Mice with Pulmonary Tuberculosis Treated with *Mycobacterium vaccae* Develop Strikingly Enhanced Recall Gamma Interferon Responses to *M. vaccae* Cell Wall Skeleton[∇]

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Whole heat-killed *Mycobacterium vaccae* is used as an immunotherapeutic agent in tuberculosis (TB), but the compound(s) that triggers its immunostimulatory ability is not known. Here, we show that among different subcellular fractions, the cell wall skeleton induced a prominent expression of gamma interferon in splenocytes from both non-TB and TB *M. vaccae*-treated mice.

Immunotherapy agents for tuberculosis (TB) should down-regulate or suppress the Th2 component and potentiate the Th1 response to manifest a more protective antibacterial immunity (14). The induction of a favorable cytokine profile, together with current chemotherapy, could improve the killing of *Mycobacterium tuberculosis*. Accordingly, it was shown that the immunization of mice with heat-killed *Mycobacterium vaccae* induces a predominantly Th1 cytokine profile and protection against *M. tuberculosis* (5). Furthermore, treatment with heat-killed *M. vaccae* after intratraqueal infection with *M. tuberculosis* also caused a 1- to 2-log-unit decrease in bacterial counts (6). In humans, numerous clinical trials using heat-killed suspensions of *M. vaccae* have been carried out (12), but to date, the main conclusion drawn is that its efficacy varies between individuals and between populations studied (13).

It would be interesting to find out which *M. vaccae* compounds induce the favorable cytokine profile. Using more purified components rather than the whole microorganism would be advantageous for avoiding the presence of immunosuppressive and/or toxic molecules or, from a pharmaceutical perspective, for achieving better standardization of the doses.

Here, we analyzed the antigenicities of different subcellular *M. vaccae* fractions by determining the cytokine production triggered by these fractions in splenocyte cultures from both noninfected mice (non-TB mice) and *M. tuberculosis*-infected mice (TB mice) that were recently treated with *M. vaccae* or given control treatment.

Initially, a rough variant of *M. vaccae* ATCC 15483[T] (used in some human studies) (12) was grown on tryptone soy agar medium, collected, and heat killed at 121°C for 15 min. It was then fractionated following a classic protocol (3). The first fraction contained mainly noncovalently attached lipids and

glycolipids, which were controlled by thin-layer chromatography (9). The second fraction, the cell wall skeleton (CWS), contained the mycolyl-arabinogalactan-peptidoglycan complex (3). Finally, the third fraction was composed of glycans, lipoglycans, and proteins (G+P). The analysis of the last fraction showed a constant content of sugars and proteins (18 to 21% and 9 to 11%, respectively) (7, 10) in all *M. vaccae* fractionations carried out. All fractions were frozen at −40°C until they were used.

Four groups of 6- to 8-week-old specific-pathogen-free BALB/c mice (Charles River, L'Arbresle Cedex, France) were studied. The first group, non-TB mice, received only phosphate-buffered saline. In the second group, *M. vaccae*-treated non-TB mice were treated with three doses of 10^7 or 10^9 *M. vaccae* bacteria (11) intraperitoneally at weeks 0, 2, and 6 and were sacrificed at week 8. The third group, untreated TB mice, were infected with *M. tuberculosis* H37Rv (ATCC 27294[T]) (2). They were sacrificed 16 weeks after infection. In the last group (*M. vaccae*-treated TB mice), TB mice were treated 8 weeks after infection with *M. vaccae*, following the same protocol as for non-TB mice. All experimental procedures were approved by the Ethical Committees for Animal Experimentation at our institutions. After the mice were sacrificed by cervical dislocation, splenocytes were cultured at 10^6 cells/ml as previously described (9). The cells were stimulated with concanavalin A (2.5 µg/ml; Sigma Chemical Co., St. Louis, MO), *M. vaccae*, or the different fractions (20 µg/ml) or with medium alone as a negative control. Culture supernatants were collected and cytokine concentrations were measured by enzyme-linked immunosorbent assays (ELISAs) using commercially available kits (OptEIA Set, Becton Dickinson Biosciences, New Jersey, and ELISA kits, Mabtech AB, Nacka Strand, Sweden) according to the manufacturer's instructions.

Mann-Whitney rank sum tests (Sigma Stat; SPSS Software) were used to perform statistical comparisons. A *P* value of <0.05 was considered significant.

In non-TB mice (Fig. 1), *M. vaccae* and the CWS and G+P fractions triggered a higher production of gamma interferon

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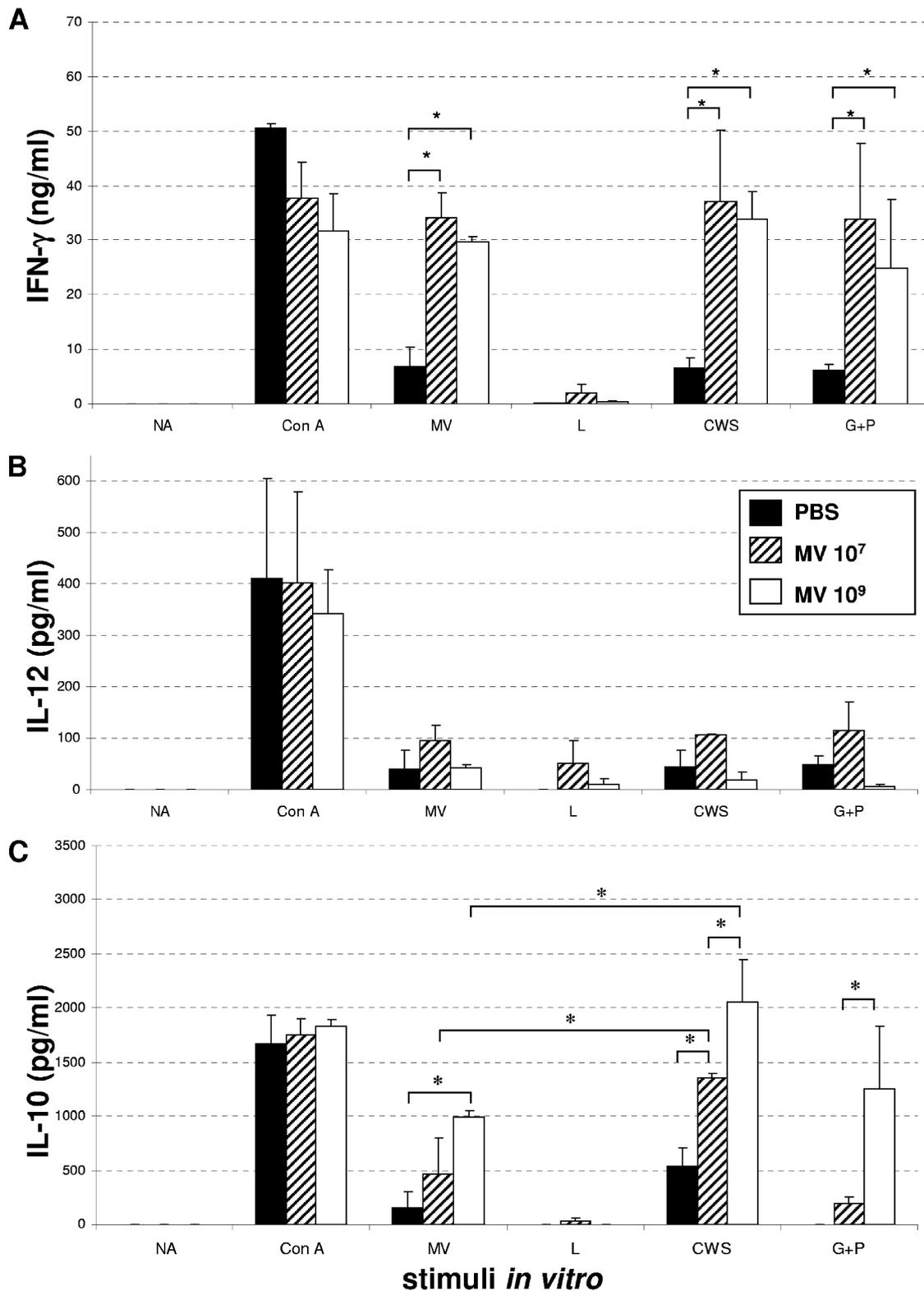
Groups of non-TB mice

FIG. 1. Higher IFN-γ and IL-10 production in splenocyte cultures stimulated with *M. vaccae* and CWS and G+P fractions in *M. vaccae*-treated mice compared to nontreated mice. Cytokine concentrations were measured in culture supernatants by ELISA. IL-12(p70) was measured after 48 h of culture and IFN-γ and IL-10 after 72 h. The data are shown as mean values plus standard deviations obtained from four mice per group. PBS, mice treated with PBS; MV 10[7], mice treated with 10[7] cells of *M. vaccae*; MV 10[9], mice treated with 10[9] cells of *M. vaccae*; NA, nonantigen; ConA, concanavalin A; MV, heat-killed *M. vaccae*; L, lipids. ∗, $P < 0.05$; Mann-Whitney rank sum test.
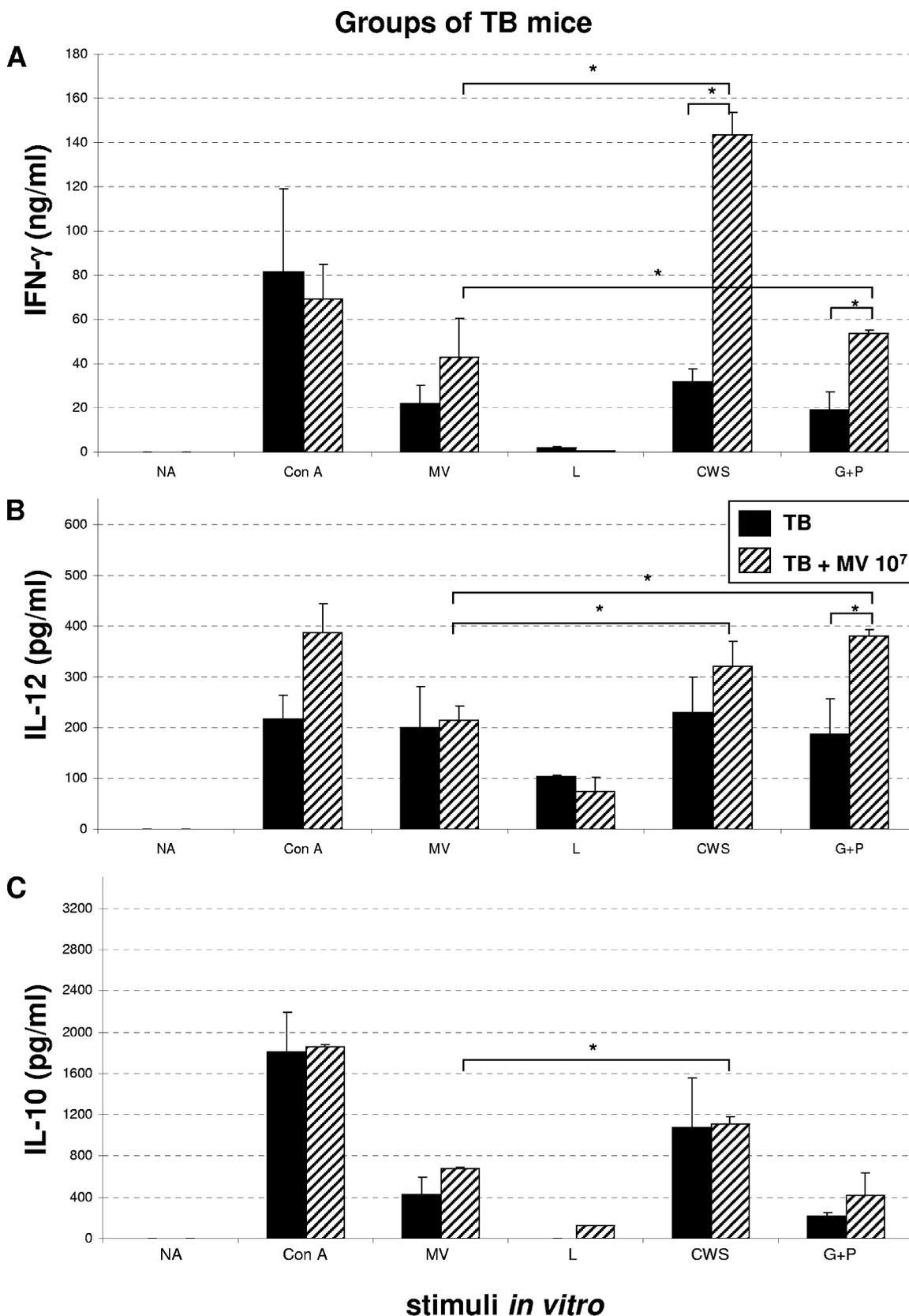
FIG. 2. Prominent IFN-γ production in splenocytes of *M. vaccae*-treated TB mice stimulated in vitro with CWS compared to that in splenocytes of *M. vaccae*-treated TB mice stimulated with *M. vaccae*. Concentrations were measured in culture supernatants by ELISA. The data are shown as mean values plus standard deviations obtained from four mice per group. TB, mice infected with *M. tuberculosis*; TB + MV 10^7, mice infected with *M. tuberculosis* and treated with 10^7 cells of *M. vaccae*. NA, nonantigen; ConA, concanavalin A; MV, heat-killed *M. vaccae*; L, lipids. ∗, $P < 0.05$; Mann-Whitney rank sum test.

(IFN-γ) and interleukin 10 (IL-10) in cultures from *M. vaccae*-treated mice than in those from non-*M. vaccae*-treated mice ($P < 0.05$). The proinflammatory/anti-inflammatory (IFN-γ/IL-10) ratio (8) obtained in cultures of mice treated with 10^7 *M. vaccae* cells was higher than in those from animals treated with 10^9 cells when cultures were stimulated with *M. vaccae* (121 versus 30, respectively), CWS (27 versus 17), and G+P (177 versus 20). Thus, the TB mice were treated only with the 10^7-*M. vaccae*-cell dose.

In TB mice, *M. vaccae*, CWS, and G+P induced higher IFN-γ production in cultures from *M. vaccae*-treated TB mice than in untreated TB mice (Fig. 2). When the responses of antigens in *M. vaccae*-treated TB mice were compared, remarkably, CWS-induced IFN-γ production was 3.3 times higher (143.5 ± 10.1 ng/ml) than that induced by *M. vaccae* (43.1 ± 17.7 ng/ml). CWS-induced IL-10 production was higher than that triggered by *M. vaccae* (1.6 times) (Fig. 2C). The IFN-γ/IL-10 ratio was clearly higher in CWS- and G+P-stimulated cultures (128 and 129, respectively) than in *M. vaccae*-stimulated cultures (a ratio of 63) in *M. vaccae*-treated TB mice.

Our results show that the CWS fraction provides antigens for recall of adaptive T-cell IFN-γ responses, essential to potentiate host resistance to *M. tuberculosis*. Interestingly, this response is especially remarkable in TB mice, being higher than that induced by the whole heat-killed mycobacteria. This could mean that the activities of the immunostimulatory components are masked in the whole *M. vaccae* by the interference of some other mycobacterial compounds present in the bacteria. The CWS fraction mainly contains the mycolyl-arabino-galactan-peptidoglycan complex (3, 16). Although the immunogenic ability of this macromolecule has not been described before for *M. vaccae*, studies of *Mycobacterium bovis* bacillus Calmette-Guérin have directly related it to the stimulation of the immune system. The peptidoglycan portion appears to be the obvious unit for the induction of Toll-like receptor 2 and 4 signaling in macrophages and dendritic cells (16), causing a cascade of events that finally promotes lymphocyte activation and the subsequent release of proinflammatory cytokines, such as IFN-γ (15).

Although to a lesser extent, *M. vaccae* treatment also elicits an increased IL-10 response. As shown in Fig. 1, immunization is required; therefore, it is probably not innate anti-inflammatory responses from macrophages. Studies using animal models of allergy have demonstrated that *M. vaccae* administration induces the production of IL-10-producing regulatory T cells (1, 17). However, IL-10 can also be produced by Th1, as has been described in TB patients (4), or Th2 cells. Thus, the cellular source of this cytokine should be further analyzed.

We demonstrated that the CWS fraction of *M. vaccae* elicits a prominent recall IFN-γ response in TB mice in comparison to the whole bacteria. These results warrant further research to test the potentially immunotherapeutic properties of this fraction.

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