b>50</b>
<b>Rv467</b>	<b>50</b>	<b>50</b>	<b>17</b>
Rv523	17	33	17
Rv655	50	33	17
Rv1174	33	33	17
Rv1253	33	50	33
<b>Rv1288</b>	<b>0</b>	<b>50</b>	<b>50</b>
<b>Rv1397</b>	<b>17</b>	<b>50</b>	<b>50</b>
<b>Rv1511</b>	<b>0</b>	<b>50</b>	<b>50</b>
<b>Rv1626</b>	<b>17</b>	<b>50</b>	<b>50</b>
<b>Rv1738</b>	<b>50</b>	<b>50</b>	<b>67</b>
Rv1818	33	67	17
Rv1884	17	50	17
<b>Rv2428</b>	<b>17</b>	<b>50</b>	<b>50</b>
Rv2624	17	50	33
Rv3478	17	67	17
Rv3619	33	50	33
Rv3620	33	33	50

<sup>a</sup> Percentage of patients with positive fold-change cytokine responses to a subset of *M. tuberculosis* antigens 6 months into antibiotic therapy. Whole blood was obtained from six TB patients at time zero (T0) and 6 months (T6) of antibiotic treatment and stimulated with individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured using multiplexing ELISA. Antigens marked in boldface induced positive changes at T6 for at least two cytokines in  $\geq 50\%$  of the patients.

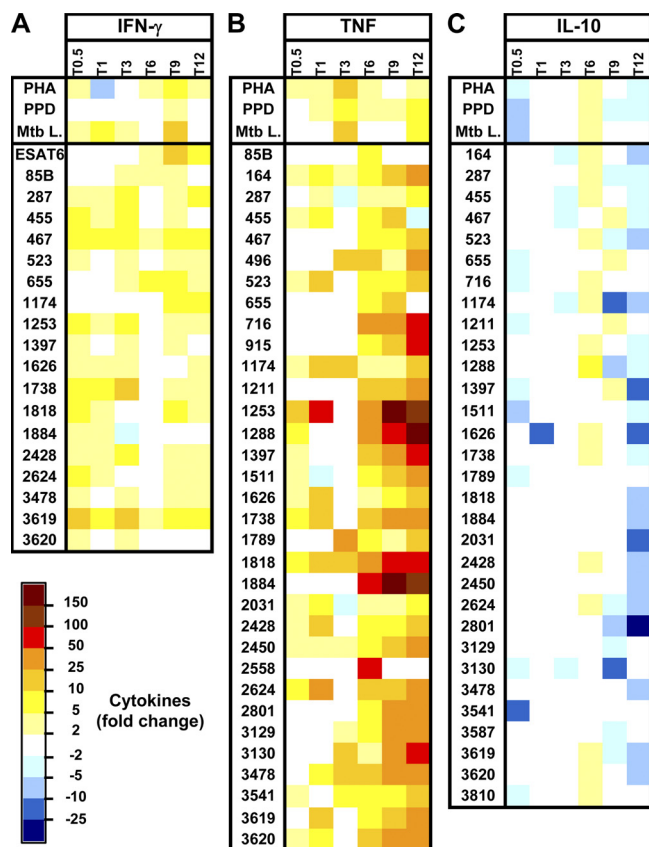


FIG. 4. Fold changes in cytokine responses to a subset of *M. tuberculosis* antigens at different times during antibiotic treatment. Whole blood was obtained from six TB patients at time 0 (T0), 2 weeks (T0.5), and 1 (T1), 3 (T3), 6 (T6), 9 (T9), and 12 months (T12) of antibiotic treatment and stimulated with Tris buffer, PHA, PPD, MtbL, or individual recombinant *M. tuberculosis* protein for 24 h at 37°C. Plasma levels of IFN- $\gamma$ , TNF, and IL-10 were measured using multiplexing ELISA. Increases (positive fold change) or decreases (negative fold change) in cytokine responses at the different time points compared to the data at T0 were calculated. For each time point and *in vitro* stimulation, data from the six subjects were averaged and color coded based on intensity.

history, chest radiography, TST, and confirmation by sputum smear or AFB culture positivity. However, an unexpectedly high number of individuals can be sputum positive (and therefore potentially infectious) without any apparent symptoms, whereas many patients who have significant symptoms remain sputum negative, thus calling this simple definition into question. The recent development of short standardized IFN- $\gamma$  release assays (QuantiFERON-TB Gold and T-SPOT.TB) based on recall responses to ESAT-6 and/or CFP-10 *M. tuberculosis* antigens offers new possibilities for the early diagnosis of active TB in adults (11, 32). The use of IFN- $\gamma$  release assays in assessing treatment efficacy in adults and children, however, has yielded conflicting results, with some studies reporting good correlations between bacterial loads and IFN- $\gamma$  responses (mainly to CFP-10) (9, 14, 20, 26, 27, 36), while other studies did not (3, 16, 31, 38, 41, 43). Our study confirms the lack of reliability of ESAT-6 as a single marker because only two patients (TBC-04 and TBC-08) of the six tested showed a moderate IFN- $\gamma$  response to this antigen at T0, while all pa-

tients had moderate to strong cytokine responses to *M. tuberculosis*-specific (PPD and MtbL) and nonspecific (PHA) stimulations. TBC-04 showed an initial increase in IFN- $\gamma$  2 weeks into therapy, consistent with previous observations (20), and both TBC-04 and TBC-08 showed further-reduced IFN- $\gamma$  responses to ESAT-6 from T1 to T6 but returned to T0 levels at T12. A similar kinetic was reported by Dominguez et al. (14). Among the four other patients, one never responded to ESAT-6, and the three others showed mild increases at T3 and onward. It is unclear why these four patients had poor correlations between the presence of bacteria and IFN- $\gamma$  responses to ESAT-6, but this observation is consistent with the considerable interindividual variation reported across studies in terms of percentage of responders and rate of decline of the response (14). When taken collectively, our data indicate that there are no differences in IFN- $\gamma$  responses to ESAT-6 during the first 3 months of treatment, followed by a moderate increase thereafter. Furthermore, our screening of 70 additional *M. tuberculosis* antigens revealed that for a majority of antigens, none of these antigens was associated with IFN- $\gamma$  declines during the course of treatment, while a small subset of antigens (the 15 antigens Ag85B, Rv0287, Rv0455, Rv467, Rv523, Rv0655, Rv1174, Rv1253, Rv1626, Rv1738, Rv1818, Rv1884, Rv2428, Rv2624, and Rv3619) induced higher IFN- $\gamma$  recall responses at T6 in  $\geq 67\%$  of TB patients. Patient responses to latency antigens were generally low, as previously observed by Leyten et al. (29), except for Rv1738. The relative discrepancy between our Rv1738 data and Leyten's might be explained by the use of whole blood instead of PBMC or by a more restricted definition of TB patients. Antigen stimulation of whole blood has the advantage of capturing the totality of the cellular response compared to that seen in PBMC, where loss of low-frequency cells during the purification process cannot be ruled out. Altogether, these observations suggest that increased rather than decreased IFN- $\gamma$  responses to a selected subset of *M. tuberculosis* antigens might better correlate with bacterial load and treatment efficacy. Furthermore, antigen stimulation of whole-blood cultures is a simple method that does not require labor-intensive PBMC purification and that might be more suitable in resource-limited laboratory settings. Nevertheless, further characterization of the T-cell response to these 15 *M. tuberculosis* antigens and phenotypic analyses at the single-cell level might provide additional information on whether higher IFN- $\gamma$  responses are associated with increased frequencies of blood antigen-specific circulating effector T cells.

In this study, we hypothesized that short (<24 h) *in vitro* blood stimulations will target circulating effector rather than memory cells. Consistent with this hypothesis, the levels of IL-2 measured in response to all single *M. tuberculosis* antigens were low (<100 pg/ml). Positive-control cultures incubated with the mitogen PHA showed elevated levels of IL-2 (500 to 1,500 pg/ml) at all time points, indicating that the lack of IL-2 responses to *M. tuberculosis* antigens were not due to an overall low T-cell response or cytokine detection problem. The IL-5 and IL-17 levels, as a measure of Th2 and Th17 cell responses, respectively, were also low (<100 pg/ml) for all *M. tuberculosis* antigens, including PPD and MtbL, in agreement with observations from Hussain et al. (23). As for IL-2, we found IL-5 and IL-17 responses to PHA stimulation (250 to 1,500 pg/ml and

100 to 1,000 pg/ml, respectively). However, PHA-driven IL-2, IL-5, and IL-17 responses were not consistently modulated during the course of therapy. Therefore, the measure of IL-2, IL-5, and IL-17 expression patterns in response to *M. tuberculosis* antigens or PHA stimulation was not predictive of sputum conversion and therapy outcome.

The ratios of IFN- $\gamma$ /IL-2 (6, 30) and IFN- $\gamma$ /IL-10 (24) have also been used with some success in the diagnosis of active TB and/or assessment of therapy outcome. In addition, Eum et al. recently demonstrated that whole-blood TNF levels in response to culture filtrate proteins were uniquely correlated with sputum conversion in patients treated for multidrug-resistant TB (15). However, they reported a decrease in TNF in sputum-negative patients at 6 months, while we observed an increase. Similarly, Harari et al. reported that subjects with active TB had elevated frequencies of antigen-specific (ESAT-6 and CFP-10) TNF-positive CD4 T cells that decreased upon completion of antibiotic treatment (19). The reasons for this difference are still unclear, but it might be related to the *M. tuberculosis* antigens tested in their and our study. Nevertheless, TNF, IL-10, and IFN- $\gamma$  responses to *M. tuberculosis* antigens were the most dynamic and likely to better predict therapy outcomes.

In summary, our results suggest that a subset of defined *M. tuberculosis* antigens can be used to monitor IFN- $\gamma$ , TNF, and IL-10 expression patterns associated with sputum conversion in pulmonary TB patients during chemotherapy. However, the sample size in the present study is too small to make a definitive conclusion, and this hypothesis will have to be validated in larger patient cohorts. These studies (i) confirm the relevance of IFN- $\gamma$ , TNF, and IL-10 expression profiles as an immune signature associated with reduction in bacterial load and (ii) define novel *M. tuberculosis* antigens with diagnostic potential. Early detection of treatment failure has significant implications for TB control programs, as it may allow the identification of TB patients whose clinical courses need to be reviewed for nonadherence, malabsorption of medications, poor penetration of TB drugs to the affected sites, or drug-resistant TB. Conversely, accurate diagnostics may allow for shortening treatment in early responders and a focusing of resources on patients with a higher likelihood of poor outcome in order to limit the chance of relapses. Accurate biomarkers are urgently needed as surrogate endpoints in clinical trials for evaluating tuberculosis medications, especially phase 2 trials.

#### ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI-044373 (S.G.R.).

We are thankful to Gregory C. Ireton for discussion and selection of *M. tuberculosis* antigens, to Jeffrey Guderian, Raodoh Mohamath, Garrett Poshusta, and Jackie Whittle for protein expression and purification, to Qiong Pan and the clinical staff of the TB Control Program, Public Health—Seattle & King County, to Anna Marie Beckmann for regulatory affairs, to Randall F. Howard for reviewing the manuscript, and to Rick Sorensen for heat map display programming.

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