



Potential for immunotherapy with heat-killed *Mycobacterium vaccae* in respiratory medicine

Immunotherapy with *Mycobacterium vaccae* has been shown to be beneficial as part of the treatment for a wide range of diseases. In the respiratory system, the late airway response in bronchial asthma is modified by a single dose and bronchial aspects of hayfever are reduced allowing a major reduction in the use of bronchial dilators. In studies of advanced adenocarcinoma of the lung survival is increased by an average of 4 months when up to five doses of *M. vaccae* are added to the course of chemotherapy. The quality of life of cancer patients receiving immunotherapy with *M. vaccae* is improved, even if survival is not increased. It is suggested that the mechanism of action of immunotherapy with heat-killed, borate-buffered *M. vaccae* is likely to be very similar in all these diseases for which human pulmonary tuberculosis provides a model. In this study, additional immunological data are reported from material stored from an earlier study of immunotherapy for pulmonary tuberculosis to help complete the information on the way that treatment with three monthly injections of heat-killed, borate-buffered *M. vaccae* (SRL172) may act.

KEYWORDS: bronchospasm ■ immune modulation ■ immunotherapy ■ lung cancer ■ *Mycobacterium vaccae* ■ respiratory disease ■ tuberculosis

The history of attempts to treat disease by enhancing or modifying immune responses has been long, complex and variably successful. The variability is attributable to a lack of understanding of the many patterns of immune reactions, some beneficial and some harmful, that occur in response to any challenge, and to a lack of technical methods to define beneficial responses and to determine the nature of responses induced by putative therapeutic agents. The situation is changing and this is illustrated by the history of the development of immunotherapy with heat-killed *Mycobacterium vaccae*. Thus, the literature contains a number of reports of the successful use of this agent in its borate-buffered formulation (SRL172) in the treatment of pulmonary tuberculosis [1-7], leprosy [8], bronchial asthma [9], hayfever [10], atopic dermatitis in childhood [11], psoriasis [12] and adenocarcinoma of the lung [13]. A phosphate-buffered preparation has been much less successful [14,15].

Although negative findings have been reported with the borate-buffered reagent in tuberculosis, these have been studies employing single doses of the agent in regions where environmental factors appear to oppose its efficacy [16,17]. A meta-analysis of the reported data on the use of single doses of SRL172 in tuberculosis [18] showed no effect, chiefly owing to the large size of the South African trial and the inclusion of HIV-seropositive patients from a study

in Zambia and Malawi [19].

Several preclinical studies of tuberculosis, bronchospasm, leishmaniasis and cancer have been carried out in mice, demonstrating the beneficial effects of this type of treatment [20-23]. With similar reagents prepared from other members of the Actinomycetales, studies have been performed in the rat model of Chagas' disease (*Trypanosoma cruzi* infection) [24] and several others are in progress. The most extensive clinical and immunological investigations of events during successful immunotherapy in humans have been in pulmonary tuberculosis [5,7].

In view of the widening scope for the potential application of this treatment to other respiratory disorders and, indeed, in other branches of medicine, we have expanded our investigations of immune mechanisms in the human pulmonary tuberculosis model. We have further analyzed the samples of cells and cell supernatants obtained from a previous study [7].

In addition to the picture already established of downregulation of Th2 mechanisms and an increase in Th1 mechanisms, we have now investigated a number of other relevant factors. These include the expression of receptors for TNF- α and IL-8 on the surfaces of mononuclear (MN) and polymorphonuclear (PMN) leukocytes, and the determination of nitrite production. The cytokines TNF- α , IFN- γ , IL-2, IL-12 and TGF- β have been measured in cell culture supernatants.

John Stanford^{1†},
Cynthia Stanford¹,
Diana Dlugovitzky²,
Gladys Fiorenza²,
Diego Martinel-Lamas²,
Dante Selenscig² &
Christina Bogue³

[†]Author for correspondence:

¹Centre for Infectious Diseases
and International Health,
Windeyer Institute of Medical
Sciences, University College
London, 46 Cleveland Street,
London W1T 4JF, UK
Tel.: +44 1892 730298

jandcstanford@btinternet.com

²Catedra de Microbiologia,
Virologia y Parasitologia,
Facultad de Ciencias Medicas,
Universidad Nacional de
Rosario, Argentina

³Hospital I. Carrasco de
Rosario, Boulevard Avellaneda
1402. 2000 Rosario, Argentina

future
medicine part of fsg

A picture emerges of a very potent, long-acting, immune modulator with considerable beneficial effects on the profile of cytokines and chemokines secreted by immune cells throughout the treatment of tuberculosis. These changes are also likely to underlie the beneficial effects seen in asthma and adenocarcinoma.

Using a quite different soluble preparation of the type strain of *M. vaccae*, Chinese researchers have reported a whole series of successful trials of their reagent in human tuberculosis [25–27] as well as in preclinical models [28,29].

Materials & methods

■ Patients

The specimens investigated were those obtained from adult patients with moderate or severe pulmonary tuberculosis from an earlier clinical study [7], who had given written consent that the samples could be subjected to immunological investigation. All patients received directly observed short-course chemotherapy with 2 months of daily rifampicin, isoniazid, ethambutol and pyrazinamide followed by 4 months of daily rifampicin and isoniazid (2RHEZ/4RH). They were randomized to receive three injections, at monthly intervals, of the immunotherapeutic agent (12 patients) or of buffered saline as placebo (10 patients). Permission to carry out the study was obtained from the Ethics Committees of the Universidad Nacional de Rosario and of Carrasco Hospital, Argentina (10 October 2001).

■ Immunotherapeutic agent & placebo

The immunotherapeutic agent was a preparation of *M. vaccae* NCTC11659 suspended in M/15 borate-buffered physiological saline at a pH of 8.0, heat-killed by autoclaving and known as SRL172. The reagent provided contained 10 mg wet-weight of bacilli per ml, of which the dose was 1 mg in 0.1 ml (approximately 1,000,000,000 killed bacilli), given by intradermal injection over the upper arm. The placebo consisted of injections of the borate buffer alone. Both immunotherapeutic and placebo, prepared to the standards of good manufacturing practice, were provided by SR Pharma PLC, UK, to whom we are grateful.

Injections were given to the randomized patients on the first day of treatment with short-course chemotherapy, and 1 month and 2 months later [7].

■ Samples available

These were separated from peripheral blood MN and PMN leukocytes collected from venous blood. Samples were collected at the beginning

of treatment (M0), just before each successive monthly injection of *M. vaccae* or placebo (M1 and M2), and 1 month after the third injection (M4). Also available were stored supernatants of 20-h cultures of these cells in RPMI 1640 medium enriched with L-glutamine, in some cases stimulated by the addition of heat-inactivated *M. tuberculosis* strain H37Rv. These supernatants had been stored at minus 70°C since the earlier investigation [7].

In addition, samples were obtained from 11 healthy volunteers to provide baselines for some of the studies on cell-surface expression and culture supernatants with which the results from patients could be compared.

■ Isolation & culture of MN & PMN cells

For isolation of MN and PMN cells, heparinized venous blood samples obtained from the patients and healthy controls were diluted with an equal volume of phosphate-buffered saline (PBS) and layered over a Ficoll–Hypaque gradient (Density 1077) in polypropylene tubes. These were centrifuged at $400 \times g$ for 30 min at room temperature (19–22°C). The following three fractions were obtained after centrifugation:

- Plasma
- Plasma–Ficoll interface
- Pellet

Mononuclear cells, comprising lymphocytes and monocytes, were recovered from the interface and PMN cells were obtained from the pellet after red blood cells had been lysed by treatment with ammonium chloride. Cells were washed three times in PBS by centrifuging at $400 \times g$ for 10 min and resuspended in RPMI 1640 (Sigma Aldrich) containing standard concentrations of L-glutamine. Cells were counted and their concentration was adjusted to 5×10^6 cells/ml with RPMI 1640. These cell suspensions were distributed in 250- μ l volumes into 6-ml polypropylene tubes. Duplicate tubes were either left unstimulated, or stimulated with 5 μ l of heat-killed *M. tuberculosis* H37Rv 1.8×10^8 bacilli per ml. These tubes were incubated for 20 h at 37°C.

Following incubation, culture supernatants were collected and stored at minus 70°C for assessment of nitrites and cytokines. The cell pellets were washed three-times with PBS, resuspended to their original volume in the same buffer and stored at minus 70°C. These cells were examined for TNF- α and IL-8 receptors.

■ Expression of TNF- α & IL-8 receptors on fresh MN & PMN cell surfaces

The expression of IL-8 and TNF- α receptors (IL-8RB [CXCR-2] and TNFR1 respectively), on the surface of the previously washed, and resuspended PMN and MN cells was determined with receptor-specific monoclonal antibodies by flow cytometry. This was carried out when the cells were fresh, but the data have not been presented until now. Expression of the receptors on each cell type was recorded as the percentages of fluorescing (positive) cells and their mean fluorescence intensity.

■ Determination of nitrite levels on cultured cells

The nitric oxide (NO) produced by cultured fresh MN and PMN cells was measured by means of the Griess reaction in culture supernatants. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 2.5% H₃PO₄ and 0.1% naphthylethylene diamine dihydrochloride in 2.5% H₃PO₄ and incubating for 10 min at room temperature. The reaction was carried out in 96-well plates (NUNC, NUNC Brand Products) with duplicate 100- μ l volumes of stimulated or unstimulated supernatants incubated for 5 min with the Griess reagent in the dark at room temperature. Absorbance was measured at 540 nm (corrected to 650 nm) in an ELISA reader (Microwell System Reader, Organon Teknika). Nitrite concentration was measured against a standard curve produced using various NaNO₂ concentrations in culture medium and data were expressed in μ M.

■ Assessment of cytokines in stored supernatants of cultured cells

Levels of TNF- α , IL-2, IL-12, IFN- γ and TGF- β in the stored culture supernatants of the MN and PMN cells were determined by ELISA, using commercial kits and following the manufacturers' instructions (R&D Systems, MN, USA). Samples were assayed in duplicate and results were expressed as the average of two readings in an ELISA reader at 450 nm. Cytokines were quantified with reference to standard curves generated using a human recombinant cytokine. The sensitivities of the assays were for TNF- α : 15.6, for IFN- γ : 15.6, for IL-2: 7.0, for IL-12: 5.0 and for TGF- β : 31.2 pg ml⁻¹.

■ Statistical analyses

Comparisons among groups were made by the Kruskal-Wallis and Mann-Whitney tests. Within group results were analysed by the

Friedman analysis of variance followed by the Wilcoxon signed rank test when applicable. Student's t test was applied to some individual results. Data were considered statistically significant if $p < 0.05$.

Results

■ TNF- α & IL-8 receptors on fresh MN & PMN cell surfaces

FIGURES 1A, 1B, 2A AND 2B SHOW the percentages of cells expressing the receptors TNFR1 and CXCR2 respectively. Expression of both receptors on both MN and PMN cells increased with time in both treatment groups. At the beginning of treatment, the percentages of patients' cells expressing either receptor were lower than that of healthy individuals, especially in the case of PMN cells.

Immunotherapy resulted in a significant increase in expression of TNFR1 and CXCR2 on MN cells in comparison with those of placebo recipients, so that by month 4, expression in the immunotherapy recipients exceeded the healthy control value.

On PMN cells of both treatment groups there were similar and steady increases of expression of TNFR1, in neither case reaching that of the healthy controls even at month 4. Expression of CXCR2 also increased with time, slightly more so in cells from immunotherapy recipients, in which the expression by month 4 almost equaled that of healthy control individuals.

■ Nitrite production of cultured cells

TABLE 1 SHOWS the results for nitrite production in stored supernatants of unstimulated and H37Rv-stimulated MN and PMN cells. Production of nitrite from both unstimulated and stimulated cell types showed little change throughout the period of the study in placebo recipients. By contrast, supernatants of unstimulated cells of both types from *M. vaccae* recipients showed a significant ($p < 0.01$) reduction by the end of the first month, increasing in significance after the third month ($p < 0.001$). Similar reductions in nitrite production were found in stimulated cells of immunotherapy patients, not quite reaching statistical significance in PMN cells. In comparison with placebo recipients, MN cells of the *M. vaccae* group produced significantly less nitrite at months 1 to 4 ($p < 0.006$), and PMN cells of the *M. vaccae* group produced significantly less nitrite at months 2 and 4 ($p < 0.0007$).

In both MN and PMN cell supernatants, the values found in healthy controls differed little from the M0 values for both groups of patients.

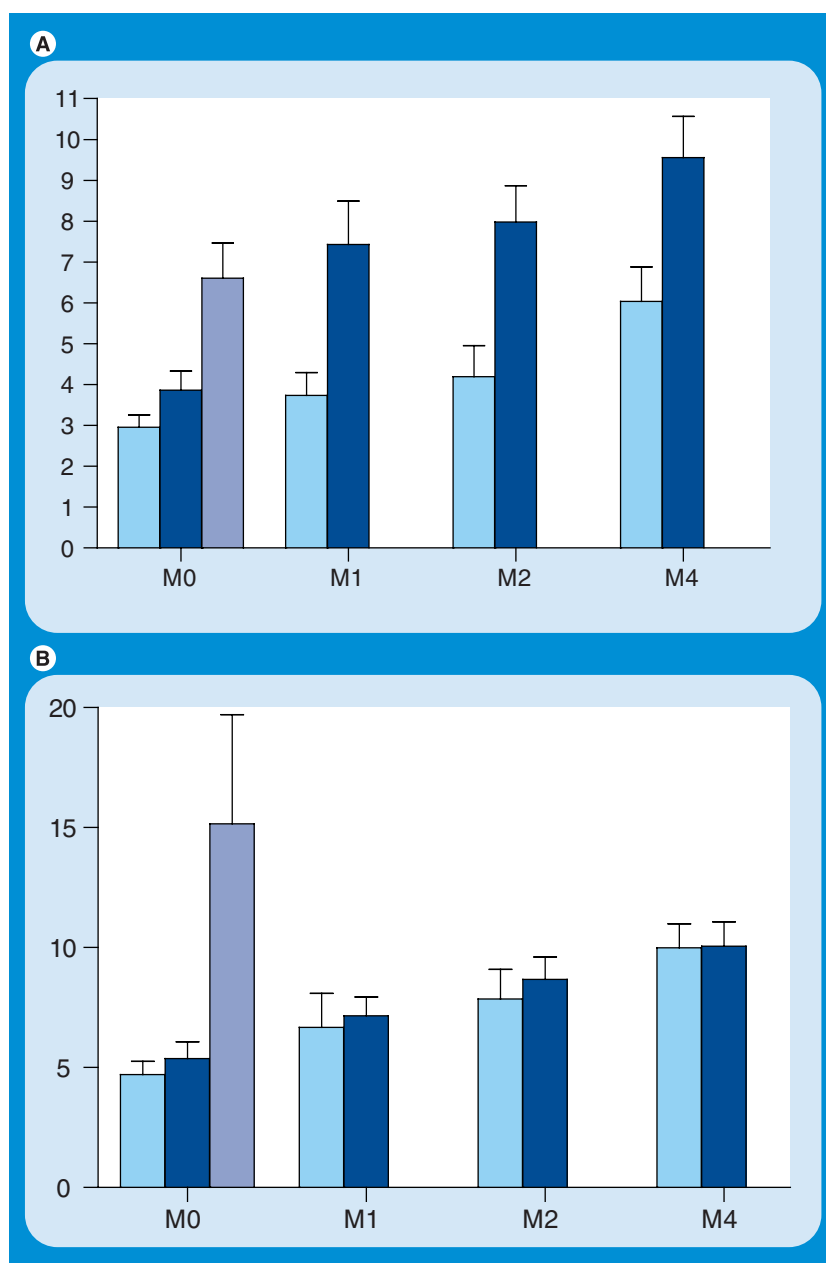


Figure 1. Percentages of (A) MN and (B) PMN cells expressing receptors for TNF- α (TNFRI) at the beginning of the treatment (M0) and after 1, 2 and 4 months of treatment (M1, 2 and 4). Data are presented as means and standard deviations. Light grey columns show results for the placebo recipient patients and dark grey columns show those for immunotherapy recipients. The mid-grey columns at M0 are the values obtained in healthy volunteers. MN: Mononuclear; PMN: Polymorphonuclear.

■ Cytokines in stored supernatants of cultured cells

The results for IL-2 in stored supernatants of unstimulated MN and PMN cells are shown in TABLE 2. In those of MN cells there is an increase with time, but with no significant differences between the treatment groups. The results in healthy control individuals were slightly less than the month 0 values for both patient groups.

PMN supernatants from both patient groups showed a steadily increasing production of IL-2 with time. This was greatest in immunotherapy recipients whose month 4 level of 6.19 ± 2.51 approached that of the healthy controls, which was 10.66 ± 2.68 . Stimulation with H37Rv slightly increased levels of IL-2, but without significantly changing the picture (data not shown).

TABLE 3 shows the levels of IFN- γ in both unstimulated and stimulated supernatants of the two cell types. Supernatants of unstimulated MN and PMN cells showed increasing levels of IFN- γ with time, somewhat greater in *M. vaccae* recipients, particularly in MN. These differences were considerably enhanced by stimulation with H37Rv. Healthy control individuals had levels of IFN- γ less than those of the patients in both groups at all time points; however, their value increased after stimulation with H37Rv.

Results for IL-12 in MN and PMN cells are shown in TABLE 4. In MN supernatants, IL-12 increased steeply ($p < 0.02$) following the first injection in *M. vaccae* recipients and remained raised ($p < 0.005$). The level hardly changed at all in MN cells of placebo recipients. Stimulation of cultures with heat-killed H37Rv in both groups of patients raised the baseline value, but did not alter the picture of increase in the immunotherapy recipients (data not shown). The healthy control value for MN cells was twice that of the placebo recipients at all time points, but only so for the *M. vaccae* recipients at month 0. Thereafter at months 1, 2 and 4, the values for the immunotherapy group exceeded that of the healthy control individuals.

In supernatants of non-stimulated PMN cells, IL-12 increased with time in both treatment groups. The increase was gradual in placebo recipients, reaching significance by month 4 ($p < 0.01$), whereas most of the increase observed in recipients of *M. vaccae* was in the first month ($p < 0.05$) and was greatest after 4 months ($p < 0.005$), at which time it did not differ significantly from that of healthy control individuals. As with MN cells, H37Rv-stimulation of PMN cells raised the IL-12 levels at each time point without altering the patterns observed in nonstimulated cells (data not shown).

Levels of TGF- β in MN and PMN supernatants from the two treatment groups are shown in TABLE 5. Those of unstimulated MN cells showed a significant increase in TGF- β in both treatment groups at months 1 and 2. Thereafter, there was a small fall by month 4 in placebo recipients and a fall to the baseline level

in *M. vaccae* recipients ($p < 0.01$). The supernatants of PMN cells showed a significant fall ($p < 0.005$) by month 4 in *M. vaccae* recipients, not seen, even as a trend, in placebo recipients. In supernatants of H37Rv-stimulated MN cells there was a steady increase in TGF- β in both patient groups ($p < 0.001$), greatest in those from *M. vaccae* recipients. Whereas levels of TGF- β steadily increased in stimulated supernatants of both cell types from placebo recipients, there was a fall after month 1 in the supernatants of PMN cells in the immunotherapy group ($p < 0.05$). Levels of TGF- β in supernatants of both MN and PMN cells of healthy control individuals were less than those of patients at all time points.

Discussion of results

Each of the studies we have carried out on tuberculosis patients in Rosario have shown better clinical improvement among those receiving *M. vaccae* as part of their treatment than among those receiving chemotherapy alone. The first two studies, of single-dose immunotherapy, which were published together [5], were carried out before the introduction of directly observed chemotherapy (DOTS) and when it was the practice at Carrasco hospital for cases to be treated for the first 2 or 3 months as in-patients. Pyrexia and cough both resolved a few days faster in immunotherapy recipients and on the basis of their clinical status these patients left hospital after 45 ± 20 days, whereas those receiving chemotherapy alone left hospital after 60 ± 15 days. In the study of three doses of immunotherapy [7], of which this is a part, DOTS had been fully implemented and ambulant treatment was the rule. A clinical scoring system was used, which showed that much the fastest improvement occurred in *M. vaccae* recipients ($p < 0.001$), notably in the severity of cough.

The clinical improvements just described associated with faster disappearance of acid-fast bacilli from the sputum, better recovery of bodyweight, better radiological clearance and a more rapid fall in erythrocyte sedimentation rate (ESR) [7]. These were accompanied by a return towards normal values for plasma TNF- α and IL-4, an increased oxidative index in both MN and PMN cells and increased expression of CD11b on cell surfaces.

Upon recognition of tubercle bacilli by phagocytic cells, rapid production of pro- and anti-inflammatory cytokines and chemokines ensues, resulting in recruitment of inflammatory cells (T cells, neutrophils and NK cells) into areas of infection. A successful outcome

to a mycobacterial infection depends upon the cytokine networks established and maintained by macrophages and T cells.

To expand the data shown in the earlier paper [7], we have now demonstrated that clinical improvement is accompanied by increases in expression of the receptors for TNF- α and IL-8 on the cell surface of both MN and PMN cells. This increase tended to be greatest in

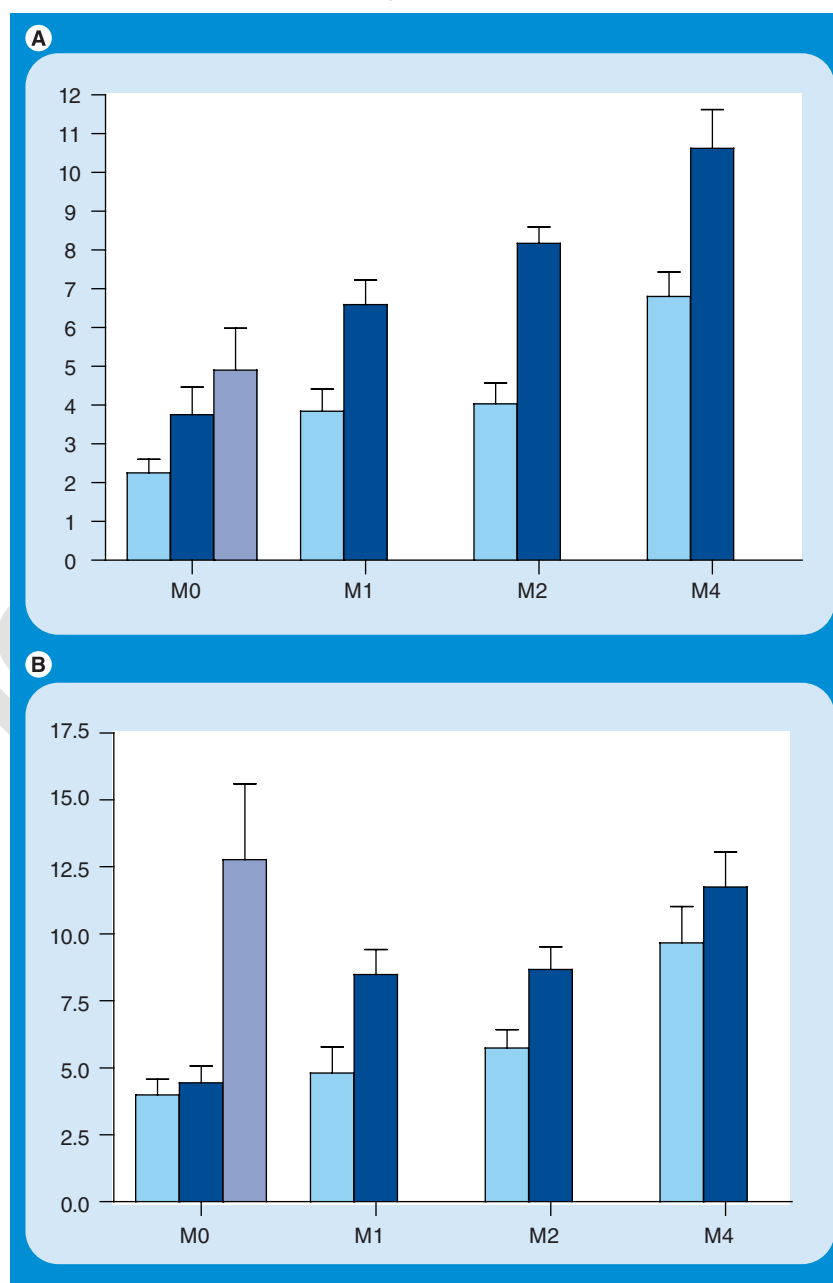


Figure 2. percentages of (A) MN and (B) PMN cells expressing receptors for IL-8 (CXCR2) at the beginning of the treatment (M0) and after 1, 2 and 4 months of treatment (M1, 2 and 4). Data are presented as means and standard deviations. Light grey columns show results for the placebo recipient patients and dark grey columns show those for immunotherapy recipients. The mid-grey columns at M0 are the values obtained in healthy volunteers. MN: Mononuclear; PMN: Polymorphonuclear.

Table 1. Nitrite concentrations in supernatants of mononuclear and polymorphonuclear cells from placebo and *Mycobacterium vaccae* recipient patients (mean $\pm$ standard deviation).

	Placebo	Difference	<i>Mycobacterium vaccae</i>
Unstimulated mononuclear cells			
M0	5.87 $\pm$ 1.46 n = 9	NS	5.70 $\pm$ 2.50 n = 9
M1	5.22 $\pm$ 1.29 n = 9	p < 0.006	3.13 $\pm$ 1.66 n = 11
M2	5.98 $\pm$ 1.66 n = 9	p < 0.0004	2.91 $\pm$ 1.24 n = 9
M4	6.28 $\pm$ 2.16 n = 7	p < 0.002	2.36 $\pm$ 1.04 n = 7
	NS*		p < 0.0002*
Healthy controls	5.70 $\pm$ 1.46 n = 9		
Unstimulated polymorphonuclear cells			
M0	6.03 $\pm$ 2.12 n = 7	NS.	5.87 $\pm$ 2.64 n = 10
M1	6.18 $\pm$ 3.21 n = 6	NS	3.56 $\pm$ 1.60 n = 10
M2	6.19 $\pm$ 1.64 n = 9	p < 0.0003	2.96 $\pm$ 0.62 n = 8
M4	5.98 $\pm$ 1.60 n = 8	p < 0.0007	2.83 $\pm$ 0.43 n = 5
	NS*		p < 0.001*
Healthy controls	6.80 $\pm$ 2.06 n = 8		
H37Rv-stimulated mononuclear cells			
M0	8.50 $\pm$ 5.01 n = 9	NS	7.01 $\pm$ 3.36 n = 9
M1	6.92 $\pm$ 3.38 n = 8	p < 0.05	4.26 $\pm$ 1.91 n = 11
M2	6.86 $\pm$ 2.80 n = 8	p < 0.05	4.19 $\pm$ 1.15 n = 7
M4	5.77 $\pm$ 2.28 n = 7	p < 0.05	2.70 $\pm$ 1.50 n = 4
	NS*		p < 0.002*
H37Rv-stimulated polymorphonuclear cells			
M0	8.81 $\pm$ 3.35 n = 7	NS	9.56 $\pm$ 4.12 n = 10
M1	12.94 $\pm$ 2.83 n = 6	p < 0.01	7.30 $\pm$ 3.45 n = 8
M2	7.44 $\pm$ 0.95 n = 7	NS	6.17 $\pm$ 3.48 n = 5
M4	7.18 $\pm$ 2.45 n = 5	NS	4.40 $\pm$ 1.44 n = 5
	NS*		ns

The cells were collected at the beginning of treatment (M0), after 1 month (M1), 2 months (M2) and 4 months of treatment (M4).

*Comparisons within groups at different time points.

NS: Not significant.

immunotherapy recipients, especially on MN cells. These changes began after the first dose of *M. vaccae* and continued after the subsequent doses. IL-8 is a chemokine produced by phagocytic cells and tissue cells after stimulation with tubercle bacilli or their products [30,31]. Increased levels of IL-8 are found in the bronchoalveolar lavage fluid of patients with pulmonary tuberculosis, and higher levels of IL-8 may be associated with increased mortality [32,33].

TNF- α is a prototypic proinflammatory cytokine produced by monocytes and macrophages exposed to certain microbial products including those derived from *M. tuberculosis*. In synergy with IFN- γ , TNF- α activates macrophages to produce inducible NO synthase (iNOS), allowing the macrophage to kill intracellularly replicating tubercle bacilli in murine experiments [34–36]. The high incidence of reactivation tuberculosis in patients being treated for rheumatoid arthritis with monoclonal antibodies against

TNF- α [37] suggests that this cytokine plays a role in preventing the development or reactivation of tuberculosis. On the other hand, in established postprimary tuberculosis, TNF- α is considered a major factor in host-mediated lung damage [38,39] when it is accompanied by type 2 cytokines such as IL-4 and IL-5 [40–42]. Furthermore, in progressive disease, TNF- α is associated with both fever and wasting [43] and levels correlate with disease activity and immunopathology [44,45].

The observed reduction in nitrite production in the culture supernatants of both cell types following immunotherapy was somewhat unexpected, but may be a result of increased breakdown of nitrites, associated with enhanced metabolic processes against tubercle bacilli. Adding heat-killed H37Rv to the cultures from *M. vaccae* recipients generally raised the level of nitrite at the beginning of treatment (M0) but made little difference to values later in treatment (TABLE 1).

Several reports indicate that alveolar macrophages from tuberculosis patients produce increased amounts of NO compared with healthy control subjects [46–48]. Wang *et al.* [49] demonstrated that the increased NO exhalation in patients with tuberculosis was due to the up-regulation of the NO synthase-2A gene (*NOS2A*) in alveolar macrophages. The role of NO in killing or limiting the growth of tubercle bacilli in humans is, in contrast to murine models of tuberculosis, controversial. It has, however, been proposed that NO produced by *M. tuberculosis*-infected human macrophages and by epithelial cells inhibits or kills *M. tuberculosis* [50,51]. In an earlier study we have demonstrated that NO production by peripheral blood MN cells from tuberculosis patients increases as the disease becomes more severe, suggesting that this molecule may also play a detrimental role in disease pathogenesis [52]. The fall in NO levels observed with time in immunotherapy recipients to levels less than those found in healthy control individuals may relate to improvement in disease status.

Levels of the type 1 cytokines IFN- γ and IL-2 increased with time in cell culture supernatants from the immunotherapy recipients, in line with the observed increase in IL-12. These changes were seen to a lesser degree in supernatants of cells obtained from placebo recipients. Quite why increases with time in IFN- γ were seen in unstimulated cultures is uncertain, but probably

reflects carry over of stimulus from the blood stream with the cells. Stimulation of cells with H37Rv markedly increased levels of IFN- γ especially in MN cells of immunotherapy patients.

IFN- γ , the prototypic cytokine of the Th1 cell response, is essential for the effective control of *M. tuberculosis* in the host. It is produced by CD4 and CD8 T cells, and NK cells, as well as by PMN cells under certain conditions [53,54] and it stimulates a mycobactericidal response in macrophages characterised by the induction of iNOS [55,56].

IL-12 is mainly secreted by phagocytic cells (monocytes, macrophages and neutrophils) and dendritic cells. It promotes T-cell differentiation into Th1 cells and production of IFN- γ [57], thereby establishing a potent Th1 response to intracellular pathogens such as *M. tuberculosis*. The role of IL-12 in disease has been confirmed by the occurrence of patients with uncommon polymorphisms or mutations causing functional defects in this cytokine that predispose to severe disseminated mycobacterial infection [58].

TGF- β is a cytokine produced especially by Th3 cells, a class of T cells involved in induction of immune tolerance and suppression of Th1 functions. This cytokine appears to deactivate macrophages, thereby enhancing intracellular growth of bacilli in human monocytes [59,60] and downregulating IFN- γ production [61]. Levels of this cytokine rose in supernatants

Table 2. Levels of IL-2 measured in the stored (-70°C) supernatants of cultured mononuclear and polymorphonuclear cells from patients with moderate or severe pulmonary tuberculosis receiving *Mycobacterium vaccae* or placebo together with short-course chemotherapy (mean $\pm$ standard deviation).

	Placebo	Difference	<i>Mycobacterium vaccae</i>
Mononuclear cells			
M0	8.37 $\pm$ 4.17; n = 10	NS	8.99 $\pm$ 7.88; n = 10
M1	12.23 $\pm$ 7.82; n = 10	NS	14.22 $\pm$ 2.82; n = 10
M2	15.90 $\pm$ 7.24; n = 8	NS	15.42 $\pm$ 5.28; n = 7
M4	17.44 $\pm$ 7.60; n = 9	NS	22.55 $\pm$ 17.09; n = 9
	p < 0.05*		p < 0.05*
Healthy controls	6.45 $\pm$ 2.18; n = 7		
Polymorphonuclear cells			
M0	1.92 $\pm$ 1.08; n = 9	NS	1.94 $\pm$ 0.77; n = 9
M1	2.72 $\pm$ 1.40; n = 9	NS	4.75 $\pm$ 4.00; n = 9
M2	3.52 $\pm$ 2.05; n = 9	NS	5.34 $\pm$ 4.32; n = 9
M4	3.62 $\pm$ 2.57; n = 8	NS	6.19 $\pm$ 2.51; n = 9
	NS*		p < 0.001*
Healthy controls	10.66 $\pm$ 2.68 n = 7		

The cells were collected at the beginning of treatment (M0), after 1 month (M1), 2 months (M2) and 4 months of treatment (M4). Stimulation with H37Rv slightly increased levels without changing the pattern of responses (data not shown).

*Comparisons within groups at different time points.

NS: Not significant.

Table 3. Levels of IFN- γ measured in the stored (-70°C) supernatants of cultured mononuclear and polymorphonuclear cells from patients with moderate or severe pulmonary tuberculosis receiving *Mycobacterium vaccae* or placebo together with short-course chemotherapy (mean $\pm$ standard deviation).

	Placebo	Difference	<i>Mycobacterium vaccae</i>
Unstimulated mononuclear cells			
M0	121.05 $\pm$ 52.67; n = 9	NS	138.61 $\pm$ 76.12; n = 9
M1	139.02 $\pm$ 80.32; n = 10	NS	262.39 $\pm$ 200.93; n = 10
M2	320.24 $\pm$ 161.06; n = 9	NS	299.08 $\pm$ 151.33; n = 9
M4	250.46 $\pm$ 110.57; n = 8	NS	341.16 $\pm$ 139.30; n = 8
	p < 0.05*		p < 0.05*
Healthy controls	56.1 $\pm$ 3.42		
Unstimulated polymorphonuclear cells			
M0	41.66 $\pm$ 8.11; n = 10	NS	39.11 $\pm$ 14.46; n = 6
M1	48.98 $\pm$ 26.31; n = 10	NS	61.45 $\pm$ 30.95; n = 6
M2	47.24 $\pm$ 25.37; n = 10	NS	72.94 $\pm$ 27.88; n = 10
M4	69.65 $\pm$ 42.39; n = 9	NS	100.25 $\pm$ 61.63; n = 9
	NS*		p < 0.05*
Healthy controls	36.89 $\pm$ 2.45		
h37Rv-stimulated mononuclear cells			
M0	157.30 $\pm$ 42.10; n = 9	p < 0.01	245.60 $\pm$ 65.21; n = 9
M1	188.30 $\pm$ 84.21; n = 10	p < 0.001	488.51 $\pm$ 65.23; n = 10
M2	401.39 $\pm$ 65.28; n = 9	p < 0.001	645.36 $\pm$ 85.26; n = 9
M4	478.65 $\pm$ 46.89; n = 8	p < 0.001	749.29 $\pm$ 87.65; n = 8
	p < 0.001*		p < 0.001*
Healthy controls	93.56 $\pm$ 6.29		
h37Rv-stimulated polymorphonuclear cells			
M0	59.52 $\pm$ 11.85; n = 10	NS	55.65 $\pm$ 15.17; n = 6
M1	68.92 $\pm$ 9.64; n = 10	NS	84.57 $\pm$ 21.95; n = 6
M2	71.54 $\pm$ 14.21; n = 10	NS	92.32 $\pm$ 29.46; n = 10
M4	96.64 $\pm$ 21.45; n = 9	NS	118.64 $\pm$ 41.54; n = 9
	NS*		p < 0.01*
Healthy controls	46.2 $\pm$ 3.45		

The cells were collected at the beginning of treatment (M0), after 1 month (M1), 2 months (M2) and 4 months of treatment (M4).

*Comparisons within groups at different time points.

NS: Not significant.

of stimulated cultures of both cell types in both treatment groups over the first 2 months, but then by the fourth month levels fell significantly in immunotherapy recipients, reflecting the expected enhancement of Th1 and down-regulation of Th2. During active tuberculosis, the monocytes of patients have been reported to exhibit enhanced production of TGF- β_1 [62]. (IL-4 also deactivates macrophages and potently downregulates iNOS [63]). As well as exerting immunosuppressive effects, TGF- β appears to play a role in the generation and functioning of regulatory T cells [64–66]. These cells are central to the regulation of the anti-infectious response, restraining immune-mediated damage but also suppressing protective responses [67,68]. In humans, TGF- β influences the induction of Th17 cells, perhaps increasing

immune regulation through T-regulatory cells [69]. The fact that IFN- γ production in *M. vaccae* recipients remained increased despite their augmented TGF- β production is more compatible with an immunoregulatory role than with an immunosuppressant action of this cytokine and perhaps also on nitrite production in patients receiving treatment with *M. vaccae*.

The immunological responses of patients with a disease – in this case, pulmonary tuberculosis – to three injections of heat-killed *M. vaccae* SRL172, are clearly shown in this and our earlier reports [5,7]. Small studies in healthy volunteers of single doses of SRL172 have failed to show significant changes except for a fleeting increase in IL-10, and it seems that the changes in cell receptor expression and cytokine induction are only seen when this immunotherapy

is applied in a disease situation. In general, our findings support and further confirm that clinical benefit resulting from multidose immunotherapy with *M. vaccae* is accompanied by a progressive switch from Th2 towards Th1. This fits with the paradigm that Th1 responses are beneficial in the recovery from tuberculosis.

General discussion

There is increasing evidence that a wide range of human diseases are associated, at least to some extent, with disorders in the regulation of immune responses and an increase in Th2 predominance. It is, therefore, highly likely that correction of such dysregulation by treatment with borate-buffered, heat-killed *M. vaccae* or related forms of immunotherapy will be beneficial. Thus a reduction in the late airway response of patients with bronchial asthma [9] and a reduction in bronchial aspects of hayfever [10] could be expected as a result of similar immunological changes leading to reduced levels of IgE and a reduction in autoimmune responses to airway tissue damage [9]. Immune regulation is also likely to have played a role in the prolongation of life in patients with adenocarcinoma of the lung receiving *M. vaccae* immunotherapy [13], although it is probably too early in our investigations to speculate on how this is achieved. Several human cancers are associated with a

'Th2 drift' [70], thus removal of colon cancer is accompanied by a switch towards Th1 [71] and improvement in malignant melanoma has also been associated with such a switch [72].

There can be little doubt that many of the immunological changes observed in the response of tuberculosis patients to immunotherapy with *M. vaccae* are due to the immunotherapy itself and it would seem reasonable to assume that these changes would accompany such treatment when applied to other conditions, and may constitute the mechanism of its action. However, part of the changes observed in tuberculosis patients are likely to be driven by antigens released from tubercle bacilli themselves, as illustrated in the effects of stimulating cells with the preparation of H37Rv. This latter effect is going to be different during the treatment of noninfectious conditions, such as bronchial asthma [9,10] and myointimal hyperplasia [73], where tissue stress proteins may be the predominant antigens that are increased. In neoplastic disease stress proteins are increased together with release of cancer related antigens, and bacteriomimetic sugars, which lack the terminal *N*-acetyl glucosamine of normal mammalian sugars and accompany rapid cell division [71]. Such unusual antigens may be both a driving force promoting Th2 mechanisms and act as targets for Th1 mechanisms promoted by the immunotherapeutic. Despite

Table 4. Levels of IL-12 measured in the stored (-70°C) supernatants of cultured mononuclear and polymorphonuclear cells from patients with moderate or severe pulmonary tuberculosis receiving *Mycobacterium vaccae* or placebo together with short-course chemotherapy (mean $\pm$ standard deviation).

	Placebo	Difference	<i>Mycobacterium vaccae</i>
Mononuclear cells			
M0	4.0 $\pm$ 2.62; n = 7	NS	3.77 $\pm$ 3.56; n = 7.
	NS		p < 0.02*
M1	4.51 $\pm$ 2.33; n = 7	p < 0.05	13.87 $\pm$ 9.07; n = 7
M2	4.00 $\pm$ 1.92; n = 7	p < 0.01	14.39 $\pm$ 5.40; n = 7
M4	4.77 $\pm$ 2.29; n = 7	p < 0.01	12.83 $\pm$ 5.86; n = 7
	NS*		p < 0.005*
Healthy controls	8.96 $\pm$ 4.73		
Polymorphonuclear cells			
M0	5.43 $\pm$ 3.42; n = 7	NS	4.72 $\pm$ 4.33; n = 7
	NS		p < 0.05
M1	9.60 $\pm$ 5.89; n = 7	NS	12.98 $\pm$ 8.17; n = 7
M2	9.82 $\pm$ 3.62; n = 7	NS	14.87 $\pm$ 5.91; n = 7
M4	11.37 $\pm$ 3.83; n = 7	NS	14.00 $\pm$ 5.21; n = 7
	p < 0.01*		p < 0.005*
Healthy controls	18.71 $\pm$ 5.86		

Stimulation of cells with H37Rv produced slightly higher values, but did not alter the picture of responses (data not shown). The cells were collected at the beginning of treatment (M0), after 1 month (M1), 2 months (M2) and 4 months of treatment (M4).

*Comparisons within groups at different time points.

NS: Not significant.

Table 5. Levels of TGF- β measured in the stored (-70°C) supernatants of cultured mononuclear and polymorphonuclear cells from patients with moderate or severe pulmonary tuberculosis receiving *Mycobacterium vaccae* or placebo together with short-course chemotherapy (mean $\pm$ standard deviation).

	Placebo	Difference	<i>Mycobacterium vaccae</i>
Unstimulated mononuclear cells			
M0	91.00 $\pm$ 24.97; n = 10	NS	98.60 $\pm$ 51.84; n = 10
	p < 0.005		p < 0.02
M1	142.30 $\pm$ 46.57; n = 10	NS	163.30 $\pm$ 59.20; n = 10
M2	143.50 $\pm$ 40.77; n = 10	NS	155.70 $\pm$ 49.01; n = 10
M4	124.90 $\pm$ 42.59; n = 10	NS	101.20 $\pm$ 17.57; n = 10
M0 to M4	p < 0.05		NS
M1 to M4	NS		p < 0.01
Healthy controls	75.11 $\pm$ 22.78		
Unstimulated polymorphonuclear cells			
M0	104.80 $\pm$ 43.7; n = 10	NS	110.70 $\pm$ 32.04; n = 10
M1	108.50 $\pm$ 19.99; n = 10	NS	105.50 $\pm$ 23.86; n = 10
M2	121.90 $\pm$ 48.80; n = 10	NS	87.00 $\pm$ 28.85; n = 10
M4	109.00 $\pm$ 18.10; n = 10	p < 0.001	65.20 $\pm$ 20.31; n = 10
M0 to M4	NS		p < 0.005
Healthy controls	36.18 $\pm$ 5.64		
h37Rv-stimulated mononuclear cells			
M0	118.60 $\pm$ 32.45; n = 10	NS	134.70 $\pm$ 46.50; n = 10
M1	169.89 $\pm$ 23.00; n = 9	p < 0.05	236.80 $\pm$ 82.81; n = 10
M2	176.21 $\pm$ 21.87; n = 10	p < 0.05	292.90 $\pm$ 157.20; n = 10
M4	185.32 $\pm$ 16.54; n = 10	p < 0.01	341.80 $\pm$ 144.95; n = 10
M0 to M4	p < 0.001		p < 0.001
Healthy controls	93.56 $\pm$ 12.54		
h37Rv-stimulated polymorphonuclear cells			
M0	117.90 $\pm$ 29.67; n = 10	NS	134.70 $\pm$ 46.50; n = 10
M1	135.32 $\pm$ 87.59; n = 10	NS	167.45 $\pm$ 32.85; n = 10
M2	176.90 $\pm$ 84.95; n = 10	p < 0.05	102.23 $\pm$ 24.65; n = 10
M4	199.34 $\pm$ 45.87; n = 10	p < 0.001	98.65 $\pm$ 22.12; n = 10
M0 to M4	p < 0.001		p < 0.05
Healthy controls	47.38 $\pm$ 13.66		

The cells were collected at the beginning of treatment (M0), after 1 month (M1), 2 months (M2) and 4 months of treatment (M4).

NS: Not significant.

these differences, the tuberculosis model demonstrates the mechanisms that may be brought to bear on other diseases with the application of *M. vaccae* immunotherapy [74].

As seen in lung cancer patients [13,75,76], improvement in quality of life has been noted in tuberculosis patients in Nigeria [4] and Vietnam with those receiving immunotherapy with *M. vaccae* returning to work earlier than those receiving placebo. Recent studies in a preclinical model [77] suggest direct actions of *M. vaccae* that might partly explain improved quality of life, and it is known that depressive psychosis, such as that associated with a cancer diagnosis, is accompanied by a Th2 preponderance [78].

The passage of adsorbed oxygen into the blood system and its distribution through the heart are

just as much parts of the physiology of respiration as are the airways and lungs. In any consideration of the place of immunotherapy in respiratory disease, the effects that it may have on circulatory physiology must also be taken into account. Preclinical studies in a rat model have shown that treatment with *M. vaccae* has significant beneficial effects reducing myointimal hyperplasia, the basis of large vessel atheroma deposition [73,79]. The parallels between immune changes constricting airways, those constricting the passage of blood and the alleviation of both by treatment with *M. vaccae* help complete the picture.

It would be very unlikely if beneficial immune-modulating properties were restricted to *M. vaccae* alone amongst the numerous species of adjuvant-rich mycobacteria and related

genera of aerobic Actinomycetales, or that an identical pattern of activity is required for the correction of immune dysregulation in a broad range of diseases. For this reason, a range of other preparations of heat-killed bacteria, including *Mycobacterium obuense*, a species very similar to *M. vaccae*, and species of the genera *Gordonia*, *Rhodococcus* and *Tsukamurella* are currently being investigated in preclinical models of human disease [24], and in naturally occurring diseases of veterinary importance.

To this point in our discourse, consideration has only taken into account published studies. However, hitherto unpublished anecdotal observations also merit consideration. Many patients receiving treatment with borate-buffered, heat-killed *M. vaccae* have observed an increased resistance to acute respiratory infections of viral origin, such as colds and influenza, presumably operative through Th1 responses to stress proteins expressed on virally infected cells [74]. Other major respiratory diseases include sarcoidosis, on which we have no anecdotal data, and chronic obstructive pulmonary disease (COPD), on which we have some information.

In studies using a borate-buffered, heat-killed preparation of *Tsukamurella incheonensis* for the treatment of sweet-itch, a serious seasonal allergy to midge bites in horses, a few animals coincidentally had respiratory airways obstruction, the equine equivalent of COPD in humans. Without exception, these few animals showed marked alleviation of their respiratory distress. Taken together with the few anecdotes that we have of patients with COPD receiving *M. vaccae* for other conditions, showing an improvement in respiratory symptoms, there would seem to be a need for a formal trial of this immunotherapy in patients with COPD.

The apparent success of immunotherapy with *M. vaccae* in the treatment of adenocarcinoma of the lung brings into question the reasons that this immunotherapy showed no extension of survival in those with squamous carcinoma of the lung, despite their improved quality of life [13,75,76]. Subsequent anecdotal data suggest that *M. vaccae* can be effective against squamous tumors, but many more doses are required to achieve success.

The reason for this difference in responsiveness for these two tumor types probably lies in the differences in antigens that they express or release. Adenocarcinomas release mucins and carbohydrates that may mimic to some degree those of bacteria, thus providing additional targets cross-reactive with bacteria to which the

modulated immune response can be directed. In addition, just as antigens released by tubercle bacilli during immunotherapy for tuberculosis may exert immunomodifying effects, both beneficial and deleterious, antigens released by tumor cells stressed by the immunotherapeutic response, may counteract those of *M. vaccae*, thus requiring repeated doses. Besides these differences in response to *M. vaccae* in the two major divisions of non-small-cell lung cancer, early studies of small-cell disease suggest that the application of additional, and perhaps more frequent, doses would also be effective [76].

Other cancer-related antigens that could be targets for bacterial immunotherapy are viruses, notably human endogenous retroviruses. Expression of a short amino-acid chain length believed to originate from such a virus has been associated with malignant melanoma, and in one patient development of cytolytic T lymphocytes to this resulted in long-term remission. This short chain has also been shown to cross-react with *M. tuberculosis* bacillus of Calmette and Guérin (BCG) [80].

Other than the local reaction to intradermal injections, similar to those following BCG vaccination and similarly resulting in a small scar, use of *M. vaccae* has few side effects. However, with the repeated injections at short intervals needed for the treatment of cancer, some patients have difficulties with compliance. Giving injections at sites other than the upper arm, for example on the thigh, tends to give larger reactions. In our past studies, mixing a low dose of new tuberculin with the *M. vaccae* reduced the scar size without reducing efficacy in tuberculosis and leprosy [81,82], although producing such a combined reagent would be very difficult under today's drug regulatory restrictions.

The future of immunomodulation with products such of the *M. vaccae* type may well lie in oral dosage and this is under active investigation.

Conclusion

New information is presented on the immunological changes following three monthly intradermal doses of borate-buffered, heat-killed *M. vaccae* (SRL172) added to short-course chemotherapy (2RHEZ/4RH) for pulmonary tuberculosis. It is suggested that this new data, much of it obtained from stored cell culture supernatants, when added to those already published, illustrates the changes that would be induced when the same immunotherapy is applied to other nonmycobacterial diseases and that tuberculosis provides a suitable model for these effects.

The data published so far on noninfectious diseases of the respiratory system are reviewed in relation to the data obtained from the tuberculosis model and unpublished anecdotal information on such diseases are discussed.

Future perspective

The information already available on immunotherapy with *M. vaccae* (SRL172) and similar preparations of other aerobic Actinomycetales indicate that this form of bacterial immune modulation is going to play a major role in future medicine. The broad spectrum of diseases that could benefit from such treatment, and the apparent nonspecificity of the approach flies in the face of concepts of modern vaccinology. Applicable in human, veterinary and agricultural medicine, at potentially low cost and with few adverse effects, widespread introduction of this treatment will have both significant

effects on mortality from disease and contribute significantly to food supply.

The development of effective oral preparations is in progress and early data show that it will have similar effects in the same range of applications.

Financial & competing interests disclosure

Cynthia Stanford and John Stanford were shareholders in SRPharma (now Silence Therapeutics), the providers of SRL172, when the study was carried out. They are now Directors of BioEos Ltd. and shareholders in Immodulon Therapeutics, the present owners of Mycobacterium vaccae NCTC 11659 as a therapeutic. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Introduction

- The scene is set and previously available data are presented.
- The availability of clinical samples from an earlier study is explained.

Materials & methods

- Treatment of the patients is described and the samples available are delineated.
- The investigation of cultured cells and their culture supernatants is explained.

Results

- These include the results for TNF- α and IL-8-receptor expression on cells and their ability to produce nitrite.
- The levels of cytokines (IL-2, IFN- γ , IL-12 and TGF- β) in culture supernatants are shown in a series of tables.

Discussion

- A summary of the findings in tuberculosis and their significance is discussed.
- It is proposed that pulmonary tuberculosis provides a suitable model for immunotherapy with *Mycobacterium vaccae* applicable to other diseases.
- These other conditions include bronchospasm and non-small-cell lung cancer.
- The difference in response of adenocarcinoma and squamous carcinoma is discussed.
- The possibilities for treating chronic obstructive pulmonary disease and myointimal hyperplasia of arteries are considered and discussed.

Bibliography

Papers of special note have been highlighted as:

▪ of interest

▪▪ of considerable interest

- 1 Bahr GM, Shaaban MA, Gabriel M *et al.*: Improved immunotherapy for pulmonary tuberculosis with *Mycobacterium vaccae*. *Tubercle* 71, 259–266 (1990).
- Compares different preparations of *Mycobacterium vaccae* in relation to their ability to induce immunological changes of apparent benefit in the treatment of tuberculosis.
- 2 Corlan E, Marica C, Macavei C, Stanford JL, Stanford CA: Immunotherapy with *Mycobacterium vaccae*. 1. In the treatment of newly diagnosed pulmonary

tuberculosis in Romania. *Respir. Med.* 91, 13–19 (1997).

- 3 Corlan E, Marica C, Macavei C, Stanford JL, Stanford CA: Immunotherapy with *Mycobacterium vaccae*. 2. In the treatment of chronic or relapsed tuberculosis in Romania. *Respir. Med.* 91, 21–29 (1997).
- 4 Onyebujoh P, Abdulmumini T, Robinson S, Rook GAW, Stanford JL: Immunotherapy for tuberculosis in African conditions. *Respir. Med.* 89, 199–207 (1995).
- First evidence that immunotherapy for tuberculosis with *M. vaccae* can work in HIV-infected patients and suggests that there could be a beneficial effect on HIV itself.
- 5 Dlugovitzky D, Bottasso O, Dominino JC *et al.*: Clinical and serological studies of

tuberculosis patients in Argentina receiving immunotherapy with *Mycobacterium vaccae* (SRL 172). *Respir. Med.* 93, 557–562 (1999).

- 6 Johnson JL, Kamya RM, Okwera A *et al.*: Randomized controlled trial of *Mycobacterium vaccae* immunotherapy in non-human immunodeficiency virus-infected Ugandan adults with newly diagnosed pulmonary tuberculosis. The Uganda-Case Western Reserve University Research Collaboration. *J. Infect. Dis.* 181, 1304–1312 (2000).
- 7 Dlugovitzky D, Fiorenza G, Farroni M, Bogue C, Stanford C, Stanford J: Immunological consequences of three doses of heat-killed *Mycobacterium vaccae* in the immunotherapy of tuberculosis. *Respir. Med.* 100, 1079–1087 (2006).

- Detailed, though small, study of three doses of *M. vaccae* against pulmonary tuberculosis. Clinical, haematological, radiological and immunological improvement are all recorded. It was from this study that the stored material for the current paper was obtained.
- 8 Ramu G, Grange J, Stanford J: Evaluation of adjunct immunotherapy with killed *Mycobacterium vaccae* in the treatment of multibacillary leprosy and erythema nodosum leprosum. In: *Clinical Mycobacteriology*. Casal M (Ed.), Barcelona, Prous Science Publishers, Barcelona, Spain 393–402 (1998).
 - 9 Camporota L, Corkhill A, Long H *et al.*: The effects of *Mycobacterium vaccae* on allergen-induced airway responses in atopic asthma. *Eur. Respir. J.* 21, 287–293 (2003).
 - 10 Hopkin JM, Ferry B, Coull P *et al.*: Mycobacterial immunization in grass pollen asthma and rhinitis. *Thorax* 53, 63 (1998).
 - 11 Arkwright PD, David TJ: Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J. Allergy Clin. Immunol.* 107, 531–534 (2001).
 - 12 Lehrer A, Bressanelli A, Wachsmann V *et al.*: Immunotherapy with *Mycobacterium vaccae* in the treatment of psoriasis. *FEMS Immunol. Med. Microbiol.* 21, 71–77 (1998).
 - 13 Stanford JL, Stanford CA, O'Brien M, Grange JM: Successful immunotherapy with *Mycobacterium vaccae* in the treatment of adenocarcinoma of the lung. *Eur. J. Cancer* 44, 224–227 (2008).
 - Important paper describing how several doses of *M. vaccae* can significantly lengthen survival in patients with advanced adenocarcinoma of the lung, while having no such effect in patients with squamous carcinoma of the lung.
 - 14 Berth-Jones J, Arkwright PD, Marasovic D, *et al.*: Killed *Mycobacterium vaccae* suspension in children with moderate-to-severe atopic dermatitis: a randomized, double-blind, placebo-controlled trial. *Clin. Exp. Allergy*. 36(9), 1115–1121 (2006).
 - 15 Davies MG, Symons C, Shaw S, Kaminski ER: An open study to assess the efficacy clinically and immunologically of *M. vaccae* vaccine in patients with atopic dermatitis. *J. Dermatolog. Treat.* 17(2), 74–77 (2006).
 - 16 Stanford JL, Stanford CA, Grange JM: Environmental echoes. *Sci. Prog.* 84, 105–124 (2001).
 - Explains how relationships with environmental mycobacteria change with time and the potential influence this could have on the prevalence of disease and the efficacy of bacterial immunotherapeutics.
 - 17 Durban Immunotherapy Trial Group: Immunotherapy with *Mycobacterium vaccae* in patients with newly diagnosed pulmonary tuberculosis: a randomised controlled trial. *Lancet* 354, 116–119 (1999).
 - 18 de Bruyn G, Garner P: *Mycobacterium vaccae* immunotherapy for treating tuberculosis. *Cochrane Database Syst. Rev.* CD001166 (2003).
 - 19 Mwinga A, Nunn A, Ngwira B *et al.*: *Mycobacterium vaccae* (SRL172) immunotherapy as an adjunct to standard antituberculosis treatment in HIV-infected adults with pulmonary tuberculosis: a randomised placebo-controlled trial. *Lancet* 360, 1050–1055 (2002).
 - 20 Hernandez-Pando R, Pavon L, Arriaga K, Orozco H, Madrid-Marina V, Rook G: Pathogenesis of tuberculosis exposed to low and high doses of an environmental mycobacterial saprophyte before infection. *Infect. Immun.* 65(8), 3317–3327 (1997).
 - 21 Zuany-Amorim C, Sawicka E, Manlius C *et al.*: Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat. Med.* 8, 625–629 (2002).
 - 22 Valian HK, Kenedy LKA, Rostami MN, Mohammadi AM, Khamesipour A: Role of *Mycobacterium vaccae* in the protection induced by first generation *Leishmania* vaccine against murine model of leishmaniasis. *Parasitol. Res.* 103, 21–28 (2008).
 - 23 Hrouda D, souberbielle BE, Kayaga J, Corbishley CM, Kirby RS, Dalgleish G: *Mycobacterium vaccae* (SRL172): a potential immunological adjuvant evaluated in rat prostate cancer. *Br. J. Urol.* 82(6), 870–876 (1998).
 - 24 Fontanella GH, Pascutti MF, Daurelio L *et al.*: Improved outcome of *Trypanosoma cruzi* infection in rats following treatment in early life with suspensions of heat-killed environmental Actinomycetales. *Vaccine* 25, 3492–3500 (2007).
 - 25 Luo Y: The immunotherapeutic effect of *Mycobacterium vaccae* vaccine on initially treated pulmonary tuberculosis. *Chin. J. Evid. based Med.* 24, 43–47 (2001).
 - 26 Luo YA, Lu SH, Guo SL *et al.*: Immunotherapeutic effect of *Mycobacterium vaccae* on multi-drug resistant pulmonary tuberculosis. *Chin. J. Tuberc. Resp. Dis.* 23(2), 85–88 (2000).
 - 27 Fan MY, Chen XR, Wang K *et al.*: Adjuvant effect of *Mycobacterium vaccae* on treatment of recurrent treated pulmonary tuberculosis: a meta-analysis. *Chin. J. Evid. based Med.* 7(6), 449–455 (2007).
 - 28 Lijun X, Yanyan W, Xiaodong Z, Xiangdong G, Lifeng T, Haiming W: Immunotherapeutic potential of *Mycobacterium vaccae* on *M. tuberculosis* infection in mice. *Cell. Molec. Immunol.* 6(1), 67–72 (2009).
 - 29 Zhao XY, Xie QM, Chen JQ, Bian RL: [Effects of *M. vaccae* on airway contraction and inflammation in asthmatic guinea pigs] *Zhonghua Jie He He Hu Xi Za Zhi* 26(4), 218–222 (2003).
 - 30 Wickremasinghe MI, Thomas LH, Friedland JS: Pulmonary epithelial cells are a source of IL-8 in the response to *Mycobacterium tuberculosis*: essential role of IL-1 from infected monocytes in a NF- κ B dependent network. *J. Immunol.* 163, 3936–3947 (1999).
 - 31 Zhang Y, Broser M, Cohen H *et al.*: Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacterium tuberculosis* and its components. *J. Clin. Invest.* 95, 586–592 (1995).
 - 32 Friedland JS, Hartley JC, Hartley CG, Shattock RJ, Griffin GE: Inhibition of *ex vivo* proinflammatory cytokine secretion in fatal *Mycobacterium tuberculosis* infection. *Clin. Exp. Immunol.* 100, 233–238 (1995).
 - 33 Sadek MI, Sada E, Toossi Z, Schwander SK, Rich EA: Chemokines induced by infection of mononuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. *Am. J. Respir. Cell. Mol. Biol.* 19, 513–521 (1998).
 - 34 Ding AH, Nathan CF, Stuehr DJ: Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* 141, 2407–2412 (1988).
 - Flesch IE, Hess JH, Oswald IP, Kaufmann SH: Growth inhibition of *Mycobacterium bovis* by IFN- γ stimulated macrophages: regulation by endogenous tumor necrosis factor- α and by IL-10. *Int. Immunol.* 6, 693–700 (1994).
 - 35 Flynn J, Goldstein MM, Chan J *et al.*: Tumor necrosis factor α is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 2, 561–572 (1995).

- 36 Gomez-Reino JJ, Carmona L, Valverde VR *et al.*: Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. *Arthritis Rheum.* 48, 2122–2127 (2003).
- 37 Rook GAW, Taverne J, Leveton C *et al.*: The role of gamma interferon, vitamin D3 metabolites and tumour necrosis factor in the pathogenesis of tuberculosis. *Immunology* 62, 229–234 (1987).
- 38 Bekker L-G, Moreira AL, Bergtold A *et al.*: Immunopathologic effects of tumor necrosis factor α in murine mycobacterial infection are dose dependent. *Infect. Immun.* 68, 6954–6961 (2000).
- 39 Hernandez-Pando R, Rook GA: The role of TNF- α in T-cell-mediated inflammation depends on the Th1/Th2 cytokine balance. *Immunology* 82, 591–595 (1994).
- 40 Hernandez-Pando R, Aguilar D, Hernandez ML, Orozco H, Rook G: Pulmonary tuberculosis in BALB/c mice with non-functional *IL-4* genes: changes in the inflammatory effects of TNF- α and in the regulation of fibrosis. *Eur. J. Immunol.* 34, 174–183 (2004).
- 41 Van Crevel R, Karyadi E, Preyers F *et al.*: Increased production of interleukin 4 by CD4⁺ and CD8⁺ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. *J. Infect. Dis.* 181, 1194–1197 (2000).
- 42 Mazzarella G, Bianco A, Perna F *et al.*: T lymphocyte phenotypic profile in lung segments affected by cavitory and non-cavitory tuberculosis. *Clin. Exp. Immunol.* 132, 283–288 (2003).
- 43 Cadranel J, Philippe C, Perez J *et al.*: *In vitro* production of tumour necrosis factor and prostaglandin E2 by peripheral blood mononuclear cells from tuberculosis patients. *Clin. Exp. Immunol.* 81, 319–324 (1990).
- 44 Condos R, Rom WN, Liu YM, Schluger NW: Local immune responses correlate with presentation and outcome in tuberculosis. *Am. J. Respir. Crit. Care Med.* 157, 729–735 (1998).
- 45 Olobo JO, Geletu M, Demissie A *et al.*: Circulating TNF- α , TGF- β , and IL-10 in tuberculosis patients and healthy contacts. *Scand. J. Immunol.* 53, 85–91 (2001).
- 46 Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR *et al.*: Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J. Exp. Med.* 183, 2293–2302 (1996).
- 47 Kuo HP, Wang CH, Huang KS *et al.*: Nitric oxide modulates interleukin-1 β and tumor necrosis factor- α synthesis by alveolar macrophages in pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* 161, 192–199 (2000).
- 48 Wang CH, Liu CY, Lin HC, Yu CT, Chung KF, Kuo HP: Increased exhaled nitric oxide in active pulmonary tuberculosis due to inducible NO synthase upregulation in alveolar macrophages. *Eur. Respir. J.* 11, 809–815 (1998).
- 49 Rich EA, Torres M, Sada E, Finegan CK, Hamilton BD, Toossi Z: *Mycobacterium tuberculosis* (MTB)-stimulated production of nitric oxide by human alveolar macrophages and relationship of nitric oxide production to growth inhibition of MTB. *Tuber. Lung Dis.* 78, 247–255 (1997).
- 50 Rockett KA, Brookes R, Udalova I, Vidal V, Hill AV, Kwiatkowski D: 1,25-dihydroxyvitamin D3 induces nitric oxide synthase and suppresses growth of *Mycobacterium tuberculosis* in a human macrophage-like cell line. *Infect. Immunol.* 66, 5314–5321 (1998).
- 51 Dlugovitzky D, Bay ML, Rateni L *et al.*: Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells from patients with pulmonary tuberculosis. *Clin. Exp. Immunol.* 122, 343–349 (2000).
- 52 Cassatella MA: The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* 16, 21 (1995).
- 53 Bogdan C, Schleicher U: Production of interferon- γ by myeloid cells – fact or fancy? *Trends Immunology* 27, 282–290 (2006).
- 54 Flynn JL, Chan J: Immunology of tuberculosis. *Annu. Rev. Immunol.* 19, 93–129 (2001).
- 55 Bottasso OA, Bay ML, Besedovsky H, del Rey A: The immuno-endocrine component in the pathogenesis of tuberculosis. *Scand. J. Immunol.* 66, 166–175 (2007).
- 56 Trinchieri G: Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3, 133–146 (2003).
- 57 Casanova JL, Abel L: Genetic dissection of immunity to mycobacteria: the human model. *Annu. Rev. Immunol.* 20, 581–620 (2002).
- 58 Hirsch CS, Yoneda T, Averill L *et al.*: Enhancement of intracellular growth of *Mycobacterium tuberculosis* in human monocytes by transforming growth factor- β 1. *J. Infect. Dis.* 170, 1229–1237 (1994).
- 59 Warwick-Davies J, Lowrie DB, Cole PJ: Selective deactivation of human monocyte functions by TGF- β . *J. Immunol.* 155, 3186–3195 (1995).
- 60 Othieno C, Hirsch CS, Hamilton BD *et al.*: Interaction of *Mycobacterium tuberculosis*-induced transforming growth factor- β 1 and interleukin-10. *Infect. Immun.* 67, 5730–5735 (1999).
- 61 Toossi Z, Gogate P, Shiratsuchi H *et al.*: Enhanced production of TGF- β by blood monocytes from patients with active tuberculosis and presence of TGF- β in tuberculous granulomatous lung lesions. *J. Immunol.* 154, 465–473 (1995).
- 62 Bogdan C, Vodovotz Y, Paik J, Xie QW, Nathan C: Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukoc. Biol.* 55, 227–233 (1994).
- 63 Marie JC, Letterio JJ, Gavin M, Rudensky AY: TGF- β 1 maintains suppressor function and Foxp3 expression in CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 201, 1061–1067 (2005).
- 64 Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L: TGF- β -dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. *Nat. Med.* 9, 1202–1208 (2003).
- 65 Bommireddy R, Doetschman T: TGF- β , T-cell tolerance and anti-CD3 therapy. *Trends Mol. Med.* 10, 3–9 (2004).
- 66 Belkaid Y: Regulatory T cells and infection: a dangerous necessity. *Nat. Rev. Immunol.* 7, 875–888 (2007).
- 67 Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A: Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 173, 803–810 (2006).
- 68 Shabaana AK, Cooper AM: IL-23 and IL-17 in tuberculosis. *Cytokine* 41, 79–83 (2008).
- 69 Ito N, Nakamura H, Tanaka Y, Ohgi S: Lung carcinoma: analysis of T helper type 1 and 2 cells and T cytotoxic type 1 and 2 cells by intracellular cytokine detection with flow cytometry. *Cancer* 85(11), 2359–2367 (1999).
- 70 Stanford JL, Stanford CA, Baban B, Grange JM: Therapeutic vaccination for cancer, the potential value of mycobacterial products. *Int. J. Pharmaceut. Med.* 12, 191–195 (1992).
- 71 Maraveyas A, Baban B, Kennard D *et al.*: Possible improved survival of patients with stage IV AJCC melanoma receiving SRL 172 immunotherapy: correlation with induction of increased levels of intracellular interleukin-2 in peripheral blood leucocytes. *Ann. Oncol.* 10, 817–824 (1999).

- 72 Stansby G, Chan YC, Berwanger CS, Shurey S, Rook GA, Stanford JL: Prevention of experimental myointimal hyperplasia by immunomodulation. *Eur. J. Vasc. Endovasc. Surg.* 23, 23–28 (2002).
- **Demonstrates that treatment with *M. vaccae* reduces the severity of carotid myointimal hyperplasia following experimental balloon angioplasty.**
- 73 Stanford J, Stanford C, Stansby G, Bottasso O, Bahr G, Grange J: The common mycobacterial antigens and their importance in the treatment of disease. *Current Pharm. Des.* 15, 1248–1260 (2009).
- **Discusses the part played by immune recognition of the common antigens of mycobacteria, including their heat-shock proteins, in many diseases and the importance of cross-reactive host proteins as targets for *M. vaccae* immunotherapy.**
- 74 O'Brien ME, Anderson H, Kaukel E *et al.*: SRL172 (killed *Mycobacterium vaccae*) in addition to standard chemotherapy improves quality of life, without affecting survival, in patients with advanced non-small-cell lung cancer: Phase III results. *Ann. Oncol.* 15, 906–914 (2004).
- 75 Harper-Wynne CL, Sumpter K, Ryan C *et al.*: Addition of SRL172 to standard chemotherapy in small-cell lung cancer (SCLC) improves symptom control. *Lung Cancer* 47, 289–290 (2005).
- 76 Rook GAW, Lowry CA: The hygiene hypothesis and psychiatric disorders. *Trends Immunology* 29, 150–158 (2008).
- 77 Reiche EM, Morimoto HK, Nunes SM: Stress and depression-induced immune dysfunction: implications for the development and progression of cancer. *Int. Rev. Psychiatry* 17, 515–527 (2005).
- 78 Shahzada A, Binder CJ: Experimental immunotherapeutic approaches for atherosclerosis. *Clin. Immunol.* (2009) (Epub ahead of print).
- 79 Schiavetti F, Thonnard J, Colao D, Boon T, Coulie PG: A human endogenous retroviral sequence encoding an antigen recognised on melanoma by cytolytic T lymphocytes. *Cancer Res.* 62, 5510–5516 (2002).
- **Firmly associates a human endogenous retrovirus in the aetiology of melanoma and shows that a short amino-acid chain length is shared between the virus-induced antigens and some bacteria.**
- 80 Bahr GM, Stanford JL, Rook GAW, Rees RJW, Abdelnoor AM, Frayha GJ: Two potential improvements to BCG and their effect on skin test reactivity in the Lebanon. *Tubercle* 67, 205–218 (1986).
- 81 Stanford JL, Terencio de las Aguas J, Torres P, Gervasoni BO, Ravioli R: Studies on the effects of a potential immunotherapeutic agent in leprosy patients. Health Cooperation Papers 7. *Proceedings of the Fourth European Leprosy Symposium* 201–206 (1987).