Polarization of PPD-Specific T-Cell Response of Patients with Tuberculosis from Th0 to Th1 Profile after Successful Antimycobacterial Therapy or *In Vitro* Conditioning with Interferon-α or Interleukin-12

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The T helper (Th) 1/Th2 balance in the T-lymphocyte response to purified protein derivative (PPD) was evaluated at the clonal level in six Italian and five Gambian patients with pulmonary tuberculosis (TB) before and after antimycobacterial therapy, as well as in five Gambian and four Italian healthy immune control subjects. In untreated patients, most PPD-specific clones derived from either peripheral blood or pleural effusions showed a Th0 cytokine profile (production of both interferon [IFN]-γ and interleukin [IL]-4/IL-5). After 6 mo of therapy and clinical healing, most PPD-specific clones showed a polarized Th1 profile (production of IFN-γ but not IL-4/IL-5) in both Italian and Gambian patients. The Th1 polarization was less marked in Gambian than in Italian patients and failed to occur in another group of four Italian patients who experienced treatment failure. The cytokine profile observed after successful therapy in patients with TB was similar to that found in healthy control subjects. T-cell clones of undefined specificity generated from PPD-stimulated cultures showed a similar Th0/Th2 bias in Gambian individuals and Italian patients with treatment failure. The Th0/Th2-biased responses in Gambian patients before therapy could be modulated *in vitro* by IFN-α or IL-12, which induced a Th1 polarization of both PPD-specific and bystander T cells. Our data show that active TB associates with a predominant Th0 response to mycobacterial antigens that could play a role in the pathogenesis of the disease. Adjunctive immunotherapy using Th1-polarizing cytokines could increase host defense against mycobacteria and accelerate healing.

Tuberculosis (TB) is the leading cause of death resulting from an infectious agent in adults. It is estimated that worldwide, 7.5 million cases occur each year, 95% of them in developing countries (1). The efficient control of the TB epidemic by standard chemotherapy is hampered by the poor adherence of many patients to therapy and the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*. Immunotherapeutic approaches aimed at stimulating protective immune responses could improve patient compliance by reducing the duration of therapy and represent an alternative treatment for MDR TB (2). The rational design of immunotherapeutic strategies will rely on our understanding of protective immunity.

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Abbreviations: bacillus Calmette-Guèrin, BCG; interferon, IFN; interleukin, IL; multidrug-resistant, MDR; peripheral blood mononuclear cell, PBMC; phytohemagglutinin, PHA; phorbol myristate acetate, PMA; purified protein derivative, PPD; recombinant IL-2, rIL-2; tuberculosis, TB; T helper, Th.

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Recent studies of patients with familial susceptibility to mycobacterial infections indicate that T helper (Th) type 1 (Th1) immune responses are essential for protection (3). Mutations affecting the expression of interferon (IFN)-γ receptors, interleukin (IL)-12, or IL-12 receptors are associated with disseminated infections with environmental mycobacteria or *Mycobacterium bovis* bacillus Calmette-Guèrin (BCG). In most cases, severe and often fatal disease is associated with the absence of production of, or response to, IFN-γ. If IFN-γ production or response is only partially defective, disease is less severe (3, 4). These observations suggest a correlation between the magnitude of the Th1 response and the control of mycobacterial infection. This concept is further supported by the fact that patients with active TB have defective production of IFN-γ *in vitro* that correlates with disease severity and improves with therapy (5–8). The mechanisms underlying this defect have not been fully elucidated. In particular, whether it is associated with a corresponding Th2 response is highly controversial, and conflicting results have been obtained by different groups studying different populations of patients with TB (9–13).

The discovery of Th1 and Th2 lymphocyte functional subsets was originally based on the study of CD4 T-cell clones in both mice and humans (14, 15). In leprosy, poor control of mycobacterial growth is associated with the detection of Th2 clones in skin lesions and Th0 clones producing both IFN-γ and IL-4 in the peripheral blood (16, 17). However, data on cytokine responses at the clonal level in TB are missing.

This study was undertaken to evaluate at the clonal level the balance between Th1 and Th2 responses to mycobacterial antigens in patients with pulmonary TB before and after antimycobacterial therapy. Healthy immune individuals were recruited as control subjects. TB affects populations from both developed and developing countries with different environmental and genetic backgrounds. As these differences could influence the quality of the immune response, the study was conducted simultaneously in Italy and The Gambia. Finally, we evaluated the possibility of achieving Th1 polarization of purified protein derivative (PPD)-reactive and bystander T cells obtained from untreated Gambian patients with TB by exposure of their T cells *in vitro* to IFN-α or IL-12 at the time of antigen presentation.

Materials and Methods

Patients and Control Subjects

This study was approved by the Ethics Committee of Florence University Medical School and the Gambia Government/Medical

Research Council (MRC) Joint Ethics Committee. Six Italian and five Gambian patients with pulmonary TB were recruited at the time of diagnosis from the Department of Internal Medicine of Florence University and the TB clinic of the MRC Laboratories, Fajara, The Gambia, respectively. Pulmonary TB was defined as the presence of acid-fast bacilli in at least two sputum samples confirmed by positive culture for *M. tuberculosis*. Chest X-ray and tuberculin skin test were done at enrollment. Gambian patients (four males and one female; age range, 25–37 yr) had more severe pulmonary disease than did Italian patients (four males and two females; age range, 18–35 yr), with more extensive infiltration of the lungs and cavities. Each of the Italian patients had a single infiltrative lesion, hilar adenopathy, and a positive tuberculin skin test (> 10 mm). In Patients 1, 2, and 3, a moderate pleural effusion was also present. In one Gambian patient (Patient 10), the tuberculin skin test was negative (< 5 mm) and in another one (Patient 9), the result of the test could not be obtained. No patient had signs of extrapulmonary TB. Anti-TB therapy included rifampin, isoniazid, pyrazinamide and etambutol for 2 mo, followed by rifampin and isoniazid three times weekly for another 4 mo. In Gambian patients, therapy was directly observed by leprosy/TB inspectors at the Serrekunda Health Center. At the end of the 6-mo therapy, clinical healing was assessed by chest X-ray, negative sputum smears, and negative culture for *M. tuberculosis*. Four PPD skin test–positive healthy Italians (BCG-vaccinated medical personnel) and five healthy immune (BCG-vaccinated) Gambian adults were enrolled as control subjects. Another group of four Italian patients with TB (three males and one female; age range, 27–46 yr) with isoniazid/rifampin–resistant pulmonary TB, who failed to heal after 4 mo of conventional therapy, were included in this study. All patients and control subjects were human immunodeficiency virus (HIV)-1/2 negative.

Reagents

Phytohemagglutinin (PHA) was purchased from GIBCO Laboratories (Grand Island, NY) and phorbol myristate acetate (PMA) from Sigma Chemical Co. (St. Louis, MO). Recombinant IL-2 (rIL-2) was kindly provided by Eurocetus (Milano, Italy). rIL-12 was kindly supplied by Genetics Institute (Boston, MA). Recombinant IFN-α-2b (rIFN-α-2b) was purchased from Schering Co. (Kenilworth, NJ); PPD was kindly provided by Istituto Sieroterapico e Vaccinogeno Sclavo (Siena, Italy).

Generation of T-Cell Clones

T cells present in the pleural effusions of three untreated Italian patients with TB (Patients 1, 2, and 3) were isolated, and *in vivo*–activated T cells were expanded for 5 d in IL-2–conditioned medium (50 U/ml) and then cloned in the presence of irradiated feeder cells and PHA (1% vol/vol) according to a protocol previously reported (18).

PPD-specific T-cell clones were generated as described previously (15). Briefly, 2×10^6 peripheral blood mononuclear cells (PBMCs) were incubated in the presence of 1 μg/ml PPD in RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% human serum in 24-well, flat-bottomed plates for 6 d. Human rIL-2 (25 U/ml) was added and cultures were continued for 6 d. In experiments involving samples obtained from Gambian patients with TB before therapy, parallel cultures of PBMCs were incubated from day 0 either with PPD alone, PPD + rIFN-α (100 U/ml), or PPD + rIL-12 (100 U/ml). To generate T-cell clones, T-cell blasts were seeded under limiting dilution conditions (0.3 cells/well) in round-bottomed, 96-well plates containing 10^5 irradiated PBMCs (as feeder cells) and PHA (1% vol/vol) in 0.2 ml RPMI 1640 supplemented with rIL-2 (25 U/ml), 2% human serum, and 10% fetal calf serum (Hyclone Laboratories, Logan, UT) as reported (15). Growing microcultures were then supplemented, at weekly intervals, with rIL-2 and 10^5 irradiated feeder cells. The phenotype of T-cell clones was examined by flow cytometry. To assess the antigen specificity of T-cell clones, 4×10^4 T-cell blasts of each clone were cocultured for 60 h with irradiated autologous PBMCs (8×10^4) in the presence of medium alone or PPD (1 μg/ml). Proliferation was measured by pulsing cultures with 0.5 μCi [^{3}H]thymidine for 16 h. Cells were then harvested and radioactivity was measured. A positive response was defined as a mitogenic index higher than 10. By this experimental protocol, each blood sample from each patient or control subject yielded two series of T-cell clones: (*1*) PPD-specific clones derived from PPD-reactive T cells and (*2*) T-cell clones with undefined specificity derived from bystander T cells present in culture.

Induction of Cytokine Production by T-Cell Clones

To induce cytokine production by PPD-reactive T-cell clones, 10^6 T-cell blasts from each clone were incubated with PPD (1 μg/ml) in 1 ml RPMI 1640 with 5×10^5 irradiated autologous non-T cells (15). Supernatants were collected after 48 h, filtered, and frozen at −70°C until cytokine concentrations were assessed. To induce cytokine production in T-cell clones with undefined specificity, 10^6 T-cell blasts from each clone were cultured in the presence of PMA (10 ng/ml) plus anti-CD3 monoclonal antibody (200 ng/ml). After 36 h, culture supernatants were collected, filtered, and stored in aliquots at −70°C until used. Duplicate samples of each clonal supernatant were assayed for IFN-γ, IL-4, and IL-5 with appropriate enzyme-linked immunosorbent assays (BioSource International, Camarillo, CA) as reported (15). Supernatants showing IFN-γ, IL-4, and IL-5 concentrations five standard deviations over the mean concentrations in control supernatants derived from irradiated feeder cells alone were regarded as positive. T-cell clones producing IFN-γ, but not IL4 or IL-5, were categorized as Th1, clones producing IL-4 and/or IL-5 but not IFN-γ were categorized as Th2, and clones producing both IFN-γ and IL-4 or IL-5 were categorized as Th0.

Statistics

The percentage of PPD-specific clones out of all clones and the percentage of clones of undefined specificity out of all clones are expressed by the median percentages. Comparisons between the Gambian and Italian groups for before therapy and after therapy responses are based on the Mann-Whitney U test. The effect of therapy was assessed using the Wilcoxon signed rank test for the Italian and Gambian groups separately. Differences between culture conditions (PPD versus PPD + IFN-α and PPD versus PPD + IL-12) for Gambian patients were assessed using the Wilcoxon signed rank test on paired data.

Results

Predominant Th0 Profile of T Cells Isolated from Pleural Effusions of Untreated Patients with TB

In vivo–activated T cells present in the pleural effusions of three untreated Italian patients with TB could be isolated and cloned in the presence of irradiated feeder cells and PHA. Among a total of 141 CD4$^+$ clones obtained, 27 (19%) proliferated in response to PPD in the presence of irradiated autologous antigen-presenting cells, whereas the antigen specificity of 114 T-cell clones remained undefined. The 27 PPD-specific clones were then stimulated with PPD under major histocompatibility complex–restricted conditions, whereas the 114 clones with undefined specificity were stimulated with PMA plus anti-CD3 antibody. Culture supernatants from both series of clones were assayed for their IFN-γ and IL-4 contents, and each clone

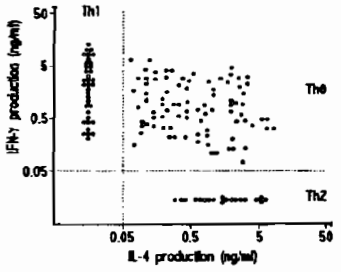

Figure 1. Predominant Th0 profile of T-cell clones isolated from pleural effusions of untreated TB patients. *In vivo*–activated T cells present in the pleural effusions of three untreated Italian TB patients were isolated, expanded in IL-2–conditioned medium, and cloned. Among the 141 $CD4^+$ clones obtained, 27 proliferated in response to PPD (*solid circles*), whereas the antigen specificity of 114 T-cell clones (*open circles*) remained undefined. The 27 PPD-specific clones were then stimulated with PPD, whereas the 114 clones with undefined specificity were stimulated with PMA plus anti-CD3 antibody, and culture supernatants were assayed for their IFN-γ and IL-4 content.

was coded according to its Th1, Th0, or Th2 profile. A Th2 profile was shown by 19 clones (only one being specific for PPD), whereas 36 clones (25.5%) were Th1 (including eight PPD-reactive clones). However, most (61%) of the T-cell clones isolated from pleural effusions (including 18 PPD-specific clones) expressed the Th0 phenotype upon antigen or mitogen activation (Figure 1). Because successful antimycobacterial therapy results in the disappearance of pleural effusion and T cells resident near the site of inflammation cannot be recovered any more, the subsequent experiments were carried out on PPD-induced T-cell lines generated from PBMCs.

Predominant Th0 Responses to PPD before Antimycobacterial Therapy in Both Italian and Gambian Patients with TB

PPD-specific T-cell clones were generated from PBMCs obtained from Italian and Gambian patients with TB before and after antimycobacterial therapy. Before treatment, a median of 21% of all clones generated from PPD-stimulated T cells from Italian patients were reactive to PPD (Table 1). A similar median proportion (31%) was observed among clones from untreated Gambian patients. In both groups, all the PPD-specific clones were $CD4^+$. Before therapy, the majority of PPD-specific clones in both Italian and Gambian patients showed a predominant Th0 profile (production of both IFN-γ and IL-4 and/or IL5) (Table 1), and the proportions of Th1 clones were not significantly different between Italian (median of 27%) and

TABLE 1

Comparison of cytokine profiles of PPD-specific T-cell clones generated from PBMCs of Italian and Gambian patients with TB at diagnosis and after therapy

	Number (%) of PPD-Specific Clones/All Clones Obtained	Number (%) of PPD-Specific Clones Showing the Indicated Cytokine Profile		
		Th1	Th0	Th2
Italian Patients				
1 Pretherapy	5/41 (12)	2 (40)	3 (60)	0
Post-therapy	39/62 (63)	35 (90)	4 (10)	0
2 Pretherapy	14/46 (30)	6 (43)	8 (57)	0
Post-therapy	29/59 (49)	24 (83)	5 (17)	0
3 Pretherapy	4/38 (11)	1 (25)	3 (75)	0
Post-therapy	22/43 (51)	19 (86)	3 (14)	0
4 Pretherapy	7/41 (17)	2 (29)	5 (71)	0
Post-therapy	25/48 (52)	21 (84)	4 (16)	0
5 Pretherapy	8/32 (25)	2 (25)	6 (75)	0
Post-therapy	28/51 (55)	23 (82)	5 (18)	0
6 Pretherapy	16/41 (39)	4 (25)	9 (56)	3 (19)
Post-therapy	15/36 (42)	10 (67)	5 (33)	0
Median % Pretherapy	21*	27‡	66	—
Median % Post-therapy	52†	83§	17	0
	* vs †: p = 0.028	‡ vs §: p = 0.028		
Gambian Patients				
7 Pretherapy	10/42 (24)	3 (30)	6 (60)	1 (10)
Post-therapy	26/53 (49)	14 (54)	12 (46)	0
8 Pretherapy	16/51 (31)	4 (25)	10 (63)	2 (12)
Post-therapy	27/48 (56)	22 (81)	5 (19)	0
9 Pretherapy	13/42 (31)	4 (31)	7 (54)	2 (15)
Post-therapy	29/51 (57)	19 (66)	9 (31)	1 (3)
10 Pretherapy	14/36 (39)	2 (14)	7 (50)	5 (36)
Post-therapy	16/34 (47)	9 (56)	6 (38)	1 (6)
11 Pretherapy	12/44 (27)	3 (25)	6 (50)	3 (25)
Post-therapy	25/47 (53)	15 (60)	9 (36)	1 (4)
Median % Pretherapy	31‖	25**	54	15
Median % Post-therapy	53¶	60††	36	3
	‖ vs ¶: p = 0.041	** vs ††: p = 0.042		

†† vs §: p = 0.009

Gambian (median of 25%) patients. In Italian patients, only a small proportion of PPD-specific clones were Th2 (6%, based on one individual where 3 of 16 PPD-specific clones were Th2), whereas in Gambian patients, the proportion of Th2 clones was higher (median of 15%).

Th1 Polarization of PPD-Specific T-Cell Response in Both Italian and Gambian Patients with TB after Successful Antimycobacterial Therapy

After 6 mo of therapy and clinical healing, the proportion of PPD-specific T-cell clones increased significantly to a median of 52% in both Italian and Gambian patients (Table 1). More importantly, in both populations, successful therapy was associated with a marked shift of PPD-specific clones from the Th0 (or Th2) to the Th1 profile. In Italian patients, the median proportion of Th1 clones was 83% after successful therapy. Interestingly, this profile was very similar to that observed in healthy immune Italian control subjects in whom the median of Th1 PPD-specific clones was 75%, whereas no Th2 clone could be detected (Figure 2). The results observed in Gambian patients with TB were similar. Also in this group, successful therapy was associated with an increase of the proportion of PPD-specific Th1 clones, although the increase was lower than that observed in Italian patients (Table 1, Figure 2). Accordingly, the median proportions of Th0 and Th2 PPD-specific clones from Gambian patients after successful therapy (36 and 3%, respectively) were higher than those found in Italian patients (17 and 0%, respectively) (Table 1). A similar difference between Gambian and Italian subjects was observed when healthy immune controls were compared. Gambian control subjects showed a significantly lower proportion of PPD-specific Th1 clones than did Gambian patients with TB after therapy. In addition, the median proportions of Th0 and Th2 clones were higher for Gambian control subjects (43 and 14%, respectively) than for Italian control subjects (25 and 0%, respectively) (Figure 2).

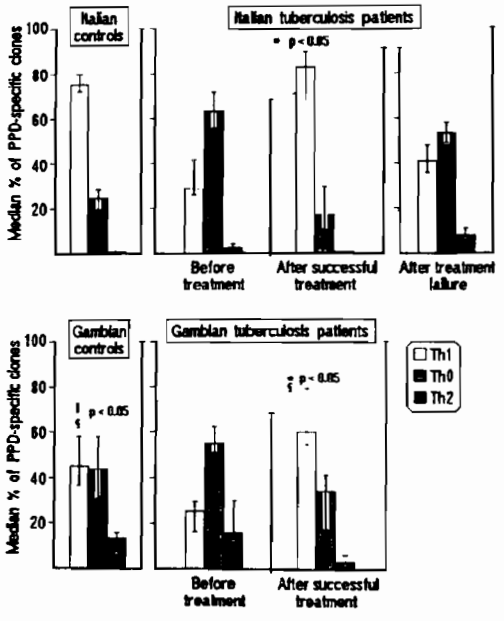

Figure 2. Comparison of cytokine profiles of PPD-specific T-cell clones generated from PPD-stimulated PBMC of Italian and Gambian PPD-reactive healthy control subjects (*left panels*), TB patients before and after successful treatment (*central panels*), and Italian TB patients with treatment failure (*upper right panel*). PPD-specific CD4 T-cell clones recovered were 106 of 171 from Italian healthy controls, 54 of 239 from Italian patients before therapy, 158 of 299 from the same patients after successful therapy, 82 of 198 from Italian patients after treatment failure, 92 of 219 from Gambian healthy controls, 65 of 215 from Gambian patients before therapy, and 123 of 233 after successful therapy. PPD-induced IFN-γ, IL-4, and IL-5 production in culture supernatant by each clone was assayed. *Bars* represent median percentages and *error bars* indicate minimum and maximum percentages. *Successful treatment versus pretreatment and successful treatment versus treatment failure. §Gambian versus the corresponding Italian individuals. ‖Gambian control subjects versus Gambian patients after successful treatment.

Higher Proportions of Th0 and Th2 Clones with Undefined Specificity from Gambian as Compared to Italian Subjects

To further characterize the differences in Th cell responses between Gambian and Italian subjects, we studied the cytokine profile of the clonal progenies of T cells present in the PPD-induced T-cell lines that failed to proliferate *in vitro* in response to PPD. Most clones with undefined specificity obtained from both Italian and Gambian patients before therapy were Th0 (median of 62 and 47%, respectively), similar to their PPD-specific counterparts. After successful therapy, however, the proportion of Th1 clones with undefined specificity increased significantly in the Italian patients (from 24 to 55%), whereas the increase observed in treated Gambian patients with TB was much lower (from 17 to 27%) (Table 2). Consequently, after successful therapy, Gambian patients showed higher proportions of Th0 (64%) and Th2 clones (9%) as compared with the healed Italian patients (Th0, 42%, and Th2, 2%) (Table 2). A similar difference was observed among T-cell clones with undefined specificity obtained from healthy Gambian or Italian control subjects, i.e., higher proportions of Th0 and Th2 clones in Gambian control subjects (53 and 12%, respectively) as compared with Italian control subjects (28 and 3%, respectively) (Figure 3).

Lack of Th1 Polarization of T-Cell Response in Italian Patients with TB Who Failed to Respond to Antimycobacterial Therapy

To further evaluate whether the Th1 polarization of T-cell response was associated with successful treatment of TB, we studied the cytokine profile of the clonal progenies of T cells present in the PPD-induced T-cell lines derived from four Italian patients with drug-resistant TB who failed to respond to therapy. The median proportion of PPD-specific clones isolated from drug-resistant TB patients was not significantly lower than that found in healed patients (44 versus 52%). However, in all the drug-resistant TB patients, the median proportions of both PPD-specific clones (Figure 2) and clones with undefined specificity (Figure 3) showing the Th1 profile were remarkably lower than those found in healed Italian patients (40 versus 83% and 30 versus 55%, respectively). On the other hand, in both the series of clones derived from patients who underwent treat-

TABLE 2

Comparison of cytokine profiles of T-cell clones with undefined specificity generated from PBMCs of Italian and Gambian patients with TB at diagnosis and after therapy

	Number (%) of Clones Undefined Specificity/All Clones Obtained	Number (%) of Clones Undefined Specificity Showing the Indicated Cytokine Profile		
		Th1	Th0	Th2
Italian Patients				
1 Pretherapy	36/41 (88)	9 (25)	23 (64)	4 (11)
Post-therapy	23/62 (37)	11 (48)	11 (48)	1 (4)
2 Pretherapy	32/46 (70)	10 (31)	17 (53)	5 (16)
Post-therapy	30/59 (51)	17 (57)	12 (40)	1 (3)
3 Pretherapy	34/38 (89)	5 (15)	24 (70)	5 (15)
Post-therapy	21/43 (49)	11 (52)	10 (48)	0
4 Pretherapy	34/41 (83)	8 (23)	21 (62)	5 (15)
Post-therapy	23/48 (48)	12 (52)	10 (44)	1 (4)
5 Pretherapy	24/32 (75)	5 (21)	16 (67)	3 (12)
Post-therapy	23/51 (45)	15 (65)	8 (35)	0
6 Pretherapy	25/41 (61)	8 (32)	14 (56)	3 (12)
Post-therapy	21/36 (58)	16 (76)	5 (24)	0
Median % Pretherapy	79*	24‡	62	14
Median % Post-therapy	49†	55§	42	2
	* vs †: p = 0.028	‡ vs §: p = 0.027		
Gambian Patients				
7 Pretherapy	32/42 (76)	9 (28)	15 (47)	8 (25)
Post-therapy	27/53 (51)	5 (19)	19 (70)	3 (11)
8 Pretherapy	35/51 (69)	11 (31)	16 (46)	8 (23)
Post-therapy	21/48 (44)	10 (48)	10 (48)	1 (4)
9 Pretherapy	29/42 (69)	5 (17)	16 (55)	8 (28)
Post-therapy	22/51 (43)	6 (27)	15 (68)	1 (5)
10 Pretherapy	22/36 (61)	3 (14)	10 (45)	9 (41)
Post-therapy	18/34 (53)	5 (28)	9 (50)	4 (22)
11 Pretherapy	32/44 (73)	4 (12)	23 (72)	5 (16)
Post-therapy	22/47 (47)	6 (27)	14 (64)	2 (9)
Median % Pretherapy	69‖	17**	47	25
Median % Post-therapy	47¶	27††	64	9
	‖ vs ¶: p = 0.041	** vs ††: p = 0.08		

¶ vs ††: p = 0.004

ment failure, the median proportions of Th0 (52 and 57%) and Th2 (7 and 12%) clones were as high as those observed in Italian patients before treatment (Figures 2 and 3).

In Vitro Th1 Polarization of T-Cell Clones from Gambian Patients with TB Induced by IFN-α or IL-12

Taken together, the data reported previously suggested that successful therapy resulted in Th1 polarization of both the PPD-specific and the bystander T-cell responses in Italian patients with TB. In contrast, in Gambian patients only PPD-specific T cells underwent Th1 polarization, whereas the general bystander T-cell responses tended to maintain the same high potential of Th2 cytokine production present in both untreated patients and healthy control subjects. Therefore, we asked whether such Th0/Th2–biased T-cell responses of Gambian patients could be modulated *in vitro* by IFN-α or IL-12, two cytokines able to induce preferential Th1 differentiation of responding T cells (19, 20). To this end, PBMCs from four untreated Gambian patients with TB were stimulated with PPD in the absence or presence of IFN-α or IL-12, and the cytokine profiles of their clonal progenies were assessed. As shown in Table 3, similar median proportions of PPD-specific T-cell clones were obtained from the T-cell lines generated in the presence of PPD + IFN-α (22%), PPD + IL-12 (24%), or PPD alone (29%). As expected, both IFN-α and IL-12 induced a marked Th1 polarization in the clonal progenies of PPD-specific T cells, with concomitant decrease of Th0 and Th2 PPD-specific clones. Higher median proportions of PPD-specific Th1 clones were obtained from T-cell lines generated in the presence of PPD + IFN-α (75%) or PPD + IL-12 (75%) as compared with PPD alone (25%). Interestingly, a similar Th1-polarizing effect was induced by both IFN-α and IL-12 in bystander T cells, whose clonal progenies showed a strong increase in the median proportions of Th1 clones (PPD + IFN-α, 73%; PPD + IL-12, 80%; PPD alone, 21%).

Discussion

This study demonstrates that patients with pulmonary TB have a predominant Th0-type, PPD-specific T-cell response, i.e., characterized by the production of both IFN-γ and IL-4/IL-5 in both the pleural cavity near the site of in-

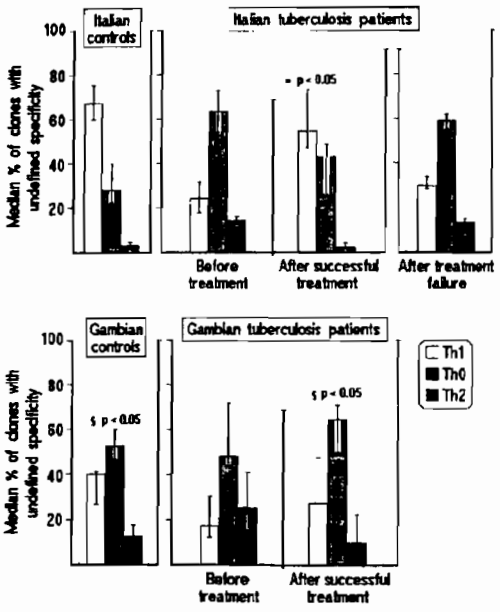

Figure 3. Comparison of cytokine profiles of T-cell clones with undefined specificity generated from PPD-stimulated PBMCs of the same subjects shown in Figure 2. The total numbers of CD4 T-cell clones with undefined specificity recovered were 65 from Italian healthy controls (*upper left panel*), 185 from Italian patients before therapy and 141 from the same patients after successful therapy (*upper central panels*), 116 from Italian patients with treatment failure (*upper right panel*), 127 from Gambian healthy controls (*lower left panel*), 150 from Gambian patients before therapy, and 110 after successful therapy (*lower central panels*). Cytokine production in culture supernatants of these T-cell clones with undefined specificity was induced by PMA plus anti-CD3 monoclonal antibody. *Bars* represent median percentages and *error bars* indicate minimum and maximum percentages. *Successful treatment versus pretreatment and successful treatment versus treatment failure. §Gambian versus the corresponding Italian individuals.

fection and peripheral blood. Healing induced by antimycobacterial therapy was associated with a shift of the T-cell reactivity to PPD toward a predominant Th1 response, resulting in a pattern resembling that found in healthy immune individuals. These data extend studies showing that patients with active TB have poor production of IFN-γ that is, however, augmented by antimycobacterial therapy (5–8). Whether decreased production of IFN-γ in untreated patients with TB associates with a concomitant increase in the production of Th2 cytokines is still controversial (9–13). The results of this study show that in active TB, the T-cell response to mycobacterial antigens includes the activation of a remarkable proportion of IL-4–producing effector T cells and that, at least in some patients, even Th2 clones specific to PPD can be detected. Several factors may account for the conflicting results obtained by different investigators. Our results are likely to be explained by the sensitivity of the clonal approach used (21). In experiments using cultures of unfractionated PBMCs, failure of detecting IL-4 in culture supernatants may be ascribed to the sensitivity of the assays used, to the IL-4 consumption by bystander cells present in culture, or to cross-inhibitory circuits. Interestingly, however, studies in which IL-4 expression could be easily detected included patients with severe disease or coming from developing countries (9, 11).

As our patients were enrolled at the time of diagnosis, we do not know whether the Th0 dominance was present before the disease onset. It is conceivable that both genetic and environmental factors may favor the development of a Th0 response to mycobacteria, which could in turn influence the disease susceptibility. The fact that all patients showed an increase in their Th1 response after successful anti-mycobacterial therapy suggests that they were not genetically conditioned to mount only Th0 responses. Another possibility is that patients with TB develop a predominant Th0 response during the course of the disease. This could be related to mechanisms favoring either the preferential expansion of Th0 cells or the decrease of the Th1 population. Recent data indicate that apoptosis of CD4 T cells could play an important role in the defective IFN-γ production observed in both TB patients (8, 22) and experimental TB (23). Indeed, several studies have shown that Th1 clones are more susceptible to apoptosis than are Th2 clones (24–26). Thus, it is possible that the Th0 dominance observed in patients with active TB is related to the death of a number of Th1 cells. Alternatively, the decreased Th1 function could also be related to suppressive cytokines. Transforming growth factor-β and IL-10 are produced during active TB and can play a role in the poor production of IFN-γ (7, 27, 28). Finally, it cannot be excluded that Th1 cells migrate more efficiently than do Th0 cells to the site of disease and are therefore not accessible through sampling of peripheral blood (29, 30). Adams and coworkers (31) recently reported that patients with TB in South Africa have elevated levels of serum immunoglobulin E (an IL-4/IL-13–dependent immunoglobulin class) that are decreased with therapy. These findings are not in conflict with our observations and suggest that the IL-4 activity of the Th0 and Th2 responses observed in the peripheral blood of patients with TB before therapy, as well as the increase of Th1 activity after healing, are not relative, rather they reflect the global immune response to the pathogen.

This study represents the first comparison of the Th1/Th2 balance between European and African individuals. Gambian individuals showed higher proportions of Th0 and Th2 PPD-specific clones as compared with Italian subjects. This Th0/Th2 bias was observed in both patients with TB and healthy immune individuals. In addition, the degree of Th1 polarization of the PPD-specific T-cell response after antimycobacterial therapy was lower in Gambian patients. Several mechanisms may be involved in this Th0/Th2 bias. First, the genetic background of the host plays an important role in regulating the immune response to infectious pathogens. Because the TB epidemic is thought to have started more recently in Sub-Saharan Africa than in Europe (32), it is possible that the Italian and the Gambian populations differ in the frequency of gene alleles involved in the control of the Th1/Th2 balance. Second, exposure to mycobacterial antigens is likely to happen much earlier in life in The Gambia, where BCG is given at birth, than in Italy, where environmental mycobacteria are less common and BCG is not given to newborns. We are cur-

TABLE 3

In vitro *effect of IFN-α or IL-12 on the cytokine profile of PPD-specific and nonspecific clones obtained from untreated Gambian patients with TB*

Patients	Culture Conditions	Number (%) of PPD-Specific Clones/All Clones Obtained	Number (%) of PPD-Specific Clones Showing the Indicated Cytokine Profile			Number (%) of Clones with Undefined Specificity/ All Clones Obtained	Number (%) of Clones with Undefined Specificity Showing the Indicated Cytokine Profile		
			Th1	Th0	Th2		Th1	Th0	Th2
7	PPD	10/42 (24)	3 (30)	6 (60)	1 (10)	32/42 (76)	9 (28)	15 (47)	8 (25)
	PPD + IFN-α	7/37 (19)	5 (71)	2 (29)	0	30/37 (81)	22 (73)	8 (27)	0
	PPD + IL-12	12/46 (26)	10 (83)	2 (17)	0	34/46 (74)	30 (88)	4 (12)	0
8	PPD	16/51 (31)	4 (25)	10 (63)	2 (12)	35/51 (69)	11 (31)	16 (46)	8 (23)
	PPD + IFN-α	9/45 (20)	7 (78)	2 (22)	0	36/45 (80)	29 (81)	7 (19)	0
	PPD + IL-12	6/39 (15)	4 (67)	2 (33)	0	33/39 (85)	23 (70)	9 (27)	1 (3)
10	PPD	14/36 (39)	2 (14)	7 (50)	5 (36)	22/36 (61)	3 (14)	10 (45)	9 (41)
	PPD + IFN-α	10/41 (24)	6 (60)	3 (30)	1 (11)	31/41 (76)	20 (64)	8 (26)	3 (10)
	PPD + IL-12	11/47 (23)	7 (64)	3 (27)	1 (9)	36/47 (77)	26 (72)	8 (22)	2 (6)
11	PPD	12/44 (27)	3 (25)	6 (50)	3 (25)	32/44 (73)	4 (12)	23 (72)	5 (16)
	PPD + IFN-α	9/38 (24)	7 (78)	2 (22)	0	29/38 (76)	21 (72)	7 (24)	1 (3)
	PPD + IL-12	13/51 (25)	11 (85)	2 (15)	0	38/51 (75)	34 (89)	4 (11)	0
Median %	PPD	29	25*	55	19	71	21§	47	24
	PPD + IFN-α	22	75†	26	0	78	73‖	25	2
	PPD + IL-12	24	75‡	22	0	76	80¶	17	2

* vs †/‡: $p = 0.07$
§ vs ‖/¶: $p = 0.07$

rently observing that the IFN-γ response induced by BCG vaccination in Gambian newborns is related to the activation of T cells showing a predominant Th0 profile (Vekemans and associates, submitted manuscript). Such a Th0 response induced by BCG in early life in Gambian subjects could persist and be responsible for the predominant Th0 response to PPD observed in healthy immune individuals in adulthood. A third mechanism may be the effect of helminth infection. Bentwich and colleagues (33) have hypothesized that the high prevalence of helminthiasis in developing countries could bias immune responses to other pathogens to the Th2 type and thereby impair protective immunity against mycobacteria. The present data obtained in T-cell clones of undefined specificity, in which the proportions of Th0 and Th2 clones were higher among Gambian in comparison to Italian subjects, may support this possibility, suggesting that populations living in developing countries are biased to preferential Th0/Th2 responses to a large number of antigens. It should be stressed that the Th phenotype of clones with undefined specificity is likely to have been influenced by the cytokine milieu generated in culture by the activated PPD-specific T cells (15). If the final outcome is represented by an abundant population of Th0/Th2 clones, one may reasonably suspect that PPD stimulation at the beginning of cultures resulted in substantial production of IL-4 and/or poor secretion of IL-12, or both (20). The observation that the bystander T-cell clones failed to polarize to Th1 in healed Gambian patients is difficult to interpret. Although the median amount of IFN-γ secreted *in vitro* by Th1 and Th0 clones from Gambian patients (2,550 pg/ml/10^6 T cells) was lower and the median amount of IL-4 (1,120 pg/ml) secreted by Gambian Th0 and Th2 was higher than those produced by the corresponding clones from Italians (median IFN-γ, 3,008 pg/ml; median IL-4, 933 pg/ml), we are reluctant to translate from the *in vitro* concentrations to the *in vivo* situation because there are innumerable factors that could contribute to those differences.

Whether increased IL-4 production may be related to the severity of the disease (10, 11) and whether the Th0 response may play a role in the pathophysiology of TB, still remains unclear. In patients with lepromatous leprosy, poor control of mycobacterial growth is associated with a Th0 response in the peripheral blood and a Th2 response in the skin lesions (16, 17). In TB, disease severity could also be associated with a Th0 response. The clinical presentations of our Gambian patients with TB were more severe and their Th0/Th2 responses were stronger compared with the Italian patients, but the two observations may well be unrelated. However, in both Italian and Gambian patients, healing was associated with a shift toward Th1 responses. Work is in progress in a large prospective study of patients with TB and contacts in Italy and West Africa based on the use of serologic markers of preferential Th1 or Th2 activation to assess whether high Th2-oriented responses favor TB susceptibility and/or increased disease severity (Lienhardt and colleagues, manuscript in preparation).

Because lack of Th1 polarization associates with treatment failure, we hypothesize that a Th0/Th2 response in TB, even if not detrimental, represents an inefficient mechanism to induce pathogen clearance and healing; thus, immunotherapeutic approaches resulting in prompt Th1 polarization of the T-cell responses could have a positive effect on the treatment of TB. In support of this, we found that the Th2-biased T-cell responses of Gambian patients could be downregulated *in vitro* by IFN-α or IL-12, which are well known for their capacity to induce preferential Th1 differentiation in responding T cells (19, 20, 34). These

data are in keeping with the observation that IL-12 improves IFN-γ production by PBMCs obtained from patients with TB (28). These *in vitro* correlates may encourage the use of IFN-α and IL-12 in the immunotherapy of TB. Clinical improvement has indeed been achieved with IFN-α administration to some patients with drug-sensitive or MDR TB (35, 36). Moreover, clinical success was achieved in one patient with IFN-γ-refractory pulmonary *Mycobacterium abscessus* by IL-12 administration (2). Further studies are required to evaluate the use of these cytokines in the immunotherapy of TB. We are currently conducting a randomized controlled trial on the safety and immunogenicity of rIL-12 in Gambian patients with drug-sensitive pulmonary TB.

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