Immune Response to *Streptomyces lividans* in Mice: A Potential Vaccine Vehicle Against TB

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Abstract: The potentialities of *Streptomyces lividans* 1326 as new live vaccine vehicle strategy have been evaluated. Immunization of mice with the *Streptomyces* mycelium induced high levels of specific antibodies against proteins released in the culture supernatant of the analyzed strain. Splenocytes from the *Streptomyces*-immunized animals were able to secrete high levels of IFN-γ (2103.9 pg/mL) and to proliferate *in vitro* on stimulation with proteins released by *Streptomyces*. The analysis of cross-reactivity against *M. tuberculosis* secreted proteins showed that the immunization of mice with *Streptomyces* led to a comparable level of cross-reacting antibodies as in the BCG immunized mice. Similarly sera antibodies from *Streptomyces* immunized group recognized whole BCG cells to the same degree as antibodies raised against BCG reacted with *Streptomyces* mycelium, indicating that these two actinobacteria cross-react immunologically.

The *Streptomyces lividans* strain was highly immunogenic in mice showing an enduring Th1-dependent immune response. This is the first report demonstrating the potential of *Streptomyces* as an attractive vehicle for developing a live TB vaccine.

Key Words: Immune response, *Streptomyces lividans, Mycobacterium tuberculosis*, live vaccine, tuberculosis strategies.

INTRODUCTION

The current vaccine against tuberculosis (TB), bacille Calmette-Guérin (BCG), is a live vaccine derived from an attenuated strain of *Mycobacterium bovis*. BCG protects against severe childhood forms of the disease, but fails to protect against adult pulmonary TB in countries where it is endemic. Also, there are 8 million people worldwide developing active TB annually causing 3 million deaths [1]. It is estimated that one-third of the world's population is latently infected with *Mycobacterium tuberculosis*. These people become a natural reservoir for the propagation of the bacilli, and cases of active tuberculosis can occur during endogenous reactivation of such a latent infection or following exogenous reinfection [2, 3].

More than one vaccine is needed: one to be delivered to infants who basically have a naive immune system against *M. tuberculosis* and the nontuberculous mycobacteria, another to treat previously infected people and a third one for therapy of people who have developed the active disease [4].

As a result of the needs, vaccine candidates against TB have been generated in the last 10 years which were tested in different laboratory assays [5], experimental animal models [6, 7], and clinical trials in human populations [8].

Recently, a new microorganism has been suggested to be a suitable candidate for developing TB vaccines, namely *Streptomyces lividans*. This bacterium belongs to one of the major branches of the Gram-positive bacteria: the high G-C organisms referred to as actinomycetes [9]. *Mycobacterium tuberculosis* belongs to a suprageneric group inside the actinomycetes genera, the *Mycobacteriaceae* family, which is phylogenetically closely related to the *Streptomycetaceae* family [10, 11]. Streptomycetes are ubiquitous having soils for natural habitat. Furthermore *Streptomyces* strains have been well-known for many years as sources of natural antibiotics and *S. lividans*, a GRAS (generally recognized as safe) microorganism, has been successfully used for the heterologous production of several proteins of eukaryotic and bacterial origin including antigens from *M. tuberculosis* [12-15]. Among human diseases caused by actinomycetes there is only one known member of the genus *Streptomyces*: *S. somaliensis*. Taking into account all these features of *S. lividans* it is important to study the immune response toward this bacterium in an animal model prior to be considering it as a tool for developing TB vaccines.

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In this paper we have assessed the immunogenicity of *S. lividans* strain 1326 in comparison with BCG in BALB/c mice, in order to determine if a live vaccine candidate would be capable of inducing appropriate humoral and cellular immune responses.

MATERIALS AND METHODS

Bacterial Strains

The commercial BCG vaccine strain from Denmark (batch: Copenhagen 1331) was used as a positive control of immunization with a bacterial strain.

S. lividans strain 1326 was obtained from the *Streptomyces* culture collection of the Jones Innes Centre, Norwich, England (kindly provided by the Professor D.A. Hopwood). This is a commonly used host strain that has no or weak restriction against heterologous DNA [16] and a low level of endogenous protease activity [17].

Preparation of the Immunogen of *Streptomyces* Cells

S. lividans 1326 was grown in an orbital shaker at 28°C for 48 h in 100 ml modified BTSB medium [18]: 10% sucrose, 1% yeast extract, 0.5% NaCl, 0.3% soy meal, 1.7% tryptone and 0.25% K_2HPO_4, pH 7.2. *Streptomyces* mycelium was harvested by centrifugation (3500 rpm, 4°C), washed with and resuspended in PBS. Aliquots (1 ml) were lyophilized and kept at 4°C until use. Three lyophilized bulbs were resuspended and plated on MRYE medium [19] to determine the total number of CFU per bulb from each lot preparation. Briefly: lyophilized bulbs were resuspended in 1 mL of PBS and 100 µL of dilutions (from 10^5 to 10^2) were plated to count the number of CFU. The following formula was used to determine the number of CFU in each bulb, $\text{No.}_{\text{CFU/bulb}} = \text{No.}_{\text{counted CFU/plate}} \times \text{Dilution factor} \times 10$.

The proteins of the resulting culture supernatant were desalted using PD-10 columns (GE Healthcare, supplied by BDC, Belgium) and PBS as exchange buffer. Then proteins were filtered (0.22 µm, Millipore) and kept at -20 °C until use. The total protein content was determined using the Bradford method [20].

Preparation of Proteins Secreted by *M. tuberculosis* and *M. bovis* Strains

The secreted proteins from *M. tuberculosis* H37Rv and *M. bovis* 1331 were obtained starting from a short-term culture filtrate (ST-CF) as previously described [21]. Briefly, the strains (8 x 10^6 CFU/ml) were grown in a modified media of Sauton without Tween 80 in orbital shaker to 37°C for 4 to 7 days. The culture supernatants were sterile filtered and proteins from them were obtained in the same way as *Streptomyces* secreted proteins.

Animals

Female BALB/c mice (18-20 g) were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). They were housed in Macrolon cages (Panlab, Barcelona, Spain), in a standard bio-clean animal room, and kept under a 12-h light-dark cycle at 22-24°C. The animals had free access to food and tap water, and were allowed to acclimatize for one week before the experiments. All experiments were carried out in accordance with the ethical guidelines for investigations with laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the Center of Biomolecular Chemistry.

Immunization Scheme

Three experimental groups (8 mice each) were immunized in the intraperitoneal region following the schedule shown in Table **1**. Mice were bled by the retro-orbital artery with a sterilized Pasteur micropipette. For the immunization with *Streptomyces* cells: the mycelium was resuspended in PBS to a concentration of 5x10^6 CFU per mL and 0.2 ml of this *Streptomyces* cell suspension was given to each animal (the volume of PBS for resuspending the lyophilized bulbs was calculated by the formula: $V\ (\text{mL}) = \text{No.}_{\text{CFU/bulb}} / 5\times10^6\ \text{CFU/mL}$). The BCG vaccine strain was resuspended in its medium to a concentration of 10^7 CFU per mL and 0.1 mL of this BCG suspension was given to each animal. This immunization experiment was done twice, data from one are presented.

Enzyme-Linked Immunosorbent Assay (ELISA) and Western Blot Assay

Antibody levels against proteins secreted by *Streptomyces*, BCG and *M. tuberculosis,* and antibodies levels against whole *Streptomyces* cells and whole BCG cells were measured by indirect ELISA (described in [18]). The nature of the antigens identified by serum antibodies of immunized animals was identified by Western blot analysis. Briefly: the antigens were separated by electrophoresis of proteins on 12.5% SDS-polyacrylamide gels. Separated proteins were visualized by Coomassie brilliant blue staining or transferred to a Hybond™-C extra membrane (GE Healthcare,

Table 1. Immunogen Groups, Doses, Immunization and Blood Extraction Intervals

Group	Immunogens	Immunogen Doses	Immunization and Extraction Intervals
1	PBS (control)	0.2 mL/mouse (i.p)	t=0: 1st blood extraction and immunization t=21days: 2nd blood extraction and immunization t=42 days: 3rd blood extraction and immunization t=63 days: Bleeding and animals sacrifice. Isolation of spleen lymphocytes
2	*S. lividans* 1326	10^6 CFU/0.2mL/mouse (i.p)	
3	BCG	10^6 CFU/0.1mL/mouse (i.p)	

supplied by BDC, Belgium) by using a semidry transfer cell (Biometra, Göttingen, Germany) according to the manufacturer's recommendations. Membranes were incubated with a pool of mice sera from the analyzed group diluted 1:10. Antimouse IgG horseradish peroxidase conjugated (Promega, Catalys AG, Switzerland) diluted 1:5000 was used as secondary antibody. Immunoreactive bands were visualized by brief exposure to 3,3-diaminobenzidine as substrate. Images were taken by the Bioimage System GENE GENIUS (SynGene, Cambridge, UK).

Antigen-Specific Lymphocyte Proliferative Response

Lymphocytes were obtained from the spleens of killed mice and were purified by Ficoll density gradient centrifugation (Histopaque SIGMA, St Louis, MO, USA). The cellular suspension was adjusted to a working dilution of 2×10^6 cells/mL. Lymphocytes were cultured in U-bottom 96-well microtiter plates (Corning, Life Science, US) in complete RPMI medium at 37°C under 5% CO_2 and challenged with the corresponding antigen at 10 µg/mL (a control without antigen stimulation was included for each group). Spleens from 3 mice in each group were pooled. In order to determine cytokine production leukocyte culture supernatants were collected after 96 h and stored at -20 ºC until use. The lymphocyte proliferation was assessed by the colorimetric assay based on the mitochondrial enzymatic reduction of the reagent MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, SIGMA, St Louis, MO, USA) to purple formazan in living cells [22]. Briefly, 15 µL of MTT (final concentration 0.5 mg/mL) were added to the wells and the cells were incubated during 4 hours. At the end, DMSO was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of the colored solution was measured at 540 nm. The experiment was done twice in duplicate and data are expressed as mean absorbance of the groups and the standard error of the mean.

Determination of IFN-γ and IL-10 Produced by Challenged Leukocytes

Cytokine determination was done using double antibody system ELISA kits (MabTech, Sweden) following manufacturer's recommendations. Positive stimulation was considered when the cytokine level was fivefold higher than in the non-stimulated wells of the corresponding group.

Statistical Analysis

Statistical differences between groups were determined by a Kruskal-Wallis test and the Dunn's multiple comparison post test**.** The analyses were conducted using the software GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California, USA).

RESULTS AND DISCUSSION

Humoral Response Against Secreted Proteins from Tested *S. lividans* Strain 1326 and *Streptomyces* Mycelium. Cross-Reactivity Against BCG whole Cells and *M. tuberculosis* Secreted Antigens

The first aim for evaluating a live candidate is to study its immunogenicity on its own. In addition a life vaccine candidate must also be non-pathogenic [23]. In this work we immunized BALB/c mice with mycelium of *Streptomyces lividans* 1326.

Using ELISA analyses, it was shown that groups of mice immunized with *S. lividans* 1326 strain or with the BCG vaccine produced significant amount of antibodies against the secreted proteins recovered from the culture supernatant of each strain respectively (Fig. **1A**). The levels of antibodies were significant from day 21 after the first immunization for the *Streptomyces* immunized group and from day 42 for the BCG immunized group (i.e. 21 days after the second immunization). Maximum levels of antibodies for all the

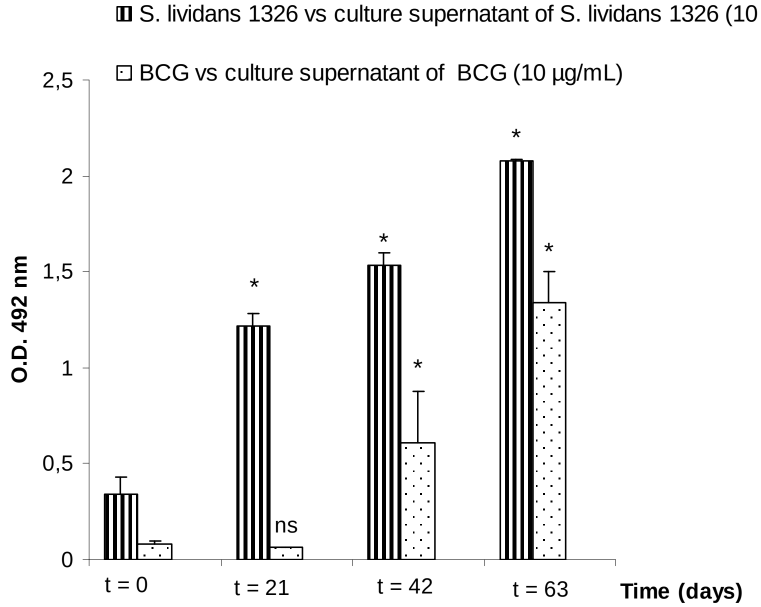

Fig. (1A). ELISA analysis of sera (diluted 1/100) from mice immunized with *S. lividans* 1326 (vertically striped bar) and BCG (dotted bar) reacting with proteins present in the culture supernatant of the respective strain. Data are represented as the mean (bar) plus the standard error of the mean (error bar) with n=8. Asterisks mean significant statistical differences ($p < 0.05$) between each bar and its respective bar at time 0 of the experiment. (ns): indicates a non significant statistical difference.

experimental groups were found at day 63 of the experiment (21 days after the third immunization). The presence of the reacting antibodies against the secreted proteins detected by ELISA in Fig. (**1A**) was confirmed by Western blot analyses (Fig. **1B**). Serum antibodies from *Streptomyces* immunized mice recognized proteins of different molecular weight present in the spent culture medium of *Streptomyces* and the sera from BCG immunized animals also reacted with different proteins secreted by this vaccine strain. No immunoreactivity against *S. lividans* 1326 or BCG was observed using sera from the PBS immunized group.

Fig. (**1**) confirms the value of *S. lividans* as a potential vehicle for delivering secreted proteins in an appropriate manner for inducing a strong humoral response against these antigens. This response is highly relevant in view of the cross reaction between the secreted proteins of *S. lividans* and *M. tuberculosis*. It is known that the high affinity phosphate-binding proteins PstS from *S. lividans* and *S. coelicolor* share 42-43% of sequence identity with PstS-1, PstS-2 and PstS-3 from *M. tuberculosis* [24], and that PstS-1 is one of the major immunodominant antigens of *M. tuberculosis* [25].

The capacity of sera from mice immunized with *Streptomyces* and BCG to react with whole cells of each strain was analyzed by means of ELISA, using *Streptomyces* mycelium or BCG cells for coating microtiter plates (Fig. **2**). It was found that antibodies obtained from *Streptomyces* and BCG immunized animals were able not only to react with the secreted proteins from each strain but also with whole *Streptomyces* and BCG cells. Moreover antibodies from *Streptomyces* immunized mice recognized whole BCG cells equally well as in the reciprocal tests with BCG induced antibodies.

The results shown in Figs. (**1** and **2**) indicate that the *Streptomyces* mycelium is strongly immunogenic in the murine model and is not pathogenic at the used doses. In previous studies, *Streptomyces* bacteria were found to be

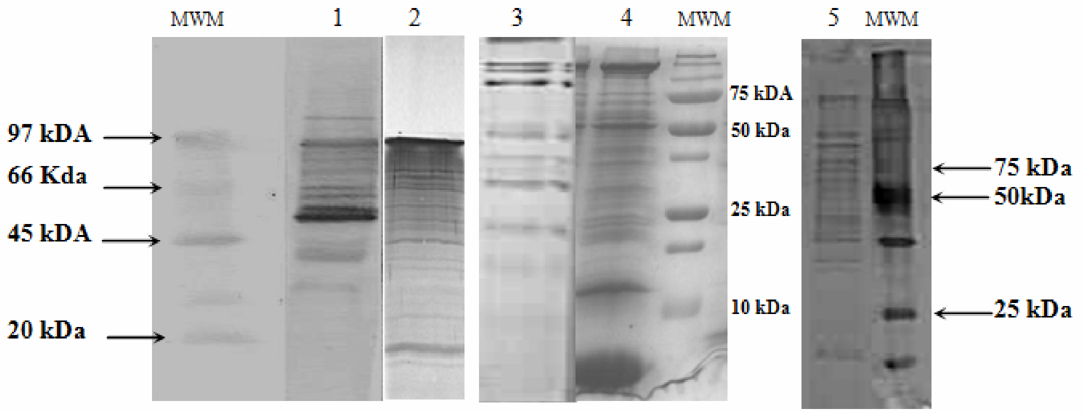

Fig. (1B). Reactivity in Western Blot of sera from mice immunized with BCG (lane-1) and *S. lividans* 1326 (lane-3) with the secreted proteins from the culture supernatant of each strain respectively at day 63. SDS-PAGE stained with Coomassie brilliant blue of the total secreted protein profile of BCG (lane-2), *S. lividans* 1326 (lane-4) and *M. tuberculosis H37Rv* (lane-5). MWM : molecular weight marker.

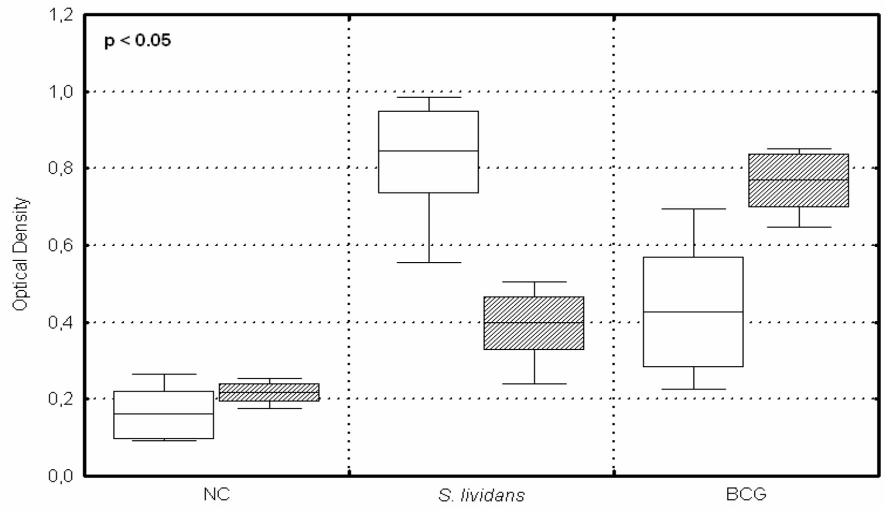

Fig. (2). Analysis of the cross-reactivity between *Streptomyces* and BCG observed in ELISA, using whole cells (coated with 10^3 CFU/well of the strains in each case). Sera diluted 1/100 from PBS (NC: negative control), *S. lividans* 1326 and BCG immunized animals reacting against *S. lividans* mycelium (open bars) and BCG whole cells (striped bars). Data are represented as the mean, 25 and 75 % percentiles, minimum and maximum with n=8. All the bacteria immunized animal responses showed significant differences with the PBS immunized group (p<0.05).

unable to colonize organs like heart, liver, kidney, lung or spleen in intranasal, intramuscular or intraperitoneal immunized BALB/c mice as indicated by microbiological analyses, and histopathological analyses revealed no lesions in these organs [26].

The degree of cross-reactivity observed between the proteins of the culture supernatants was the same irrespective of whether antibodies induced by *Streptomyces* or BCG were used (Fig. **3**). The antibody responses against *M. tuberculosis* secreted proteins were similar in the groups of animals immunized with the *Mycobacterium bovis* vaccine strain and the *Streptomyces* strain, indicating cross-reactivity between *Streptomyces* and the *Mycobacterium* genus. However, the antibody reactivity obtained against the *M. tuberculosis* secreted proteins was not as high as the antibody reactivity obtained against the secreted protein pattern of *Streptomyces* or BCG (Fig. **1A**). Phylogenetic proximity analysis has shown that these two species of bacteria belonging to the Actinomycete order, are closely related [27].

Antigen-Specific Lymphocyte Proliferative Response and Cytokine Production in Splenocytes of Immunized Mice

In order to evaluate the capacity of immunization with *Streptomyces* mycelium to raise a cellular response, the lymphocytes isolated from the spleens of experimental animals were re-exposed to the secreted antigenic proteins. The proliferation of the lymphocyte populations from the bacteria-immunized mice (Fig. **4**) was significant when challenged with secreted proteins of both *Streptomyces* and

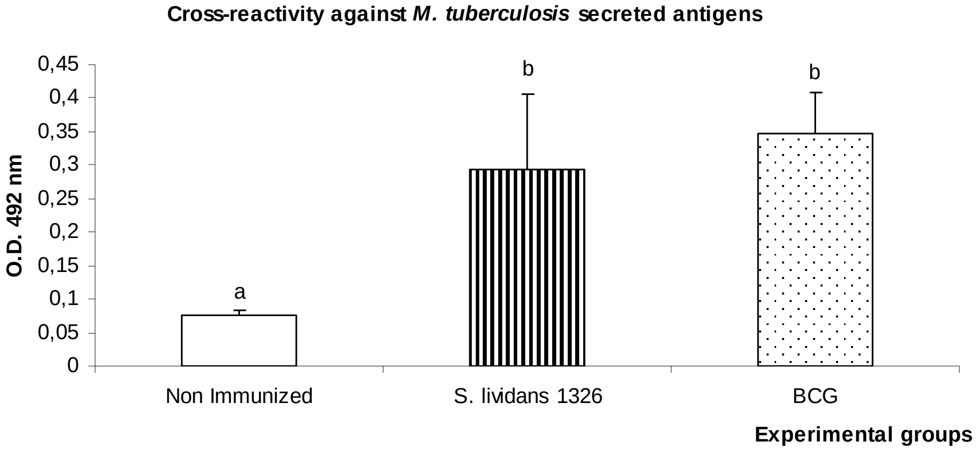

Fig. (3). ELISA analysis of cross-reactivity with *Mycobacterium tuberculosis*. 1/100 diluted sera from mice immunized with PBS, *S. lividans* 1326 and BCG reacting against *M. tuberculosis* H37Rv secreted proteins (10µg/mL) at day 63. Data are represented as the mean (bar) plus the standard error of the mean (error bar) with n=8. Letters represent significant statistical differences (p< 0.05) between groups.

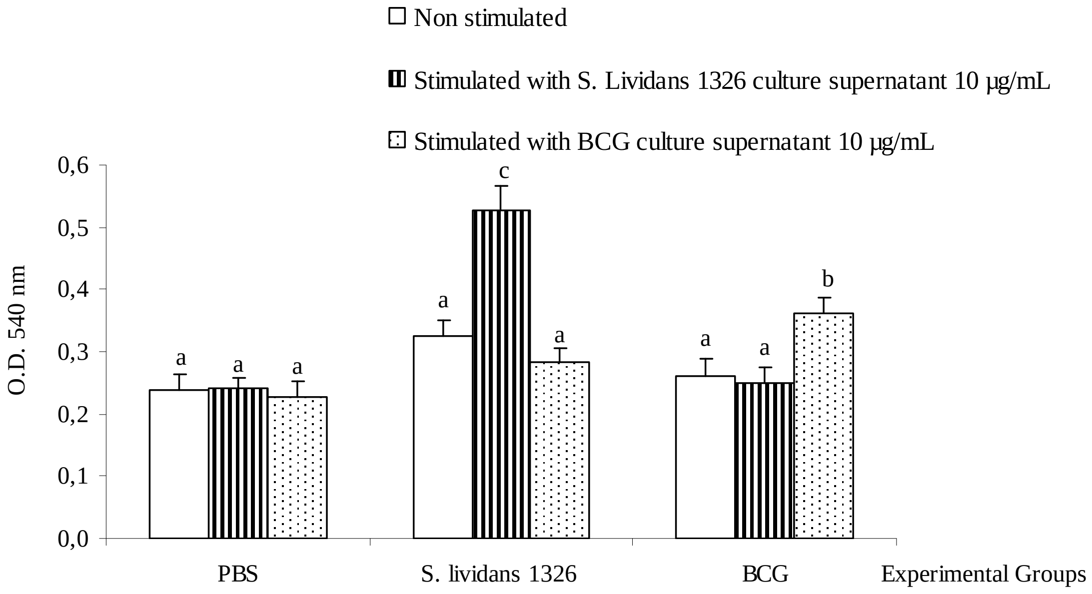

Fig. (4). Lymphoproliferative antigen-specific response in splenocytes isolated from mice immunized with PBS, *Streptomyces lividans* 1326 and BCG. Splenocytes were challenged *in vitro* for 96 hours with *S. lividans* 1326 secreted proteins and BCG secreted proteins (10µg/mL of total proteins respectively) at day 63. Proliferation of splenocytes was determined after 4 hours incubation with the reagent MTT and results were compared to non-stimulated conditions. Data are represented as the mean (bar) and the standard error of the mean (error bar). Different letters indicate significant statistical differences (p< 0.05) between groups.

Table 2. Levels of IFN$_\gamma$ and IL-10 (pg/mL) from Lymphocytes Isolated from Spleen of BALB/c Mice Immunized with PBS, *S. lividans* 1326 and BCG (rows) in Response to Exposition/Challenge *in vitro* to Proteins from Culture Supernatant of *S. lividans* 1326 and BCG. Data are Expressed as the Mean and Standard Deviation in Parenthesis. Values in boldface Correspond to a Positive Antigen-Specific Stimulation Five Fold Higher than in Non-Stimulated Wells

Immunized Group with	Cytokine (pg/mL)	Exposition *in vitro* with Antigen During 96h (10μg/mL Total Proteins)	
		Proteins from Culture Supernatant of *S. Lividans* 1326	Proteins from Culture Supernatant of BCG
PBS	IFNγ	30.1 (15)	85.0 (11)
	IL-10	**40** (35)	**65** (60)
S. lividans 1326	IFNγ	**2103.9** (117)	**225.8** (17)
	IL-10	543.3 (24)	**268.3** (130)
BCG	IFNγ	3.4	**267.2** (2)
	IL-10	0	**310** (71)

BCG strains, with the strongest response observed when lymphocytes from *S. lividans* 1326 immunized mice were challenged with *Streptomyces* secreted proteins.

Beside the lymphocyte proliferation, the levels of IFN$_\gamma$, and IL-10 secreted *in vitro* from the re-stimulated lymphocytes were also measured by ELISA (Table **2**). The immunization with *S. lividans* 1326 in mice was found to elicit a strong cellular immune response leading to levels of 2103.9 pg/ml of the major Th1-type cytokine IFN-γ, which are as high as those observed by other authors [28]. In contrast, immunization with BCG produced a lower Th1 response than with the *Streptomyces* strain. Similarly to the humoral response, lymphocytes from the *Streptomyces* immunized group, when challenged with BCG secreted antigens, secreted similar quantities of IFN-γ and IL-10 as the lymphocytes from BCG immunized animals.

During normal phagocytosis, the contents of the bacillus-containing phagosome are degraded upon fusion with lysosomes, but during infection with *M. tuberculosis* this process is blocked [29, 30]. The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, such as IFN-γ and TNF-α [31]; and in this respect *S. lividans* seems to create a favorable response with cross-reacting proteins promoting a Th1 immune response. Since the tubercle bacilli reside inside a compartment within the macrophage, their antigens are presented by MHC class II molecules to CD4+ T lymphocytes. The main function of CD4+ T cells is the production of cytokines including IFN-γ, which activates macrophages and promotes bacilli destruction. Recently, another function has been ascribed to these cells, i.e., helping to develop the CD8+ T cell mediated response [32, 33]. In the same way, CD4+ T cells may participate in the induction of apoptosis of infected cells and subsequent reduction of bacterial viability through the CD95 Fas ligand system [34]. Therefore, formulations that induce the production of enduring Th1 responses are desirable and are an essential element of a successful TB vaccine.

CONCLUSION

The *S. lividans* strain 1326 possessed adequate immunogenicity in BALB/c mice when used under the conditions described in this paper, confirming the potential of this bacterium as a delivering system for its secreted proteins. The cellular immune response toward *Streptomyces* was in some cases stronger than the response obtained with BCG immunized animals, as shown by the proliferation assays and levels of IFN-γ secreted by leukocytes against homologous antigens. Genetic variation between BCG strains used in different trials may explain the differences in efficacy that have been reported [35].

This first study of the murine immune response toward *Streptomyces* suggests that it may be worthwhile to characterize the immune response and protective efficacy of others *Streptomyces* strains, such as wild-type strains and recombinant strains secreting *Mycobacterium tuberculosis* antigens.

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