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Impaired Gamma Interferon Response to *Mycobacterium vaccae* Antigens in Patients with Cavitary Pulmonary Tuberculosis▽

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The ability of tuberculosis patients to recognize *Mycobacterium vaccae*-specific antigens before starting chemotherapy and according to disease severity was analyzed. We report that the *M. vaccae* cell wall skeleton fraction triggers more enhanced cytokine production than the whole bacterium. Moreover, a tendency was observed for a lower gamma interferon/interleukin-10 ratio in patients with cavitary disease induced by *M. vaccae* antigens.

The saprophytic *Mycobacterium vaccae* is being tested as an immunotherapeutic agent in tuberculosis (TB). It is supposed to boost a cross-protective immune response to epitopes shared with *Mycobacterium tuberculosis*. However, the efficiency of a single injected dose of *M. vaccae* varied between different clinical trials (14). The causes of this variability are not clear, but the immune status background of the patients (11, 14) or differences in the disease severity (6) could have been influential. Evidence from some of these trials suggests that *M. vaccae* immunotherapy induces an enhancement of protective gamma interferon (IFN-γ) levels, together with a reduction in circulating interleukin-10 (IL-10) levels (3). In fact, a reverse relationship between the *M. tuberculosis* antigen-induced IFN-γ/IL-10 ratio and disease severity in TB (5) and also a direct relationship between the IFN-γ/IL-10 ratio with TB cure (1, 12) have been demonstrated in a large-scale analysis. However, no data are available on the ability of the immune systems of TB patients to recognize *M. vaccae*-specific antigens before starting anti-TB chemotherapy and according to TB severity.

Herein, we analyze the IFN-γ- and IL-10-induced responses in peripheral blood mononuclear cells (PBMC) of TB patients, using both *M. tuberculosis* and, for the first time, *M. vaccae* and its cell wall skeleton (CWS) fraction. We have recently demonstrated that the CWS fraction elicits a prominent recall IFN-γ response in splenocyte cultures from mice with TB in comparison with the whole bacterium (10).

Blood samples were collected from adult pulmonary TB patients admitted to the Tuberculosis Unit-Infectious Diseases Service of the Bellvitge Hospital (Spain), before starting chemotherapy. In all cases the disease was confirmed by isolation of *M. tuberculosis* in cultures. All patients were tuberculin skin test positive and had not been previously *Mycobacterium bovis* BCG vaccinated. None of the patients had human immunodeficiency virus coinfection, *M. tuberculosis* reinfection, or other diseases that could affect the immune response. According to the presence of lung cavitation at the time of chest X-ray examination, the patients were classified as affected by non-cavitary ($n = 4$ patients) or cavitary ($n = 9$ patients) pulmonary TB. Ten blood samples were also taken from healthy tuberculin skin test-negative employees at the hospital. The study was approved by the Ethics Committee of Clinical Investigation of Bellvitge Hospital, and written informed consent was obtained from all participants.

Cells of a stable rough variant of *M. vaccae* strain ATCC 15483^T and *M. tuberculosis* H37Rv (ATCC 27294^T) were heat killed and diluted as described previously (9). The CWS fraction was obtained as described previously (10). All antigens were frozen at −40°C until use. Isolated PBMC were cultured at 2×10^6 cells/ml in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and were stimulated using phytohemagglutinin (Sigma, St. Louis, MO) at 1:300 as a positive control, *M. tuberculosis* (10 μg/ml), *M. vaccae* (10 μg/ml), CWS (5 μg/ml), or medium alone as a negative control. Culture supernatants were collected after 48 h of culture (for IL-10 quantification) or 120 h (for IFN-γ), and cytokine concentrations were measured by enzyme-linked immunosorbent assays using commercially available kits (Mabtech AB, Stockholm, Sweden).

Cytokine levels were compared using a two-tailed Mann-Whitney rank sum or Wilcoxon signed rank test, linear regression analysis, and Spearman's rank correlation, as indicated in the figure legends and table footnotes. A P value of <0.05 was considered significant.

Analyzing the response for each TB patient individually, we observed a strong positive correlation between *M. tuberculosis*- and *M. vaccae*-induced IFN-γ production ($r = 0.937$; $P < 0.001$) and IL-10 production ($r = 0.923$; $P < 0.001$) and also between *M. tuberculosis*- and CWS-induced IFN-γ production ($r = 0.916$; $P < 0.001$) and IL-10 production ($r = 0.895$; $P < 0.001$). When comparing *M. vaccae*- and CWS-induced IFN-γ or IL-10 production, there was again a significant positive correlation in each TB patient (Fig. 1). However, although each patient reacted in the same way to both *M. vaccae* and CWS, remarkably, the CWS not only retained the immune-

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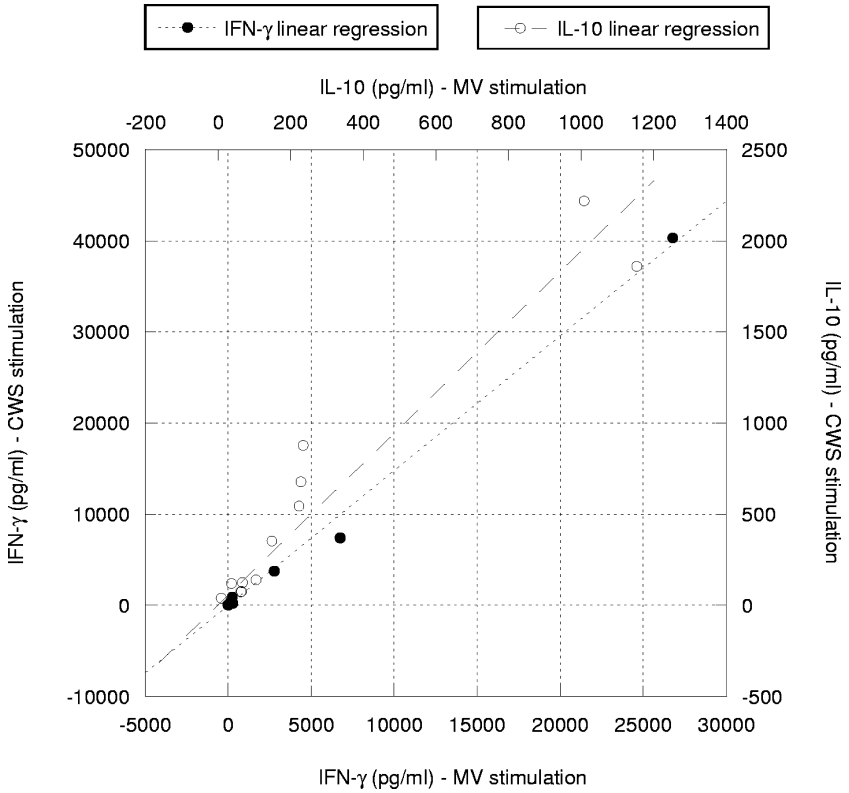

FIG. 1. Correlation of IFN-γ and IL-10 production in TB patients for each stimulus (*M. vaccae* and CWS antigens). A significant correlation was found between IFN-γ and IL-10 production after in vitro stimulation with the *M. vaccae* or CWS antigen. This was confirmed by Spearman's rank correlation coefficients ($r = 0.93$ and $P < 0.001$ for IFN-γ production; $r = 0.972$ and $P < 0.001$ for IL-10 production).

stimulatory ability of the whole bacterium but also triggered enhanced cytokine production in TB patients (Table 1). In all cases the cytokine levels in cultures from healthy controls were lower than those obtained from TB patients (Table 1).

Our results also showed different patterns of responses to *M. vaccae* and CWS antigens among TB patients. Seven out of 12 patients showed impaired IFN-γ production compared to IL-10 production, i.e., samples F to L (Table 1), showing an

TABLE 1. IFN-γ and IL-10 production in PBMC cultures from pulmonary TB patients and healthy subjects

Subject(s)	Cytokine production induced by indicated antigen						IFN-γ/IL-10 production ratio[b]			TB form
	IFN-γ (pg/ml)[a]			IL-10 (pg/ml)[a]						
	M. tuberculosis	*M. vaccae*	CWS	*M. tuberculosis*	*M. vaccae*	CWS	*M. tuberculosis*	*M. vaccae*	CWS	
TB patients										
A	162,250.4	26,786.6	40,313.7	2,407.0	1,008.9	2,218.3	67.4	26.6	18.2	Cavitary
B	32,222.2	2,791.5	3,768.4	424.0	148.4	352.6	76.0	18.8	10.7	Noncavitary
C	22,423.7	6,742.5	7,394.9	379.3	228.7	675.9	59.1	29.5	10.9	Noncavitary
D	3,143.0	258.2	922.0	210.0	68.0	125.3	15.0	3.8	7.4	Noncavitary
E	1,001.0	270.9	229.9	256.4	63.6	73.4	3.9	4.3	3.1	Cavitary
F	2,758.8	47.8	74.7	2,272.4	1,153.0	1,859.9	1.2	0.0	0.0	Noncavitary
G	1,423.1	94.5	185.8	740.4	223.3	543.4	1.9	0.4	0.3	Cavitary
H	526.2	2.8	26.6	187.7	105.9	138.9	2.8	0.0	0.2	Cavitary
I	325.9	13.8	7.9	861.4	235.2	876.1	0.4	0.1	0.0	Cavitary
J	155.8	0.9	0.0	45.3	10.0	37.6	3.4	0.1	0.0	Cavitary
K	27.8	0.7	1.0	131.5	66.2	75.1	0.2	0.0	0.0	Cavitary
L	25.9	0.0	10.6	85.8	37.5	119.4	0.3	0.0	0.1	Cavitary
Median[c]	1,212.0[&,¶]	71.1	130.3*	317.9[&]	127.1	245.8*				
Healthy subjects (median)	61.5	20.6	10.7	101.3	57.8	79.7				

[a] Minimum detectable concentrations were 2 pg/ml for IFN-γ and 0.5 pg/ml for IL-10.
[b] Ratios are the average IFN-γ values divided by the IL-10 value for each individual (5).
[c] Median levels of CWS-induced cytokines were significantly increased compared to those produced by *M. vaccae* (*, $P < 0.05$). Median levels of *M. tuberculosis*-induced cytokines were higher than those produced by *M. vaccae* (&, $P < 0.001$) or CWS (¶, $P < 0.001$), measured using the two-tailed Wilcoxon signed rank test.

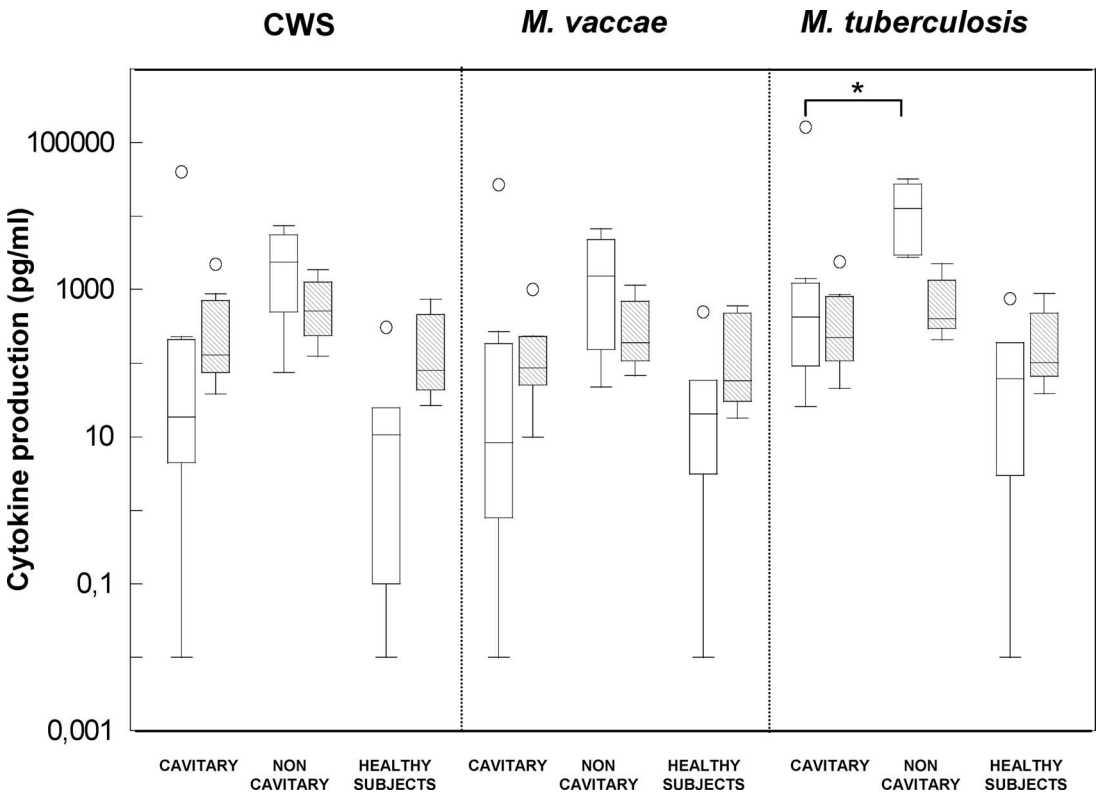

FIG. 2. IFN-γ and IL-10 production in pulmonary TB patients and healthy controls in response to CWS, *M. vaccae*, or *M. tuberculosis* antigens. The boxes extend from the 25th to the 75th percentiles, with a line at the median, and the whiskers show the highest and lowest values. Empty boxes represent IFN-γ production and scattered boxes show IL-10 production. A dot represents one out-of-order value. Median levels of *M. tuberculosis*-induced IFN-γ in noncavitary TB patients were significantly higher than those produced in cavitary TB patients (*, $P < 0.05$; measured using the two-tailed Mann-Whitney rank sum test).

IFN-γ/IL-10 index lower than 1. However, the remainder of the patients (samples A to E) had an index higher than 1. When we correlated these results with the radiographic manifestation of the disease, a tendency towards a lower IFN-γ/IL-10 ratio in patients with cavitary disease was observed, i.e., 85.7% of the patients (six out of seven) with a ratio lower than 1 had cavitation in their lungs (Table 1).

A clear increase in cytokine levels in noncavitary versus cavitary TB patients was observed (Fig. 2). Although the median ratio showed a log difference among groups, the differences were statistically significant only in the case of *M. tuberculosis*-induced IFN-γ detection (Fig. 2). Although the specific cytokine response against *M. vaccae* antigens in cavitary and noncavitary TB patients has not been studied before, diminished Th1 responses to *M. tuberculosis* antigens in PBMC from TB patients have been directly correlated with the severity of the disease (2, 8, 13, 15). Our findings further support these previous observations (Fig. 2). This is an interesting point because one of the initially reported benefits of *M. vaccae* immunotherapy was the improvement in chest radiographic healing or cavity closure (14). Johnson et al. (7) specifically analyzed the effect of one injected dose of *M. vaccae* on radiographic response in three large trials and did not observe any benefit. On the contrary, studies in which three or five doses of *M. vaccae* were administered showed its efficacy for healing and closing cavities in TB patients (4, 14). These previous studies together with our results lead us to believe that patients showing an impaired *M. vaccae*-induced IFN-γ response in vitro will not respond to one dose of *M. vaccae* administration in vivo. Therefore, the in vitro analysis of the *M. vaccae*-specific immune responses in each TB patient could be indicative of the outcome of the in vivo administration, which may be of interest in understanding the unsuccessful results and the requirement of many injected doses among patients whose Th1-type responses are decreased.

Although larger studies are needed to confirm these preliminary findings, our results showed that the CWS fraction appeared to be a possible immunotherapeutic agent candidate, confirming the previous results obtained in mouse experiments (10). Furthermore, the results suggest an association between the in vitro reactions to *M. vaccae* antigens with disease severity, suggesting a different capability to respond to in vivo immunotherapy.

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