Orally Administered *Mycobacterium vaccae* Modulates Expression of Immunoregulatory Molecules in BALB/c Mice with Pulmonary Tuberculosis

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The environmental saprophyte *Mycobacterium vaccae* induces a Th1 response and cytotoxic T cells that recognize *M. tuberculosis*, and by subcutaneous injection, it is therapeutic for pulmonary tuberculosis (TB) induced by high-dose challenge in BALB/c mice. However, *M. vaccae* also drives regulatory T cells that inhibit Th2 responses, and this is seen in allergy models, not only following subcutaneous injection but also after oral administration. An oral immunotherapeutic for TB would be clinically useful, so we investigated *M. vaccae* given orally by gavage at 28-day intervals in the TB model. We used two different protocols: starting the oral *M. vaccae* either 1 day before or 32 days after infection with *M. tuberculosis*. Throughout the infection (until 120 days), we monitored outcome (CFU), molecules involved in the development of immunoregulation (Foxp3, hemoxygenase 1, idoleamine 2,3-dioxygenase, and transforming growth factor β [TGF-β]), and indicators of cytokine balance (tumor necrosis factor, inducible nitric oxide synthase, interleukin-4 [IL-4], and IL-4 inhibitory splice variant of IL-4 associated with improved outcome in human TB). Oral *M. vaccae* had a significant effect on CFU and led to increased expression of Th1 markers and of IL-4, while suppressing IL-4, Foxp3, and TGF-β. When administered 1 day before infection, oral *M. vaccae* induced a striking peak of expression of hemoxygenase 1. In conclusion, we show novel information about the expression in TB of murine *M. vaccae* in extensively drug-resistant TB might be justified.

The immediate reason for the renewed interest in immunotherapy for tuberculosis (TB) is the emergence of extensively drug-resistant TB (XDR-TB) (2). If drugs on their own fail to treat TB, modulation of the host immune response with immunotherapeutic agents may provide additional therapeutic support. In addition, immunotherapy would also be valuable for drug-sensitive TB. The current 6-month treatment regimens are too long and cause problems with logistics and compliance. Immunotherapy might act in synergy with the currently available drugs and so improve their efficacy. Moreover, if immunotherapy can switch the immune system away from immunopathology and the Koch phenomenon toward an optimal protective response (although this remains poorly defined), it might reduce the duration of chemotherapy.

An injectable preparation of killed *Mycobacterium vaccae* was previously shown to be therapeutic in a high-dose intracheal challenge model of pulmonary TB in BALB/c mice, even if given when the disease was already well advanced (14, 24). In this model, disease is characterized by an initial phase of partial immunity dominated by T-helper 1 cytokines plus tumor necrosis factor (TNF) and interleukin-1 (IL-1), followed by a phase of progressive disease accompanied by increasing expression of IL-4, diminishing expression of IL-1 and TNF (11), and exquisite sensitivity to the toxic effects of injected recombinant TNF (13). When mice in this late progressive phase were treated with two injections (day 60 and day 90) of 0.1 or 1.0 mg of heat-killed *M. vaccae*, there were a fall in CFU, a fall in IL-4 levels, a return to a type 1 cytokine profile, a switch from pneumonia to granuloma, and restoration of expression of IL-1 and TNF without any apparent toxicity or any increase in the toxicity of injected recombinant TNF (14). Mechanisms likely to contribute to these protective effects of *M. vaccae* include enhanced Th1 activity and induction of CD8+ cytotoxic T cells that can lyse macrophages infected with *Mycobacterium tuberculosis* (28). However, studies with IL-4−/− mice (10) and with neutralizing antibodies to IL-4 indicate that in high-dose TB challenge models, reduction in IL-4 production is also therapeutic (14, 26), and the suppression of IL-4 expression induced by *M. vaccae* in the BALB/c TB model was striking, suggesting a third protective mode of action (14). This ability of *M. vaccae* to suppress even a preexisting IL-4/Th2 response has been investigated in experimental allergy models (35, 36) and clinical trials (21) and is mediated by CD25+ CD45RBlow regulatory T cells (Tregs) (1, 35, 36).

Recently it was found that in a mouse model of allergic...
pulmonary infection killed \textit{M. vaccae} is as effective when given as an oral immunotherapeutic as it is when administered subcutaneously (s.c.) (16). Since the oral route would clearly have enormous advantages for multidose immunotherapeutic strategies in humans, we have now tested the efficacy of oral \textit{M. vaccae} in the BALB/c model of pulmonary TB in which \textit{M. vaccae} was previously shown to be effective by the s.c. route. Moreover, in view of the performance of the \textit{M. vaccae} to drive the development of Tregs (35, 36), we also investigated the time course of expression of several molecules associated with induction of Tregs. These molecules include indoleamine 2,3-dioxygenase (IDO) (15) and heme oxygenase-1 (HO-1) (33), which are enzymes involved in the maturation of CD4+ CD25^hi Tregs, in addition to Foxp3, transforming growth factor \(\beta\) (TGF-\(\beta\)), IL-4, and IL-482. The latter is a splice variant of IL-4 thought to be a natural inhibitor of IL-4 in humans and mice (30, 34) and is associated with protection and with stable latent TB in humans (5).

MATERIALS AND METHODS

Murine model of progressive pulmonary TB. The experimental model of progressive pulmonary TB has been described in detail (11, 13, 14). Briefly, the virulent strain of \textit{M. tuberculosis} H37Rv was cultured in 7H9 liquid medium. After 1 month of culture, mycobacteria were harvested and adjusted to 2.5 \(\times\) 10^7 cells in 100 \(\mu\)l of phosphate-buffered saline (PBS), aliquoted, and maintained at \(\sim\)70°C until used. Before use, bacteria were recounted and viability was checked (11, 13, 14). Pathogen-free male BALB/c mice, 6 to 8 weeks old, were anesthetized (sevoflurane; Abbott Laboratories, Abbott Park, IL), and the trachea was exposed via midline incision. The animals were infected using an insulin syringe administering 2.5 \(\times\) 10^7 viable bacteria suspended in 100 \(\mu\)l of PBS. After suturing of the incision, mice were maintained in vertical position until the effect of anesthesia waned. Infected mice were kept in groups of five in cages fitted with microisolators connected to negative pressure. All procedures were performed in a biological security cabinet in a biosafety level III facility. The protocol was approved by the Ethics Committee for Experimentation in Animals of the Instituto Nacional de Ciencias Médicas y Nutrición “Salvador Zubirán.”

To evaluate the effect of administration of heat-killed \textit{M. vaccae} on the course of infection, two different experimental protocols were used, and each was performed twice. In the first protocol, heat-killed \textit{M. vaccae} cells at a dose of 0.1 \(\mu\)g suspended in 100 \(\mu\)l of saline or saline alone was administered using an intragastric cannula. Then 25 h later, the animals were infected with H37Rv via the trachea as described above. Further similar doses of heat-killed \textit{M. vaccae} were administered intragastrically on days 28, 56, and 112 after H37Rv infection. In the second type of experiment, animals received heat-killed \textit{M. vaccae} using the same dose and route of administration, but starting after the pulmonary TB infection was well established. Thus, intragastric heat-killed \textit{M. vaccae} was given 32, 60, and 88 days after infection with H37Rv, and groups of six animals were sacrificed 68, 75, 90, and 120 days after infection.

Assessment of CFU in infected lungs. Right or left lungs from three mice per group at each time of killing were homogenized with a Polytron (Kinematica, Lucerne, Switzerland) in sterile tubes containing 2 ml of PBS-Tween 80 at 0.05%. Groups of six animals were sacrificed 68, 75, 90, and 120 days after infection. The same paraffin-embedded material was used to determine the presence of HO-1 by immunohistochemistry. Lung sections from treated and control mice at each time point were mounted on silane-covered slides and deparaffinized, and the endogenous peroxidase was quenched with 0.03% \(\text{H}_2\text{O}_2\) in absolute methanol. Sections were incubated overnight at room temperature with rabbit-specific polyclonal antibodies against HO-1 (BioVision, CA) diluted 1/300 in PBS. Bound antibodies were detected with biotinylated antibodies (biotin-conjugated anti-rabbit immunoglobulin G [Vectorstain system; Vector Laboratories]) diluted 1/200, followed by incubation with horseradish peroxidase-conjugated avidin for 30 min. The reaction was revealed by 3,3-diaminobenzidine–hydrogen peroxide for 5 to 10 min at room temperature. Tissue sections were counterstained with hematoxylin. The negative controls consisted of performing the usual rabbit serum or an irrelevant antibody instead of the primary antibody.

Real-time RT-PCR analysis of cytokines and iNOS expression in lung homogenates. Three lungs, left or right, from the same number of animals in two different experiments were used to isolate RNA at each time of killing using the RNeasy mini kit (Qiagen, CA), according to the manufacturer’s instructions. The quality and quantity of RNA were evaluated through spectrophotometry (260/280 nm) and on agarose gels. Reverse transcription (RT) of the mRNA was performed using 5 \(\mu\)g RNA, oligo(dT), and the Omniscript kit (Qiagen, CA). Real-time PCR was performed using the 7500 real-time PCR system (Applied Biosystems, CA) and QuantiTect SYBR green master mix kit (Qiagen, CA). Standard curves of the desired RT-PCR product, as well as negative controls, were included in each PCR run. Specific primers were designed using the program Primer Express (Applied Biosystems, CA) for the following targets: glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'–CATTTGGAAGGCTGCTGA-3' and 5'–GGAAGCCATG CACGTGAC-3'; inducible nitric oxide synthase (iNOS), 5'-AGCCAGAGAC AGGTGAAAG-3' and 5'-CATTTGCGTCTGCTCCCAAAA-3'; TNF-\(\alpha\), 5'-TGTG GCTTCTGGAACCTTACCC-3' and 5'-GGCGAGAAAAGGCTGTTG-3'; IL-4, 5'- CAGGAGAAGGCAACAC-3' and 5'-GCTGTITTAGGCTTCCAGGAAG-3'; Foxp3, 5'-GGCCGAGCACAACACTGAC-3' and 5'-GAAGCTCG ACCGGACATTG-3'; IDO, 5'-GACTTGGGATGCCCAGA-3' and 5'-ACCCCTCT ACAAGCCACTCT-3'; and HO-1, 5'-GTGATGGAGCCTCCCAA GC-3' and 5'-TCTCAGCTGATGTTACCT-3'. The following cycling conditions were used: initial denaturation at 95°C for 15 min, followed by 40 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 34 s. Quantities of the specific mRNA in the sample were measured according to the corresponding gene-specific standard. The mRNA copy number of each cytokine was related to one million copies of mRNA encoding G3PDH.

RESULTS

Effects of oral heat-killed \textit{M. vaccae} on the bacterial load in the lung. When \textit{M. vaccae} was administered at 28-day intervals from the day before infection, and the number of CFU/lung was analyzed by two-way analysis of variance (ANOVA) with Bonferroni posttests (Fig. 1), the overall growth curves were significantly different (\(P = 0.0001\)), and from day 30 the CFU at individual time points were significantly reduced in the \textit{M. vaccae}-treated animals (\(P < 0.001\)). When \textit{M. vaccae} was administered monthly from day 32 and numbers of CFU were assessed from day 68 (i.e., from 8 days after the second oral treatment), the overall growth curves differed significantly (\(P = 0.0096\), pooled data from two experiments), although differences at individual time points did not achieve significance.

Effects of oral heat-killed \textit{M. vaccae} on expression of cytokines and iNOS in the lung. Compared to controls, mice treated with \textit{M. vaccae} administered at 28-day intervals from the day before infection showed increased expression of mRNAs encoding TNF and iNOS (Fig. 2). In contrast, mRNA for IL-4 was decreased. For these three molecules, the curves differed significantly (\(P < 0.0001\)). \(P\) values for individual time points are indicated on the graphs. Expression of IL-462 was
low and was not affected by treatment with \textit{M. vaccae}. Since
TNF (and gamma interferon [IFN-\gamma], which was not mea-
sured) enhances expression of iNOS, while IL-4 inhibits it,
these results are compatible with a downregulation of the Th2
response that occurs in this model of TB and a return to a Th1
phenotype.

When given from day 32, the effect of oral \textit{M. vaccae} on
cytokines was similar to that seen when it was given from the
day before infection. Expression of mRNAs encoding TNF and
iNOS was increased (Fig. 3). Moreover, there were decreased
IL-4 and increased IL-4\textsubscript{2} on days 68, 75, and 90, although
IL-4 itself was more abundant than the splice variant at all
times. These effects on the IL-4 variants were lost by day 120.

E
c-oral \textit{M. vaccae} on expression in the lung of Foxp3,
TGF-\beta, HO-1, and IDO. In untreated BALB/c mice with pul-
monary TB, there occurred a biphasic increase in expression of
Foxp3 (Fig. 4), consistent with flow cytometry data obtained by
others (18), and a progressive rise in TGF-\beta as reported pre-
viously (10). Interestingly, intragastric \textit{M. vaccae} given monthly
from the day before infection caused a sustained reduction in
expression of Foxp3 and TGF-\beta. In addition, the treatment
cased early increases in expression of mRNA encoding HO-1
and IDO. For HO-1, this was confirmed by immunohistochem-
istry in several lung tissue compartments, and the increases
were very significant (Fig. 5 and 6). Despite the later fall in
mRNA, protein levels as detected by immunohistochemistry
remained elevated in the \textit{M. vaccae}-treated animals at day 120
(Fig. 5). Activity of these enzymes is associated with increased
development of Foxp3\textsuperscript{Tregs} (15, 33), so it is interesting that
the mRNA levels had returned almost to control levels by the
time the reduction in Foxp3 and TGF-\beta became striking in the
\textit{M. vaccae}-treated animals, although the protein levels had not
(Fig. 5 and 6).

When given from day 32, intragastric \textit{M. vaccae} caused an
inhibition of expression of Foxp3 mRNA (Fig. 7), just as ob-
served when given from day 0. There was also a minimal,
though statistically significant, increase in IDO mRNA, similar
to that seen after day 30 when treatment was given from day 0.
Expression of HO-1 was consistently suppressed.
DISCUSSION

Although the reductions in CFU that we have observed in this model using intragastric M. vaccae are modest, the effects in humans, over longer treatment durations, cannot be predicted. These effects were seen in the absence of chemotherapy, whereas in humans, immunotherapy would be used as an adjunct to available chemotherapy, even if only second- and third-line drugs were appropriate, as may be the case for patients with XDR-TB. Synergy between immunotherapy and chemotherapy is likely. Such synergy between multidose M. vaccae and chemotherapy was observed in one preliminary study in Argentina, despite the use of patients infected with fully drug-sensitive organisms who were therefore responding rapidly to the chemotherapy itself (6).

The induced changes in cytokine balance and in regulatory molecules were striking and suggest diminution of the IL-4 response that develops in this model during established disease and a switch back to protective Th1 responses with consequent inhibition of TGF-β. The concomitant fall in expression in TGF-β (Fig. 4b) might be secondary to the fall in IL-4, because the rising TGF-β levels are largely secondary to IL-4 release in this model (10).

We have pointed out previously that high IL-4 levels occur in TB most often in developing countries close to the equator, particularly in low-lying areas (22). We have suggested that this is due to the combined influence of high exposure to environmental mycobacteria and to helminths, followed by high-dose challenge with M. tuberculosis (23, 25). A study in Cameroon showed that even in the normal population, the magnitude of the IL-4 response to purified protein derivative increases with age, at least up to the age of 16 years, at which time recruitment in this study ceased (29). IL-4, together with TGF-β, is able to oppose apoptosis, autophagy, and the induction and function of cytotoxic lymphocytes (3, 9, 17, 23). These are crucial mechanisms providing the immune system with mycobacterial pathways that circumvent the ability of M. tuberculosis to damage phagosome function. This might explain why a neutralizing antibody to IL-4 is therapeutic in another high-dose challenge mouse model of TB (26) and suggests that reducing IL-4 is likely to be therapeutically useful. Similarly inhibition of TGF-β by administering soluble type III TGF-β receptors is also therapeutic in this model (12).

We have not monitored IFN-γ expression in this study because protection is not quantitatively related to levels of this cytokine, which is always expressed at high levels in our model (11, 13, 14). This lack of correlation may be because the classical IFN-γ-mediated macrophage activation pathway is inhibited by M. tuberculosis, so that protection depends on an unknown extent on “rescue” mechanisms such as apoptosis, autophagy, and cytotoxic lymphocytes that can kill via bacterial peptides or recycle the damaged phagosome into fresh macrophages (discussed and referenced in reference 25).

The role of IDO in TB has not been fully evaluated. We were intrigued by this molecule because IDO metabolizes tryptophan, releasing kynurenine and metabolites such as 3-hydroxyanthranilic acid and quinolinic acid that modulate T-cell differentiation, function, and survival (8). Moreover, IDO-positive dendritic cells drive maturation of Treg (15). In view of the small but significant increases in expression of IDO induced by M. vaccae, particularly during the first 3 weeks, it will
be interesting to test the effects of an inhibitor of the enzyme in this model.

HO-1 degrades heme to biliverdin, free divalent iron, and carbon monoxide (CO), resulting in immunomodulatory effects, perhaps mediated by CO (20). We show that expression of HO-1 increases steadily to very high levels in this TB model and that oral \textit{M. vaccae} causes a striking (and precisely reproducible [data not shown]) early increase in HO-1 expression between days 7 and 21. Further work will be required to determine whether this peak of HO-1 activity results in the expansion of a beneficial subset of regulatory cells. Interestingly, activation of HO-1 had a protective effect in an allergy model that was mediated by Foxp3$^+$ CD4$^+$ CD25$^+$ Tregs, IL-10, and membrane-bound TGF-β (33). However, other data suggest that enhanced HO-1 activity can drive Foxp3$^+$ Tregs that facilitate graft tolerance across a powerful histocompatibility barrier, so there is no evidence of preferential downregulation of Th2 (19). Since the activity of this enzyme can be modulated in vivo, the relevance of the early peak of HO-1 induced by oral \textit{M. vaccae} can be investigated experimentally.

In conclusion, we have used a well-established model of progressive pulmonary TB to provide new information on the time course of expression of several molecules that are involved in immunoregulation, and we have shown that a potential immunotherapeutic agent, already shown to be active in this model when given s.c., not only reduces CFU when given orally, but also modulates expression of these regulatory molecules. There is an urgent need for safe immunotherapeutics, and in a recent report, the Special Programme for Research & Training in Tropical Diseases of WHO endorsed this approach (4). Protocols for the production of \textit{M. vaccae} in accordance with good manufacturing
practice guidelines have been established and used successfully in the past. Moreover, *M. vaccae* has a proven safety record, and these findings should encourage further investigation and clinical trials. The initial data from a multidose study of *M. vaccae* given by the intratracheal route were encouraging (6), and we feel that trials of the oral route in XDR-TB are now justified. This route would have major advantages under conditions in developing countries.

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