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Original article

Expression of MPB83 from *Mycobacterium bovis* in *Brucella abortus* S19 induces specific cellular immune response against the recombinant antigen in BALB/c mice

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Abstract

Immunodominant MPB83 antigen from *Mycobacterium bovis* was expressed as a chimeric protein fused to either β-galacotosidase, outer membrane lipoprotein OMP19 or periplasmic protein BP26 in gram-negative *Brucella abortus* S19, in all cases driven by each gene's own promoter. All fusion proteins were successfully expressed and localized in the expected subcellular fraction. Moreover, OMP19-MPB83 was processed as a lipoprotein when expressed in *B. abortus*. Splenocytes from BALB/c mice immunized with the recombinant S19 strains carrying the genes coding for the heterologous antigens in replicative plasmids, showed equally specific INF-γ production in response to MPB83 stimulation. Association to the lipid moiety of OMP19 presented no advantage in terms of immunogenicity for MPB83. In contrast, fusion to BP26, which was encoded by an integrative plasmid, resulted in a weaker immune response. None of the constructions affected the survival rate or the infection pattern of *Brucella*. We concluded that *B. abortus* S19 is an appropriate candidate for the expression of *M. bovis* antigens both associated to the membrane or cytosolic fraction and may provide the basis for a future combined vaccine for bovine brucellosis and tuberculosis.

Keywords: *Brucella*; Heterologous expression; Mycobacteria

1. Introduction

Bovine tuberculosis (bTB) remains an important animal and zoonotic disease in many countries, causing both significant economic losses and acting as a public health hazard. *Mycobacterium bovis*, the causative agent of bTB, is closely related to *Mycobacterium tuberculosis*, the agent responsible for human tuberculosis [1]. While the main host of *M. bovis* is cattle, other animals, including humans, may also be infected [2]. The presence of bTB undermines the development of the dairy and meat industry and it is an impediment to international trade. At present there is no vaccine available for bovine tuberculosis that does not interfere with diagnosis. Therefore, controlling and eventually eradicating this disease is essential. New vaccine candidates and novel immunization strategies are strongly needed to improve the control of the global tuberculosis epidemic.

Among the many *M. bovis* antigens that have been characterized, MPB83 has generated good T-cell and B-cell responses in mice and in cattle when used as nucleic acid based vaccine [3–5]. Even though in a later study vaccination of calves with MPB83 DNA vaccine did not induce protection against bovine tuberculosis [6], the combined DNA (Ag85B, MPT64, and MPT-83a)/BCG vaccine increased the protective efficacy by more than 10–100-fold in calves challenged with *M. bovis* compared to the combined DNA and BCG groups [7]. MPB83 is a glycosylated lipoprotein processed by signal peptidase II and located at the surface [8], possibly with the lipid tail coupled to the N-terminal cysteine embedded in the mycobacterial outer membrane [9]. In SDS-PAGE followed by

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Western blot of mycobacteria whole cell protein extracts, two bands of 25 and 23 kDa that react with monoclonal antibody MPB83 have been detected. It has been proposed that they correspond to different glycosylated forms of MPB83 [10]. These characteristics make MPB83 an attractive antigen to use as a model for heterlogous expression.

On the other hand, *Brucella abortus* is a facultative intracellular pathogen which causes brucellosis, a major zoonosis, in several countries. Brucellosis causes abortion and infertility in cattle and a clinical manifestation known as undulant fever in humans [11]. The vaccines used for control of bovine brucellosis are attenuated *B. abortus* S19 (smooth strain) and RB51 (rough strain) [12]. Both strains induce a strong Th1 response and generate cell-mediated immunity (CMI), while S19 also induces a strong humoral response against surface antigens, particularly O antigen [13]. S19 vaccine has been widely used for more than 50 years and the immune response it elicits, both in animal models as well as in the natural host, has been extensively studied. Animal models for brucellosis are also well established and characterized.

For these reasons, *B. abortus* is an attractive vector for the expression of heterologous antigens of veterinary importance allowing expression from different promoters [14–18]. Furthermore, we have previously shown that *B. abortus* S19 can efficiently express RAP1 from *Babesia bovis* and is immunostimulatory in the mouse model of infection [19]. Taken together, these features form a strong basis for using *B. abortus* as an antigen carrier to induce a Th1 biased immune response.

In this work, we have generated recombinant *B. abortus* S19 strains expressing MPB83 antigen from *M. bovis* in either the membrane or cytosolic fractions and analyzed their ability to elicit a specific immune response towards the recombinant antigen as well as the infection parameters of the recombinant strains in BALB/c mice.

This study provides a strong basis for expressing protective antigens of *M. bovis* in *B. abortus* S19 and supports further investigation on this strategy for the development of heterologous vaccines for the control of bovine tuberculosis.

2. Materials and methods

2.1. Bacterial culture conditions

B. abortus strain 19 was obtained from our strain collection. *Escherichia coli* DH5α were purchased from Invitrogen. *E. coli* derived strains were grown in either liquid or solid Luria Bertani (LB) medium containing appropriate antibiotics at the following concentrations: ampicillin 100 μg/ml; kanamycin 50 μg/ml. *Brucella* cultures were grown at 37 °C in trypticase soy broth (TSB) or tryptose agar (TA) (DIFCO), supplemented with the appropriate antibiotics (kanamycin 25 μg/ml, ampicillin 50 μg/ml).

2.2. Plasmids

In order to construct vectors for MPB83 expression in *B. abortus*, the *mpb83* gene was amplified by PCR from the genomic DNA of reference strain *M. bovis* AN5, generously provided by Dr. Angel Cataldi. Primers 5′ctgcagcgatgatcaacgttcaggc3′ and 5′ctgcaggaggcaaaccgcctacac3′ were used, with PstI restriction sites (underlined) incorporated into both primers to allow the subcloning of the full length *mpb83* gene into pBBomp19 [19]. In order to clone *mpb83* into PBBR1MCS2 [20] and pKS26 [21], primers: forward 5′gcgcgcatgatcaacgttcagg3′ and reverse 5′gcgcgcgaggcaaaccgcctacact3′ were used. BssHII sites (underlined) were incorporated into both primers. Amplification was performed at 95 °C, 2 min, followed by 35 cycles of 95 °C, 1 min; 58 °C, 1 min and 72 °C, 1.5 min. The amplified gene fragments were cloned in pGEM-T vector (Promega) and then subcloned into PstI of pBBomp19 to generate pomp19-83, in BssHII site of pKS26 for p26-83 or into EcoRI of pBBR1MCS2 to make p83. Recombinant plasmids were transformed into *E. coli* DH5α for cloning and amplification. Correct framing and orientation were analyzed by DNA sequencing.

2.3. Generation of recombinant B. abortus S19 expressing MPB83

Selected purified plasmids were used to electroporate *B. abortus* S19 according to methods previously described [22]. Recombinant colonies were confirmed by colony PCR using *mpb83* specific oligonucleotides. Finally, total proteins from the kanamycin or ampicillin-resistant *B. abortus* S19 colonies (rS19) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot (WB) with a specific monoclonal antibody (Mab) for MPB83. *B. abortus* S19 strains harbouring plasmids pBBR1MCS, pBBomp19, pKS26, p83, p26-83 pomp19-83 were designated Sp, Spomp19, Sp26, Sp83, Sp26-83 and Spomp19-83, respectively.

2.4. Subcellular localization of recombinant MPB83 in B. abortus

Subcellular fractioning of Sp83, Sp26-83 and Spomp19-83 was performed as previously described [23]. The fractions were analyzed by Western blot (WB) using monoclonal antibody anti-rMPB83 (kindly provided by Dr. H. Wiker).

2.5. Determination of lipid association of OMP19-MPB83

Liquid cultures of *B. abortus* and *E. coli* recombinant strains were treated with or without globomycin in order to assess inhibition of processing by signal peptidase II according to previously described methods [19]. Globomycin was kindly provided by M. Inukai (Sankyo Co. Tokyo, Japan). It was dissolved in ethanol and used at 100 μg/ml (final ethanol concentration of 2%) in the exponential phase of growth of the bacterial cultures. As a negative control, a culture was treated

with ethanol 2%. After 12 h (for Brucellae) or 2 h (for *E. coli*) of incubation, bacteria were washed in phosphate-buffered saline (PBS) and suspended in SDS-PAGE sample buffer. These lysates were analyzed by SDS-PAGE and Western Blot with the corresponding anti-MPB83 monoclonal antibodies to evaluate accumulation of lipoprotein precursors.

2.6. Mice assays

Female BALB/c mice of 12 weeks of age were used. They were raised in the animal house of the Instituto de Biotecnología (INTA), under appropriate conditions of temperature, water and feeding. Animals were inoculated intraperitoneally (i.p.) with 1×10^6 colony forming units (CFUs) of the indicated strains or with purified recombinant MPB83 (rMPB83) (25 μg/ml in Freund's adjuvant, Sigma). Negative control mice were treated with saline alone (PBS). Mice were euthanized by CO_2 asphyxiation and serum and spleens were collected. For determining bacterial spleen colonization and plasmid stability, spleens were weighed, homogenized in 5 ml of PBS and used for colony forming unit (CFU) determination by serial dilution followed by plating on TA with or without the corresponding antibiotics. Colonies were examined after 3 days at 37 °C. Animal experiments were approved by the institute's bioethics committee.

2.7. IFN-γ production determination

For cellular immune response assessment, splenocytes were obtained by a previously described method [23]. Cells were incubated at 37 °C in 5% CO_2 in the presence of different antigens indicated in the text (1 or 5 μg of recombinant MPB83, 5 μg of *B. abortus* S19 crude heat inactivated extract, 0.5 μg of concanavalin A, or culture media RPMI 1640 alone). After 72 h, the supernatant was collected and IFN-γ production was determined by ELISA using 0.1 μg of capture-purified anti-mouse IFN-γ (clone R4-6A2) (BD Pharmingen, San Diego, CA) and 0.05 μg of detection-biotinylated anti-mouse IFN-γ (BD Pharmingen) per well according to the manufacturer's instructions. The assays were performed in triplicate.

2.8. Statistical analysis

Spleen weight and bacterial CFU in the spleen were analyzed, due to the dispersion observed, by the non-parametric test of Kruskale Wallis after which a multiple comparisons test was undertaken. The data for IFN-γ production were subjected to analysis of variance, and the means were compared using Tukey's test.

3. Results

3.1. Expression and cellular localization of MPB83 in B. abortus S19

MPB83 antigen from *M. bovis* was successfully expressed in *B. abortus* S19 as a full length fusion to the first 36 aminoacids of β-galactosidase (Fig. 1a), to the first 38 aminoacids of *Brucella* lipoprotein OMP19 and to the first 39 aminoacids of *Brucella* periplasmic protein BP26 (Fig. 1b) using vectors pBBRMCS2 [20], pBBomp19 [19] and pKS26 [21], respectively. Western blot analysis using monoclonal anti-rMPB83 antibody revealed reactive bands with apparent molecular masses of 24 kDa for OMP19-MPB83 and BP26-MPB83 and of 27 kDa for β-galactosidase-MPB83 in whole extracts of recombinant S19 strains, as expected. As a control, supernatant of *M. bovis* AN5 was used (reviewed in [24]) (Fig. 1). Plasmids pBBRMCS2 and its derivative pBBomp19 are both replicative vectors while pKS26 is an integrative vector for *Brucella*. Based on our previous experience with BP26 which showed it may be toxic when over-expressed in high copy number plasmids [21,22], we decided to use an integrative vector for expression of MPB83 as a fusion to BP26.

Subcellular fractioning of the recombinant strains generated (rS19) revealed that MPB83 was localized in association to the membrane fraction when expressed as a fusion protein to OMP19 (Fig. 2b), while it was localized in the cytoplasm fraction when expressed as a fusion protein to β-galactosidase (Fig. 2a). Recombinant fusion protein BP26-MPB83 was detected in the soluble fraction supporting the idea that MPB83 as a fusion to BP26 is not anchored to membranes. The purity of the membrane fractions was confirmed by the detection of OMP19 using anti-OMP19 antiserum and the absence of any bands using anti-L7/L12 antiserum (data not shown).

3.2. OMP19-MPB83 is partially processed in presence of globomycin

Globomycin is a potent and specific inhibitor of lipoprotein signal peptidases. When cells are grown in its presence, there is an accumulation of pro-lipopeptides [25,26]. To evaluate whether OMP19-MPB83 is successfully expressed as a lipoprotein, both *E. coli* (data not shown) and *B. abortus* S19 expressing OMP19-MPB83 (Epomp19-83 and Spomp19-83, respectively) were grown with globomycin, and cell extracts were analyzed by WB (Fig. 3). The appearance of a band corresponding to the unprocessed precursor was detected in both cases, indicating the accumulation of a precursor when treated with globomycin. In untreated cells, only the mature chimeric protein was detected. Inhibition of the maturation by globomycin confirms the lipoprotein nature of this chimeric protein. As controls, Sp26-83 and Sp83 were also treated with globomycin, but no differences were detected when treated with the solvent (ethanol 2%) alone (data not shown).

3.3. Expression of MPB83 does not affect infection characteristics of B. abortus S19 in the mouse model of infection

In order to evaluate whether expression of MPB83 affects *B. abortus* pattern of infection as well as the stability of the replicative vectors carrying *mpb83* gene constructs, groups of five mice were infected with each strain and, at different time points, spleens were collected, disrupted and serial dilutions

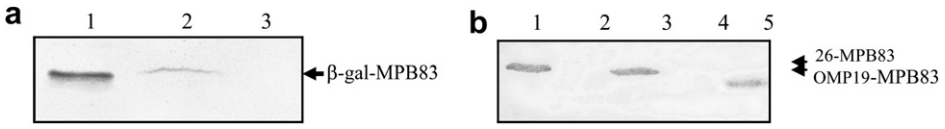

Fig. 1. Expression of MPB83 fusion proteins in *B. abortus* S19. Detection of chimeric proteins in *B. abortus* S19 whole cell extracts by Western blot using anti-MPB83 monoclonal antibody (dilution1:200). Panel (a) β-gal-MPB83. Lanes: 1: supernatant of strain *M. bovis* AN5; 2: Sp83; 3: Sp (b) OMP 19-MPB83 and 26-MPB83. Lanes: 1: Sp26-83; 2: Sp26; 3: Somp19-83; 4: Somp19; 5: supernatant of strain *M. bovis* AN5.

were plated for bacterial counts. Vector stability was assessed by comparing the number of colony forming units (CFUs) from each strain recovered from mice spleen on solid media, supplemented or not, with the selective antibiotic at 3 and 4 weeks post-infection (Fig. 4a). Although there is a slightly lower count of CFU in selective antibiotics for strains bearing replicative plasmids (Sp, Sp83 and Spomp19-83), these differences were not statistically significant either at 3 or 4 weeks post-infection, indicating a high stability of recombinant plasmids in vivo. No difference in CFU was observed for Sp26-83 with or without selective antibiotic at any time point which was expected as pKS26 is an integrative vector for Brucellae. Furthermore, when these strains were grown in liquid culture with and without antibiotics, no differences in growth parameters were observed (data not shown).

Regarding infection pattern, S19 recombinant strains presented the same levels of spleen colonization as S19 carrying empty pBB (Sp) which also correlates with expected bacterial counts for this kind of experimental infection [21,22]. Accordingly, splenomegaly was observed in all mice groups respect of control uninfected group, and correlates with infection parameters expected for S19. No differences in the spleen weight among infected mice groups were observed at any time point (Fig. 4b). These results suggest that the expression of MPB83 in S19 does not alter the infection characteristics of S19 in the mouse model of infection. Expression of MPB83 following passage in vivo was confirmed by PAGE followed by WB of whole protein extracts of randomly picked rS19 colonies recovered from the spleen of immunized mice at all time points (data not shown).

3.4. B. abortus *S19 strains expressing MPB83 induce a specific cellular immune response to MPB83 in BALB/c mice*

The next question we addressed was whether the heterologous proteins expressed by the recombinant *B. abortus* strains were able to generate an immune response in the course of an experimental infection in the mouse model. Groups of five mice were intraperitoneally infected with Sp, Sp83, Sp26-83 and Spomp19-83. As control, another group of mice was injected with saline solution (PBS). In order to study specific cellular immune response stimulation, splenocytes were collected from animals at 3 weeks post-inoculation and were stimulated for 72 h with purified recombinant MPB83 (rMPB83) pretreated with polymyxin B to reduce potential LPS contamination and INF-γ production was measured (Fig. 5). Splenocytes from mice immunized with Sp83 and Spomp19-83 produced significantly equally higher levels of IFN-γ than splenocytes from mice infected with Sp, when stimulated with rMPB83 at a concentration of 5 μg/ml. Although splenocytes from mice immunized with Sp26-83 also produced significantly higher levels of IFN-γ compared to splenocytes from mice infected with Sp, this was significantly lower than the response of the other groups. Coincidently, at lower concentrations of stimulating antigen rMPB83 (1 μg/ml) only splenocytes from Sp83 and Spomp19-83 immunized groups presented statistically significant higher levels of IFN-γ ($p < 0.001$) than the control group, Sp. Similar results were obtained in another independent experiment (data not shown). The level of IFN-γ produced by ConA or heat-killed S19 stimulated splenocytes was high in all cases. No rMPB83 specific cellular immune responses were detected in the animals inoculated with PBS or rMPB83 (data not shown).

These data confirm the induction of a specific cellular immune response against MPB83, using *B. abortus* S19 as a live carrier.

4. Discussion

The most convenient and practical means to control bovine tuberculosis is vaccination. *M. bovis* BCG is the most well-known vaccine, though it is not currently used as a bTB vaccine. Moreover, BCG vaccination failed to induce protection in the majority of field experiments against natural infection (reviewed in [27]). One of the biggest obstacles to the introduction of BCG vaccination as a mechanism for bTB control in

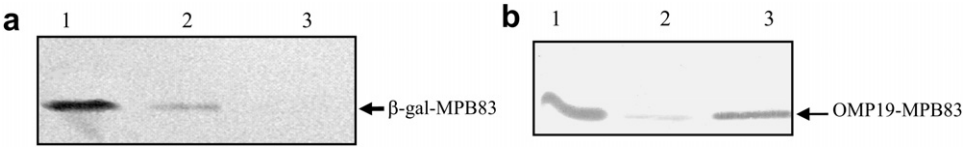

Fig. 2. Subcellular localization of MPB83 fusion proteins expressed in *B. abortus* S19. Detection of chimeric proteins in *B. abortus* S19 subcellular fractions by Western blot using anti-MPB83 monoclonal antibody (dilution 1:200). Panel (a) Sp83. 1: whole cell extract, 2: cytoplasmic fraction, S150000, 3: membrane fraction, ultracentrifugation pellet P150000. Panel (b) Spomp19-83. 1: whole cell extract, 2: cytoplasmic fraction, S150000, 3: membrane fraction, ultracentrifugation pellet P150000.

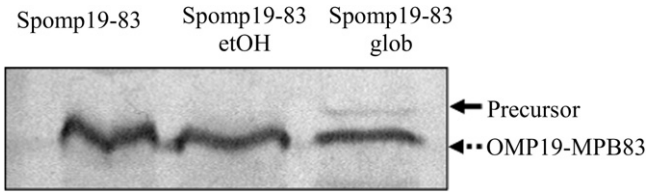

Fig. 3. Lipidation of OMP19-MPB83 fusion protein. Western blot of *B. abortus* S19 extracts using anti-MPB83 monoclonal antibody. *B. abortus* pomp19-83 cells were treated with 100 ng/ml globomycin B in ethanol 2% for 12 h. They were also treated with ethanol 2% as a negative control. The solid arrow indicates the unprocessed precursor; the dashed arrow shows OMP19-MPB83. References: Spomp19-83: Spomp19-83 non-treated; Spomp19-83 etOH, treated with ethanol, Spomp19-83 glob, treated with globomycin diluted in ethanol.

cattle has been that BCG vaccination sensitises animals to the tuberculin skin test, such that vaccinated animals respond positively to the skin test. The potential use of live recombinant bacteria expressing selected mycobacterial antigens as a platform for vaccine development is therefore highly attractive. In this regard, *B. abortus* has proved to be a useful vector for the expression of heterologous antigens [14–19].

In order to test this hypothesis, we have expressed MPB83 antigen of *M. bovis* in *B. abortus* S19. S19 has been used as a vaccine for bovine brucellosis for more than 50 years now in many countries of Latin America, and the immune response it elicits is very well characterized both in mice and in its natural host, the bovine. This makes it a strong candidate for model studies of heterologous expression. Surveillance and diagnosis of brucellosis in countries where S19 is the vaccine of choice is done by classical serology tests that include agglutination and Bengal Rose [11,28]. This is accompanied with a proper vaccine protocol that involves early vaccination of heifers. On the other hand, RB51 is a rough strain also in use in many countries. We have chosen to work with S19 because LPS is a strong humoral antigen and may serve as a natural adjuvant.

MPB83 is a glycosylated lipoprotein processed by signal peptidase II and located at the surface in mycobacteria [8]. We have cloned *mpb83* full length gene in three different vectors with the objective of generating recombinant *Brucella* strains that would express the chimeric protein in different subcellular localizations. MPB83 antigen was successfully expressed as a fusion to β-galactosidase, localized in the cytoplasmic fraction, and as a fusion to the first aminoacids of *B. abortus* OMP19 lipoprotein, associated to the membrane fraction. As to the chimeric BP26-MPB83 protein, the band detected by Western Blot essays coincides with the predicted molecular weight of the processed polypeptide and was detected in the soluble fraction. This suggests that the first 28 aminoacids of the BP26 aminoterminal sequence were cleaved as expected and the resulting polypeptide is not associated to membranes. Nevertheless, purification of periplasmic *Brucella* fraction could not be achieved and periplasmic localization of recombinant protein was not confirmed.

It is well known that lipoproteins are key antigens in immunity to many bacterial diseases [29–32]. It also has been found that non-lipoprotein antigens from the parasite *Leishmania major* in fusion to bacterial lipoproteins can enhance immunity [33]. Lipoproteins are synthesised with an N-terminal hydrophobic signal peptide that is cleaved from the mature polypeptide by lipoprotein signal peptidase (LSP) prior to covalent linkage of a fatty acid [33]. The presence of the lipid moiety would allow the protein to take up the correct membrane topology [34] and would confer immunostimulatory properties [35,36]. Molecular analysis of phylogenetically diverse bacterial pathogens has demonstrated divergence of the LSP recognition site. Mycobacterial recognition sequences appear to have undergone genetic drift [37]. Heterologous expression of lipoproteins in *E. coli* with divergent LSP recognition sequences often results in expression of recombinant protein with lipid modification being incomplete or entirely absent [38–40]. Specifically, MPB83 is not lipidated

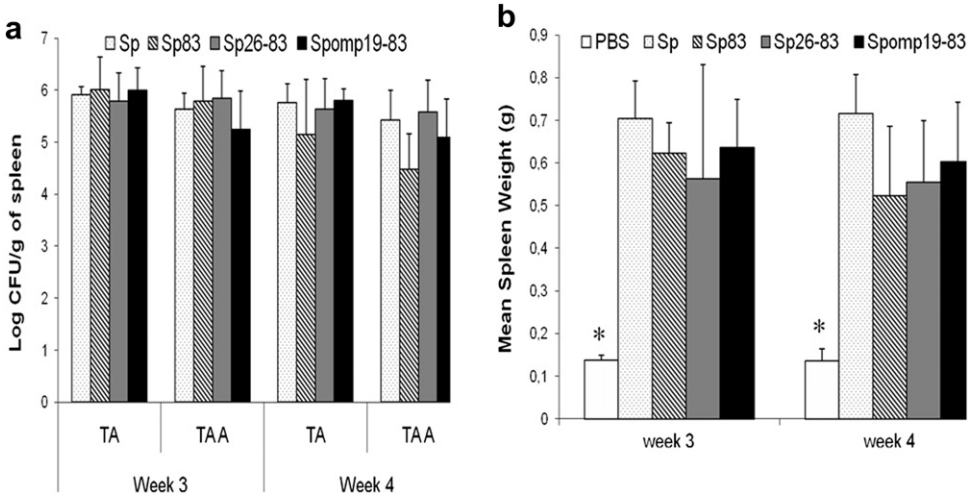

Fig. 4. Plasmid stability and spleen colonization of recombinant strains in immunized mice. BALB/c mice were inoculated intraperitoneally with each strain indicated. Spleens were disrupted and serial dilutions were plated with or without the corresponding antibiotic in order to determine plasmid stability. The data presented are means and standard deviations of the number of brucellae detected in the spleens (a) and spleen weight (b) of five mice infected with each strain at 3 and 4 weeks after immunization in a single experiment. *, significantly different from the other groups ($p < 0.05$). TA: tryptose agar, TAA: tryptose agar supplemented with antibiotics: kanamycin or ampicillin, according to the plasmid used.

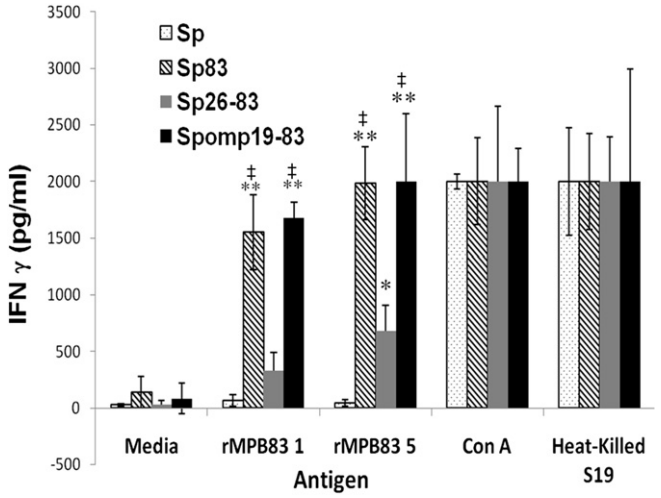

Fig. 5. Mice splenocytes IFN-γ production at 3 weeks post-infection. Quantitative ELISA analysis of IFN-γ secreted by lymphocytes upon stimulation with different antigens for 72 h. Spleen cells (4×10^6/ml) from mice inoculated with Sp, Sp83, Sp26-83, Spomp19-83 or PBS (negative control) were stimulated with 1 μg or 5 μg of MPB83 (rMPB83.1 and rMPB83.5, respectively); concanavalin A (Con A); inactivated whole cell extract of *B. abortus* S19 (heat-killed S19) or RPMI 1640 culture media (media) as negative control. Each bar represents the geometric mean ± standard deviation (error bars) of the responses in spleen cells from five individual mice (each by triplicate). **, statistically significant differences compared to Sp group ($p < 0.001$). *, statistically significant difference compared to Sp group ($p < 0.05$), ‡ statistically significant differences compared to Sp26-83 ($p < 0.05$).

or processed when expressed in *E. coli* [41], which correlates with our results that is not lipidated in *B. abortus* either. This inconvenience may be overcome including an LSP recognition sequence that is efficiently processed by Gram-negative bacteria when expressing mycobacterial antigens in Gram-negative bacteria [41–43]. In this work, we have expressed MPB83 as a fusion to OMP19 lipoprotein [44], therefore conferring a valid lipidation signal that would be recognized in *Brucella*. When Spomp19-83 were treated with globomycin, a potent and specific inhibitor of lipoprotein signal peptidases that induces accumulation of the lipoprotein precursors [19,26], a band corresponding to the unprocessed precursor was detected by Western blot, suggesting the lipidic nature of the chimeric protein.

We tested the immunizing capacity of the recombinant *B. abortus* (rS19) strains and the stability of the corresponding plasmids in BALB/c mice. Stability of recombinant bacteria within the vaccinated host is a prerequisite for any potential live recombinant vaccine. There were no statistically significant differences between the CFUs of any rS19 recovered from the spleen of mice on solid medium either with or without the selective antibiotic, indicating that even under no selective pressure, recombinant bacteria did not lose the plasmids they were carrying. Importantly, rS19 colonies recovered from the intracellular environments maintained their ability to express MPB83 in all cases. In previous studies, when RapI form *B. bovis* was expressed as a fusion to OMP19, the recombinant plasmid was more stable than the other constructions under study [19]. In this work, such difference is not observed as all constructions show similar levels of stability, probably due to the small size of the chimeric protein expressed which may result in lower metabolic burden. Therefore, the stability of plasmids coding for recombinant antigens may be highly influenced by the nature of the antigen that is being expressed.

In order to evaluate the specific immunogenicity of the heterologous protein expressed in *Brucella*, groups of BALB/c mice were immunized with Sp83, Sp26-83 or Spomp19-83. Splenocytes from mice of the different groups were obtained and evaluated for their capacity to secrete IFN-γ in response to MPB83 stimulation. When stimulated with 5 μg of rMPB83, splenocytes form groups of mice immunized with the recombinant strains expressing the three different fusion proteins secreted higher levels of IFN-γ than the control group. Nevertheless, significantly lower stimulation was achieved with splenocytes from Sp26-83 immunized mice. This may be explained by the fact that the heterologous *bp26-mpb83* gene construction was carried by an integrative plasmid, resulting in only one copy in the chromosome. Whether the gene copy number resulted in lower protein expression levels remains to be determined. Coincidently, with lower concentration of the stimulating rMPB83 antigen, only splenocytes from mice immunized with Sp83 and Spomp19-83 were able to stimulate statistically significant secretion of IFN-γ. It is well known that one major drawback of integrative vectors is the reduced level of heterologous expression [45], which counter balances their higher stability. For MPB83 expression in the vectors tested, the improvement in stability of the integrative vector was not significant whilst there was an important loss of immunizing capacity, suggesting that replicative vectors would be more suitable for further work with this antigen.

When pathogen-molecular patterns bind to mammalian Toll-like receptors (TLRs), they stimulate an innate immune response so that an adaptive immune response can arise. *B. abortus* LPS and mycobacterial lipoproteins are well studied examples of immune cells activation through interaction with TLR4 and TLR2, respectively [46–48]. In particular, the role of MPB83 in the stimulation of IL-12 and TNFα production through binding to TLR2 has been demonstrated in dendritic cell from cattle [49]. In this regard, the expression of MPB83 as lipoprotein in a Gram-negative bacterial species, as *B. abortus*, was highly promising towards designing a novel vaccine. However, no significant differences were observed in IFN-γ production from splenocytes stimulated with rMPB83 from either Sp83 or Spomp19-83 immunized groups suggesting that association to the lipid moiety did not result in a significant advantage in terms of immunogenicity for this antigen, at least under the conditions assayed.

Although MPB83 is a valid model antigen candidate, vaccination of cattle with *Brucella* expressing MPB83 may compromise the specificity of the tuberculin skin test since PPD contains this antigen. When assayed in cattle, this issue should be addressed. MPB83 is considered an antigen that might be useful for differentiation of vaccinated and infected animals – DIVA diagnosis – in BCG vaccinated animals [50]. Therefore, the use of a candidate vaccine expressing MPB83 to control the bovine tuberculosis in cattle would most probably need to be accompanied with a diagnosis based on a PPD

devoid of MPB83 and any other antigen present in the vaccine formulations.

In summary, expression of mycobacterial antigen MPB83 in *B. abortus* S19 was achieved and cellular immune responsiveness specific to the recombinant antigen was demonstrated. These results indicate that *B. abortus* is an adequate vector to express and present antigens of *M. bovis*. Development of a *B. abortus* S19-based multivalent vaccine that could confer protection against brucellosis and tuberculosis would be highly beneficial for controlling these two important diseases in livestock and certain wild animals. Studies presented in this paper indicate that the development of such multivalent vaccine is certainly feasible.

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