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Development of a new DNA vaccine based on mycobacterial ESAT-6 antigen delivered by recombinant invasive *Lactococcus lactis* FnBPA+

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Abstract The use of the food-grade bacterium *Lactococcus lactis* as a vehicle for the oral delivery of DNA vaccine plasmids constitutes a promising strategy for vaccination. The delivery of DNA plasmids into eukaryotic cells is of critical importance for subsequent DNA expression and effectiveness of the vaccine. In this context, the use of the recombinant invasive *L. lactis* FnBPA+ (fibronectin-binding protein A) strain for the oral delivery of the eukaryotic expression vector vaccination using lactic acid bacteria (pValac), coding for the 6-kDa early secreted antigenic target (ESAT-6) gene of *Mycobacterium tuberculosis*, could represent a new DNA vaccine strategy against tuberculosis. To this end, the ESAT-6 sequence was cloned into the pValac vector; the *L. lactis* fibronectin-binding protein A (FnBPA)+ (pValac:*ESAT*-6) strain was obtained, and its immunological profile was checked in BALB/c mice. This strain was able to significantly increase interferon gamma (IFN-γ) production in spleen cells, showing a systemic T helper 1 (Th1) cell response. The mice also showed a significant increase in specific secretory immunoglobulin A (sIgA) production in colon tissue and fecal extracts. Thus, this is the first time that *L. lactis* has been used to deliver a plasmid DNA harboring a gene that encodes an antigen against tuberculosis through mucous membranes.

Keywords *Lactococcus lactis* · DNA delivery system · Intestinal mucosa · Tuberculosis · ESAT-6

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Introduction

Mucosal administration of bacterial carriers to deliver plasmid DNA constitutes a promising vaccination strategy. However, most of the bacteria used to deliver DNA vaccines into mammalian cells are pathogens, such as *Listeria monocytogenes*, *Salmonella typhi*, and *Shigella flexneri* (Schoen et al. 2004). Thus, since there is a risk associated with possible reversion to their virulent phenotype (Dunham 2002), food and commensal lactic acid bacteria (LAB) have been used as alternative mucosal DNA delivery vehicles (Wells and Mercenier 2008).

Lactococcus lactis (*L. lactis*), the model LAB, has generally recognized as safe (GRAS) status due to its lack of pathogenicity and has been extensively used for the production and delivery of antigens and cytokines and more recently as a vehicle for the oral delivery of DNA vaccines (Wells and

Mercenier 2008; Bermúdez-Humarán et al. 2011; Pereira et al. 2014).

Internalization of the bacterial carrier is a fundamental step to achieve efficient DNA delivery in eukaryotic cells (Pereira et al. 2014). To increase the delivery, an invasive *L. lactis* strain (*L. lactis* FnBPA+) that expresses the fibronectin-binding protein A (FnBPA) of *Staphylococcus aureus*, a bacterial invasin that is involved in intracellular spreading in the host, was used (Que et al. 2001; Innocentin et al. 2009). Furthermore, a new plasmid called pValac, which contains the cytomegalovirus promoter (pCMV) and the polyadenylation sequence (polyA) from bovine growth hormone (BGH), was also constructed for eukaryotic DNA vaccination using LAB. Its applicability was confirmed when green fluorescent protein (GFP) was expressed in PK15 and Caco-2 cells transfected with the pValac:*gfp* plasmid (Guimarães et al. 2009).

The *L. lactis* FnBPA+ strain was also tested for its internalization ability and potential as a DNA vaccine delivery vehicle, and it was shown to be invasive and capable of transferring the pValac:*gfp* plasmid to Caco-2 cells with an increased GFP expression when compared to native lactococci (Innocentin et al. 2009). Furthermore, in mice, oral administration of the invasive *L. lactis* FnBPA+ (pValac:*gfp*) strain showed GFP expression in epithelial cells of the small and large intestines by fluorescent microscopy, confirming the capacity of this invasive recombinant LAB as a DNA delivery vector in vivo (Pontes et al. 2012; del Carmen et al. 2013).

In this regard, it is believed that the use of this invasive *L. lactis* strain, harboring the pValac vector, for the eukaryotic expression of specific antigens could represent a new strategy for controlling infectious diseases, such as tuberculosis (TB). TB, which is caused by *Mycobacterium tuberculosis*, is the leading cause of death of young adults due to a single infectious agent worldwide. It results in more than eight million new TB cases and approximately two million deaths annually. *Mycobacterium bovis* Bacille Calmette Guerin (*M. bovis* BCG), the only available TB vaccine for the last 80 years, is ineffective against pulmonary TB in adults (Liu et al. 2009). Hence, the development of effective vaccination strategies against TB still represents one of the prime objectives of TB research.

The 6-kDa early secreted antigenic target (ESAT-6) protein, an immunodominant secretory protein of *M. tuberculosis*, is an attractive target for a new TB vaccine. This antigen is present in all pathogenic members of the *M. tuberculosis* complex and absent in the *M. bovis* BCG vaccine (Pym et al. 2002). Several studies have been conducted using ESAT-6 for the development of new vaccines against TB. It was demonstrated that ESAT-6 can induce immunological protection as a DNA vaccine, inducing an immune response alone (Xu et al. 2008) or in combination or fusion with other antigens of *M. tuberculosis* (Chang-hong et al. 2008; Yuan et al. 2012; Yu et al. 2012). ESAT-6 has been used as a DNA boost for the *M. bovis* BCG prime vaccine (Wang et al. 2009; Lu et al. 2011; Cervantes-Villagrana et al. 2013) and in the development of recombinant forms of *M. bovis* BCG (Xu et al. 2010).

In this context, this study aimed to construct the pValac:*ESAT*-6 plasmid, verify its functionality in vitro, construct the invasive *L. lactis* FnBPA+ (pValac:*ESAT*-6) strain to orally immunize BALB/c mice, and evaluate the resulting immunological cellular and humoral response against the ESAT-6 antigen.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *L. lactis* subsp. *cremoris* strains were grown in M17 (Difco, Sparks, MD, USA) medium supplemented with 0.5 % glucose (GM17) at 30 °C without agitation. *Escherichia coli* (*E. coli*) strains were aerobically grown in Luria-Bertani (LB, Accumedia) medium at 37 °C with vigorous shaking. Bacteria were selected by the addition of antibiotics as necessary: for *L. lactis* FnBPA+ (pValac:*ESAT*-6), erythromycin (Ery; Sigma Aldrich) at 5 μg/mL and chloramphenicol (Cm; Sigma Aldrich) at 10 μg/mL; for *E. coli* (pValac:*ESAT*-6), Cm at 10 μg/mL.

DNA manipulations

General DNA manipulation techniques were carried out according to standard procedures. Unless otherwise indicated, DNA restriction and modification enzymes were used as recommended by the suppliers. Plasmid DNAs from *E. coli* and *L. lactis* were isolated as previously described (Green and Sambrook 2012) with the following modifications: for plasmid DNA extraction from *L. lactis*, the first step included lysozyme (10 mg/mL, Serva) for 60 min at 37 °C to prepare protoplasts. Electroporation of *L. lactis* was performed as previously described (Langella et al. 1993). *L. lactis* transformants were plated onto GM17 agar plates containing the required antibiotic and were counted after 24 hours of incubation at 30 °C.

pValac:*ESAT*-6 and *L. lactis* FnBPA+ (pValac:*ESAT*-6) construction

The ESAT-6 open reading frame (ORF) was amplified from the genomic DNA of the *M. tuberculosis* H37Rv strain (ATCC 27294) using the Pfx Platinum® DNA Polymerase (Invitrogen) and the specific oligonucleotides for ESAT-6: 5′-GGATCCACCATGGAGCAGCAGTGGAATTTCGCG-3′ (ESAT-6 Fwd) and 5′-GAATTCCTATGCGAACATCCCA

Table 1 Bacterial strains and plasmids used in this work

Bacterial strain	Characteristics	Source
Escherichia coli TG1	*E. coli* K-12-derived strain; F′ [*traD36 proAB*$^{+}$ *lacI*^{q} *lacZ*Δ*M15*] *supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5*, ($r_K^- m_K^-$)	Lucigen
Escherichia coli TG1 pValac:*gfp*	*E. coli* TG1 strain carrying the pValac:*gfp* plasmid	Guimarães et al. 2009
Escherichia coli TOP10	*E. coli* K-12-derived strain; F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^{R}) endA1 λ^{-}	Invitrogen
Escherichia coli TOP10 pBAD:*ESAT*-6	*E. coli* TOP10 strain carrying the pBAD:*ESAT*-6 plasmid	This work
Lactococcus lactis MG1363	*L. lactis* MG1363 wild-type strain	Gasson 1983
Lactococcus lactis MG1363 (pValac:*ESAT*-6)	*L. lactis* MG1363 strain carrying the pValac:*ESAT*-6 plasmid	This work
Lactococcus lactis MG1363 FnBPA+	*L. lactis* MG1363 strain expressing FnBPA of *S. aureus*	Que et al. 2001
Lactococcus lactis MG1363 FnBPA+ (pValac:*ESAT*-6)	*L. lactis*MG1363 strain expressing FnBPA of *S. aureus* and carrying the pValac:*ESAT*-6 plasmid	This work
Plasmid	Characteristics	Source
pBAD202/D-TOPO®	Prokaryote expression vector (Km^{r}; pBAD; araC gene; pUC ori)	Invitrogen
pBAD:*ESAT*-6	pBAD containing ESAT-6 ORF	This work
pValac	Eukaryotic expression vector (pCMV/Cm^{r}/RepA/RepC)	Guimarães et al. 2009
pValac:*gfp*	pValac vector containing *gfp* ORF	Guimarães et al. 2009
pValac:*ESAT*-6	pValac vector containing *ESAT*-6 ORF	This work

pBAD araBAD promoter, *Km^{r}* kanamycin resistance gene, *pUC ori* origin of replication pUC, *araC gene* encodes a regulatory protein for pBAD, *ESAT*-6 6-kDa early secreted antigenic target, *pValac* vaccination using lactic acid bacteria, *pCMV* cytomegalovirus promoter, *RepA* and *RepC* replication origins, *Cm^{r}* chloramphenicol resistance gene, *gfp* green fluorescent protein coding sequence

GTGACG-3′ (ESAT-6 Rev). The amplified product was purified (Kit illustra™ GFX™ PCR DNA and Gel Band Purification—GE Healthcare), cloned into the Zero Blunt® TOPO® vector (Invitrogen), and transformed into *E. coli* TOP10. Next, the 300-bp ESAT-6 ORF and the pValac vector were both digested with *Bam*HI and *Eco*RI restriction enzymes (Invitrogen), gel purified, ligated by using the T4 DNA ligase (Promega), and transformed into *E. coli* TG1 to obtain the *E. coli* TG1 (pValac:*ESAT*-6) strain. The integrity of the insert was confirmed by sequencing by the use of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and the ABI3130 sequencing equipment (Applied Biosystems).

Then, the pValac:*ESAT*-6 plasmid was transformed into the *L. lactis* FnBPA+ strain. To confirm the *L. lactis* FnBPA+ (pValac:*ESAT*-6) construction, specific primers for the pValac vector (ValF-5′GCTTATCGAAATTAATACGACTCACTATAGGG-3′ and ValR 5′-GGCTGATCAGCGGGTTTAAACG-3′) and for part of the fibronectin ORF (FnAF-5′-TCAGCTATTGATATCGATTA-3′ and FnAR-5′ CAACACTATTGTGTCCACCG 3′) were used.

Transfection assays of mammalian CHO cells with pValac:*ESAT*-6

The pValac:*ESAT*-6 plasmid was assayed for ESAT-6 expression by transfection into the Chinese hamster ovary cell line [Flp-In™-CHO (Invitrogen)]. CHO cells were cultured in complete Nutrient Mixture F12 Ham media (Sigma Aldrich) supplemented with 10 % fetal calf serum (Gibco), 1 % L-glutamine (Sigma Aldrich), 100 ng/mL zeocin (Invitrogen), and 2.5 % Hepes (Sigma Aldrich). Ninety to 95 % confluent CHO cells were then transfected with 4 μg of pValac:*ESAT*-6 or no plasmid (negative control) previously complexed with Lipofectamine 2000 (Invitrogen), as described by the supplier. The standardization of the transfection was performed with pValac:*gfp*, being the transfection efficiency of GFP-producing cells visualized by a fluorescence microscope (Zeiss Axiovert 200).

Analysis of the ESAT-6 protein by immunofluorescence

Cells transfected or not with the pValac:*ESAT*-6 plasmid were washed twice in phosphate-buffered saline (PBS) solution, fixed for 15 min with paraformaldehyde (Sigma Aldrich, 4 % in PBS), and permeabilized with Triton X-100 (Sigma Aldrich, 0.1 % in PBS) for 10 min at room temperature. The cells were incubated in a 1/50 dilution of specific rabbit polyclonal immunoglobulin G (IgG) anti-ESAT-6 antibody (Abcam, 10 μg/mL) in 1 % bovine serum albumin (BSA; Sigma Aldrich) in PBS for 120 min at room temperature and rinsed three times with PBS. The cells were then incubated in a 1/500 dilution of anti-rabbit IgG Alexa Fluor 488 (Invitrogen, 4 μg/mL) in 1 % BSA in PBS and 4′,6-

diamidino-2-phenylindole (DAPI 2 μg/mL; Invitrogen) for 60 min at room temperature in the dark. After washing three times, the samples were mounted and the images were captured using a Zeiss LSM 510 META inverted confocal laser-scanning microscope equipped with an argon laser and blue filter with maximum emission of 520 nm; a filter with emission of approximately 461 nm was used for DAPI detection. Images of each sample were collected and analyzed using Zeiss LSM Image Browser software. Cells transfected with the pValac:*gfp* plasmid were used as positive control.

Cells transfected or not with the pValac:*ESAT*-6 plasmid were examined through flow cytometry. Through the use of the Foxp3 Staining Buffer Set Kit (eBioscience), 10^6 cells were incubated with fixation buffer, washed, and incubated with the permeabilization buffer. Thereafter, the pellet was incubated with the rabbit polyclonal IgG anti-ESAT-6 antibody (Abcam, 10 μg/mL) diluted in 1 % BSA in PBS for 30 min at room temperature and then washed. Next, the cells were incubated with the anti-rabbit IgG Alexa Fluor 488 antibody (Invitrogen, 4 μg/mL) for 30 min at room temperature in the dark. After being washed twice, the cells were fixed with paraformaldehyde (Sigma Aldrich), and quantification of the ESAT-6-producing CHO cells was performed by the use of the FACScan (Becton Dickinson Bioscience) equipment; the acquired data were analyzed with the aid of the the FlowJo program (TreeStar, Ashland, OR, USA). Cells transfected with the pValac:*gfp* plasmid were used as positive control.

Mice

Conventional BALB/c mice of 4–5 weeks of age were obtained from Centro de Bioterismo (CEBIO) of Universidade Federal de Minas Gerais (UFMG—Belo Horizonte, Brazil) and used for the immunization assays. Procedures and manipulation of animals followed the rules of the Ethical Principles in Animal Experimentation, adopted by the Ethics Committee on Animal Experimentation (CEUA/UFMG/Brazil). All animals were maintained in collective cages (five animals/cage) in an environmentally controlled room with a 12-hour light/dark cycle and given free access to water and food.

Immunization assay with *L. lactis* FnBPA+ (pValac:*ESAT*-6)

BALB/c mice were divided into the following experimental groups: saline (negative control), *L. lactis* MG1363 (wild-type *L. lactis* strain, negative control), *L. lactis* FnBPA+ (invasive *L. lactis* strain, negative control), *L. lactis* MG1363 (pValac:*ESAT*-6), and *L. lactis* FnBPA+ (pValac:*ESAT*-6). Mice were orally immunized by gavage with a 1×10^8 CFU bacterial suspension in a final volume of 100 μL of saline. Immunizations were administered at three different time points (days 1, 15, and 29), and at each time, the mice were immunized for three consecutive days. Animals were then sacrificed on day 42, after being anesthetized with a ketamine and xylazine mixture (Agener União). Two independent experiments were performed with five mice in each group.

Characterization of the humoral immune response profile

Blood collection was performed on days 14, 28, and 42, and the day of sacrifice. Serum was separated to evaluate anti-ESAT-6 immunoglobulins (IgG, IgG1, IgG2a, and IgA), being mucosal secretory immunoglobulin A (sIgA) also measured from colon tissues and fecal extracts. These Igs were measured by capture enzyme-linked immunosorbent assay (ELISA) (Green and Sambrook 2012), coating the plates with 5 μg/mL of recombinant ESAT-6 (rESAT-6). The rESAT-6 protein was obtained in this work, using the commercial vector araBAD promoter (pBAD)202/D-TOPO® (Invitrogen) and *E. coli* TOP10 to obtain the *E. coli* TOP10 (pBAD:*ESAT*-6) strain, according to the manufacturer's instructions. rESAT-6 was purified by the use of the FPLC ÄKTAPRIME plus (GE Healthcare) (Fig. S1).

Characterization of the cellular immune response profile

On the day of sacrifice, the spleens were removed and macerated. A total of 1×10^6 cells/spleen were plated in complete RPMI 1640 (Sigma Aldrich) medium supplemented with 10 % of fetal bovine serum (Gibco), sodium pyruvate 1 mM (Sigma Aldrich), non-essential amino acids 1 mM (MEM NEAA—Gibco), gentamicin 25 μg/mL (Gibco), and L-glutamine 2 mM (LGC Biotecnologia) and maintained at 37 °C and 5 % CO_2. The spleen of each animal was seeded in 96-well microtiter plates and treated by three different methods: experimental samples were stimulated with 5 μg/mL of rESAT-6 (Fig. S1), positive control samples were stimulated with 16 μg/mL concanavalin A (ConA; Sigma Aldrich), and only complete RPMI medium was added to negative control samples (non-stimulated cells). After 60 h of stimuli, the cell supernatant was collected to measure the cytokines IFN-γ, tumor necrosis factor alpha (TNF-α), and the interleukins 12 (IL-12), IL-10, and IL-4 through sandwich ELISA (Green and Sambrook 2012). The results reflect the subtraction of non-stimulated culture from the stimulated rESAT-6 culture.

Statistical analysis

Statistical variance analysis was performed using GraphPad Prism 5.0 software (San Diego, CA, USA), and all results are expressed as the mean±standard error of the mean (SEM). Variance analyses were performed by one-way ANOVA followed by Bonferroni posttest. A

95 % confidence limit was considered to be significant ($p<0.05$).

Results

Construction of *L. lactis* FnBPA+ (pValac:*ESAT*-6)

The ESAT-6 ORF was successfully cloned into the pValac vector (Table 1) between the pCMV and the BGH polyA site, as required for gene expression by host eukaryotic cells (Fig. 1). The construction of the pValac:*ESAT*-6 plasmid (Table 1) was confirmed by digestion, PCR, and sequencing (Fig. S2). The pValac:*ESAT*-6 plasmid was then transferred to the invasive *L. lactis* FnBPA+ strain, a recombinant strain that is capable of adhering to and invading eukaryotic cells, resulting in the recombinant *L. lactis* FnBPA+ (pValac:*ESAT*-6) (Table 1).

Functionality of pValac:*ESAT*-6 plasmid in eukaryotic cells in vitro

In order to confirm the functionality of pValac:*ESAT*-6, this plasmid was transfected into CHO cells to determine the expression and the protein production (ESAT-6 antigen) by eukaryotic cells. Through confocal microscopy and labeling of the transfected cells with the specific antibodies, it was possible to visualize the expression of ESAT-6 by these cells after 72-h post-transfection with the pValac:*ESAT*-6 plasmid.

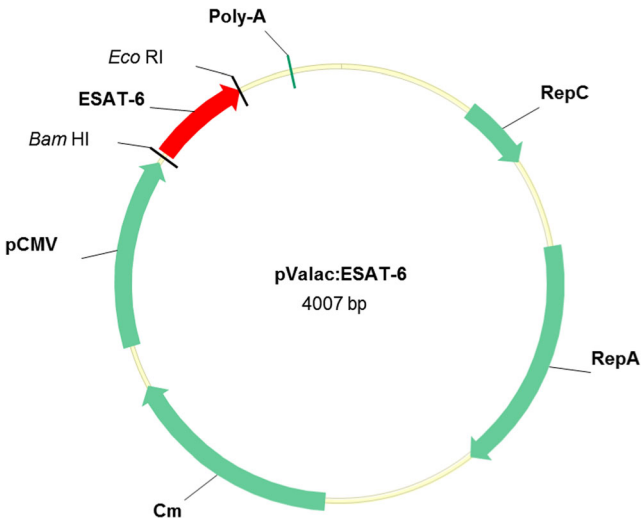

Fig. 1 Structure of the pValac:*ESAT*-6 plasmid. *Tick marks* indicate *Bam*HI and *Eco*RI restriction sites and BGH polyadenylation region (polyA). *Arrows* indicate cytomegalovirus promoter (pCMV), ORF of the 6-kDa early secreted antigenic target (ESAT-6), and replication origin of *E. coli* (RepC) and *L. lactis* (RepA) and chloramphenicol resistance gene (Cm)

The ESAT-6 protein in green (Fig. 2a), the nucleus in blue (Fig. 2b), and the overlap of these two labels (Fig. 2c) could be observed. No fluorescence was visualized in the negative control of non-transfected cells labeled with the primary and secondary antibodies (Fig. 2d) neither in the negative control of the transfected cells labeled with the secondary antibody only (Fig. 2e). As a positive control, cells were transfected with pValac:*gfp* (Fig. 2f).

These results were also verified through flow cytometry (Fig. 3). By representation of the results in dot plot graphics, it was possible to confirm the expression of the ESAT-6 protein by CHO cells since approximately 34 % of pValac:*ESAT*-6-transfected cells emitted fluorescence (Fig. 3a) compared to no fluorescence by non-transfected cells (Fig. 3b). As a positive control, cells were transfected with pValac:*gfp* (Fig. 3c). Considering all of these observations, the results demonstrated that the pValac:*ESAT*-6 plasmid is functional.

L. lactis FnBPA+ (pValac:*ESAT*-6) was able to induce significant sIgA

The administration of *L. lactis* FnBPA+ (pValac:*ESAT*-6) to BALB/c mice was able to increase anti-ESAT-6 sIgA in the colon and fecal extracts. A statistically significant increase in specific sIgA production in colonic tissues (Fig. 4a) and fecal extracts (Fig. 4b) was observed in this group when compared to all the other groups tested. These mice, immunized with *L. lactis* FnBPA+ (pValac:*ESAT*-6), had approximately three times more anti-ESAT-6 sIgA in their colon compared to other groups. Regarding the humoral systemic response, levels of IgG and their isotypes IgG2a and IgG1 were measured in the serum of immunized mice, but no significant difference was observed during the experiment (Fig. S3). Despite this, an increase of serum IgA was observed 2 weeks after the last immunization of the experimental *L. lactis* FnBPA+ (pValac:*ESAT*-6) group, but it was not significantly different when compared to the *L. lactis* FnBPA+ control group, in which it was not possible to confirm an increase in systemic IgA (Fig. 4c).

L. lactis FnBPA+ (pValac:*ESAT*-6) was able to induce significant IFN-γ production

The administration of *L. lactis* FnBPA+ (pValac:*ESAT*-6) to BALB/c mice was able to increase IFN-γ production by rESAT-6-stimulated spleen cells. After 60 h of stimulus, a significant increase in IFN-γ production was observed in the group that received *L. lactis* FnBPA+ (pValac:*ESAT*-6), with a production of approximately 1.5 ng/mL (Fig. 5a). Regarding the other pro-

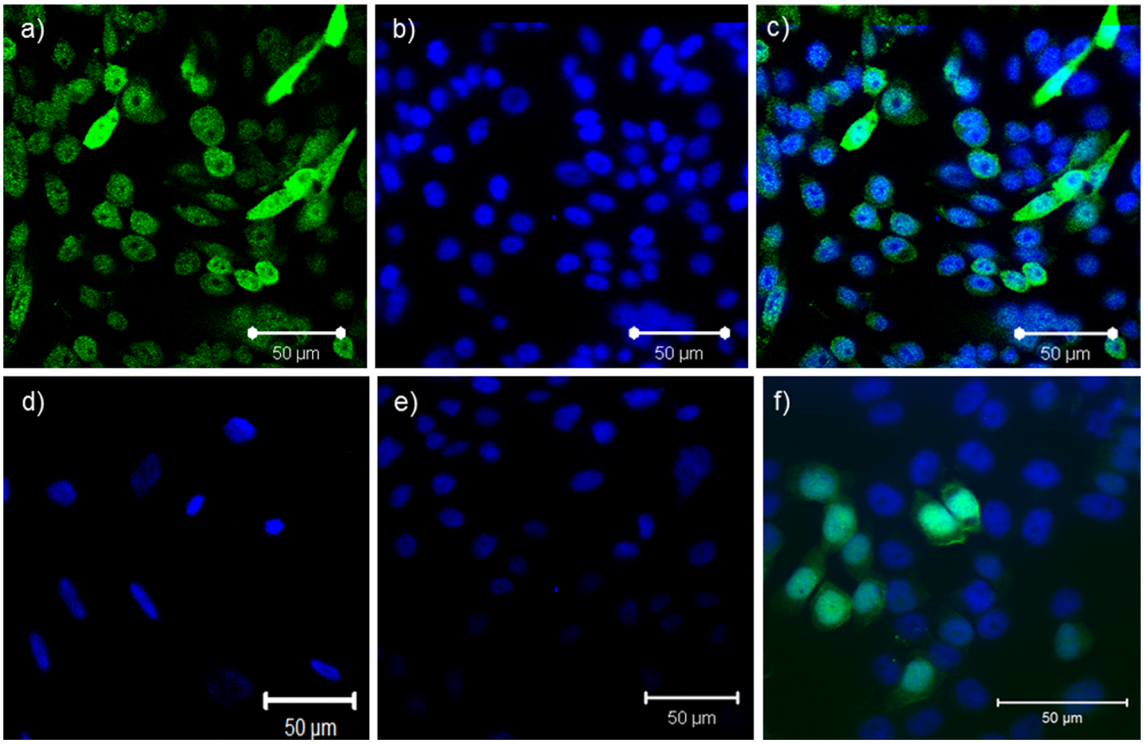

Fig. 2 Expression of the ESAT-6 protein by CHO cells transfected with the pValac:*ESAT*-6 plasmid. pValac:*ESAT*-6 transfected cells: **a** capture of Alexa 488, **b** capture of DAPI, **c** overlap of images **a** and **b**, **d** negative control of the primary and secondary antibodies, **e** negative control of the secondary antibody, and **f** positive control pValac:*gfp*-transfected cells. Images obtained using a Zeiss LSM 510 META inverted confocal laser-scanning microscope with a ×63 objective

inflammatory cytokines tested, TNF-α and IL-12, no significant difference was observed among the experimental groups tested (Fig. 5b, c). Furthermore, no significant difference was observed for IL-10 and the Th2 cytokine IL-4, with no statistically significant difference in IL-10 among the groups tested and no detection of IL-4 (S4).

Discussion

The use of LAB, especially *L. lactis*, for the production of heterologous proteins of biotechnological interest is a consolidated reality. With new information regarding these microorganisms and new genetic tools, new possibilities of use are being envisioned. In this context, currently, the use of *L. lactis*

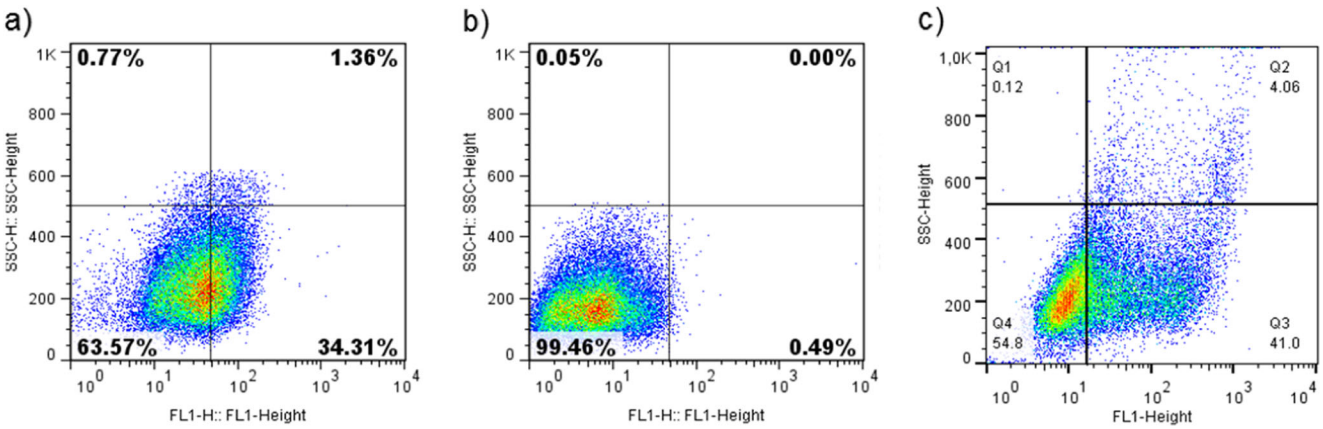

Fig. 3 Expression of the ESAT-6 protein by CHO cells transfected with the pValac:*ESAT*-6 plasmid. **a** Transfected cells labeled with specific ESAT-6 antibodies. **b** Non-transfected cells labeled with the specific primary anti-ESAT-6 and secondary Alexa 488 antibodies (negative control). **c** pValac:*gfp* transfected cells (positive control). Dot Plots showing the cell count on the Y axis and the FL1 detector (Argon laser, 488 nm) on the X axis examined through Flow cytometry (FACScan - Becton Dickinson Bioscience). Images obtained using FlowJo software

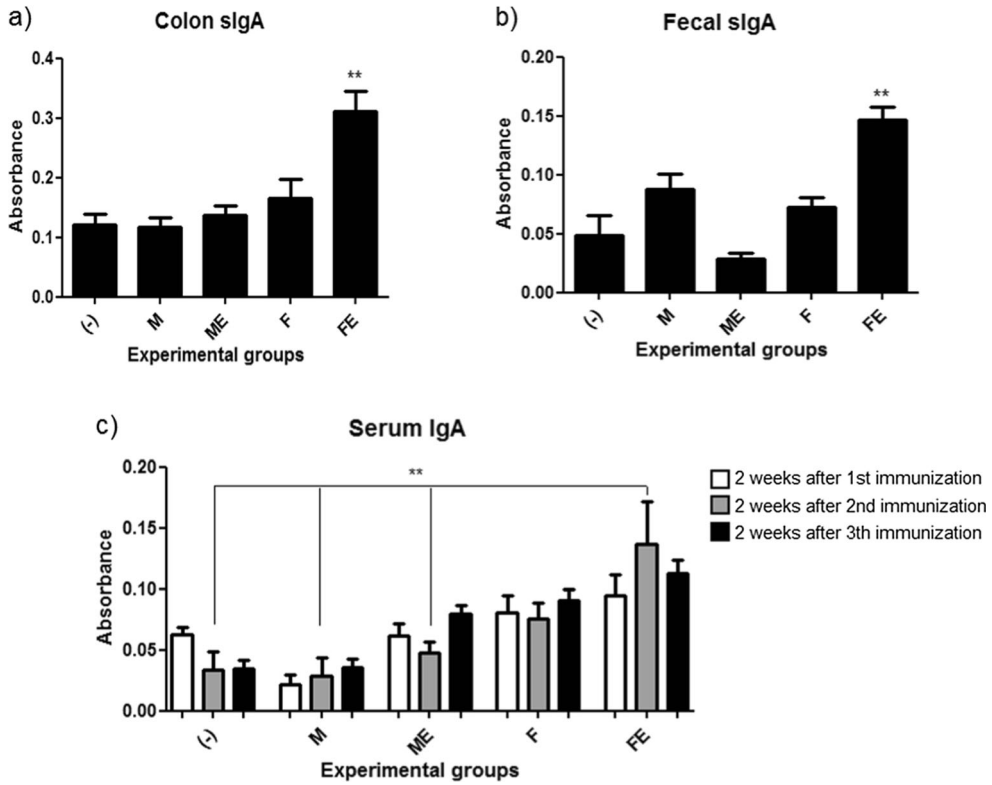

Fig. 4 Production of anti-ESAT-6 IgA in immunized animals. **a** sIgA detection in colonic tissues and **b** sIgA detection in fecal extracts of immunized animals, both at the end of the experiment. **c** Serum IgA detection in immunized animals at three different time points of the experiment. Experimental groups: (−): saline; *M*: *L. lactis* MG1363; *ME*: *L. lactis* MG1363 (pValac:*ESAT*-6) (non-invasive experimental group); *F*: *L. lactis* FnBPA+ (invasive control); *FE*: *L. lactis* FnBPA+ (pValac:*ESAT*-6) (invasive experimental group). Data are shown as the mean± SED and are from two independent experiments (n=10). p value: *p<0.05 and **p<0.01 versus all groups or as displayed on the figure and analyzed at the same immunization/collection time

as a mucosal delivery system for DNA vaccines may represent the next step to be achieved in this area.

Wild-type strains of *L. lactis* are able to deliver eukaryotic expression vectors in vitro and in vivo (Guimarães et al. 2006; Chatel et al. 2008). However, to increase the efficiency of eukaryotic expression cassette delivery to the host cells, the *L. lactis* FnBPA+ recombinant strain is used, being able to invade and deliver DNA vaccines into human epithelial cells. Following cell invasion, *L. lactis* FnBPA+ is probably internalized by lysosomal phagosome vesicles and lysed; the released DNA is transferred to the nucleus, and the ORF of interest is finally expressed. This way, Innocentin et al. (2009) demonstrated that *L. lactis* FnBPA+ internalization capacity is higher than that of the wild-type strain of *L. lactis*. Thus, this work has opened up new and promising possibilities for the use of *L. lactis* for DNA mucosal vaccine delivery.

The system presented here uses oral administration, a non-invasive route that can be easily used in large immunization programs. Moreover, this immunization system could be used for the control of many infectious diseases whose pathogens invade the host through the mucosal surface, as is the case for TB.

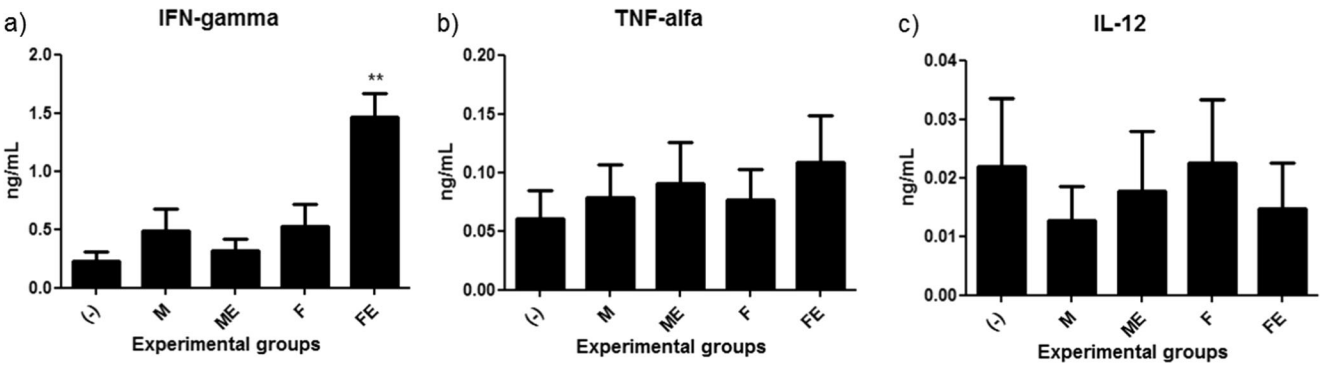

Fig. 5 Production of cytokines from spleen cells stimulated with recombinant ESAT-6. Production levels analyzed by ELISA of **a** IFN-γ, **b** TNF-α, and **c** IL-12 after 60 h of culturing supernatants of spleen cells. Experimental groups: (−): saline; *M*: *L. lactis* MG1363; *ME*: *L. lactis* MG1363 (pValac:*ESAT*-6) (non-invasive experimental group); *F*: *L. lactis* FnBPA+ (invasive control); *FE*: *L. lactis* FnBPA+ (pValac:*ESAT*-6) (invasive experimental group). The *bar* represents the stimulated culture minus the non-stimulated culture for each animal. Data are shown as the mean±SED and are from two independent experiments (n=10). p value: **p<0.01 versus all groups

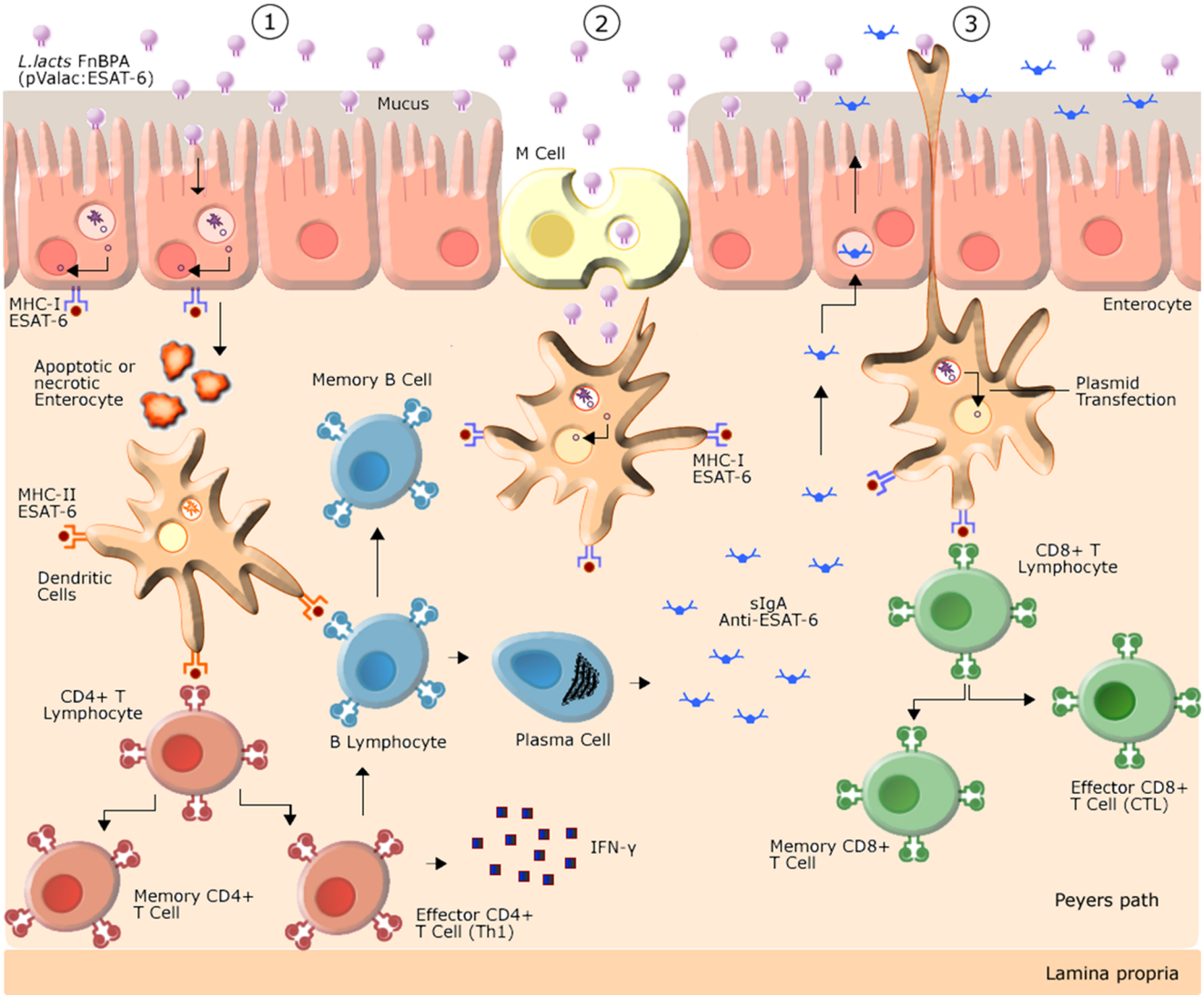

Fig. 6 The proposed mechanism of action of *Lactococcus lactis* FnBPA+ (pValac:*ESAT*-6) DNA delivery system in intestinal membrane. *L. lactis* FnBPA+ carrying pValac:*ESAT*-6 is internalized by intestinal cells through FnBPA invasion (*1*), or by dendritic cells (DC) through bacterial M cells transport (*2*), or by dendrites that capture lumen bacteria (*3*), where the plasmid can reach the nucleus, leading to the expression of ESAT-6 and its presentation by the major histocompatibility complex I (MHC-I) pathway. DCs can acquire apoptotic or necrotic enterocytes producing ESAT-6, presenting them by a phagosomal MHC-II pathway. This describes the activation of specific T helper 1 (Th1) lymphocytes that produce IFN-γ, plasma cells that produce sIgA (anti-ESAT-6), and specific effector cytotoxic T lymphocytes (CTLs)

In this regard, a new plasmid called pValac:*ESAT*-6 was constructed in the present work (Fig. 1). Its functionality was confirmed (Figs. 2 and 3) and the pValac:*ESAT*-6 was then inserted into the *L. lactis* FnBPA+ strain. This new construction, *L. lactis* FnBPA+ (pValac:*ESAT*-6), was analyzed in vivo, testing the immune profile of this new DNA vaccine.

The immunization of mice with *L. lactis* FnBPA+ (pValac:*ESAT*-6) demonstrated that this strain leads to a specific immune response to ESAT-6, with significant mucous anti-ESAT-6 sIgA in colonic tissue and fecal extract, demonstrating a specific mucosal immune response after its oral administration (Fig. 4a, b). Furthermore, the immunization with *L. lactis* FnBPA+ (pValac:*ESAT*-6) was able to generate a T helper 1 (Th1) systemic cellular immune response, detected by an increase in the secretion of IFN-γ in the supernatant of murine splenocytes after rESAT-6 stimulation (Fig. 5a). These results suggest that the recombinant invasive *L. lactis* FnBPA+ can be used as an alternative method for mucosal DNA vaccines delivery.

The immune response obtained using the immunodominant antigen ESAT-6 was as expected because many authors have shown that this antigen has an important role in the T cell response in the first stage of the infection, and a large number of epitopes have already been described (Mustafa et al. 2000; Lalvani et al. 2001; Mustafa et al. 2003). Several studies have also demonstrated the ability of ESAT-6, when used as a DNA vaccine, to generate a predominantly Th1 response

with high IFN-γ production and consequent protection to TB challenge (Xu et al. 2008; Yuan et al. 2012). It has already been shown that ESAT-6 acts by increasing the immunogenicity and protection of the BCG vaccine when used in a prime-boosting vaccination regime (Wang et al. 2004; Fan et al. 2007).

However, the route of immunization used for DNA vaccination in these studies was intramuscular (Wang et al. 2004; Fan et al. 2007; Xu et al. 2008; Yuan et al. 2012). The intramuscular route is less efficient in stimulating mucosal immunity, which is important for protection against TB since the mucosa is the first surface that comes into contact with *M. tuberculosis*.

Thus, due to the need to develop TB vaccines that activate a full immune response, including mucosal immunity, some studies have used this route for the administration of DNA vaccines. The use of an intranasal naked DNA vaccine expressing the fusion protein ESAT-6/Ag85A/IL-21 as a primer and intranasal BCG as a booster showed promising results for IFN-γ and increased sIgA in bronchoalveolar lavage and diminution of bacterial presence in the lungs of immunized mice after challenge (Dou et al. 2012). However, the administration of naked DNA via mucosa requires large amounts of plasmid per dose, which complicates and increases the cost related to lipopolysaccharide (LPS)-free plasmid purification.

To optimize the delivery of DNA directly into the intestinal epithelial cells, Wang et al. (2009) developed a DNA vaccine encoding the ESAT-6/Ag85B fusion protein, in which the oral mucosa route and the bacteria DNA delivery system were used. This oral DNA vaccine, delivered by the attenuated bacteria *Salmonella typhimurium*, developed a strong Th1 immune response with a high amount of IFN-γ-producing cells. The increase in specific IgA in the serum and tissues of the intestine, stomach, and lungs were higher leveled compared to the responses observed when BCG alone was used.

Moreover, when combining this DNA vaccine with BCG, higher production of sIgA, IFN-γ, and protection after TB challenge was observed.

Regarding the invasive system presented here (*L. lactis* FnBPA+), its applicability for oral DNA delivery was displayed through the immune response generated after immunization. The production of specific sIgA in colonic tissues was approximately three times higher in the *L. lactis* FnBPA+ (pValac:*ESAT*-6) group than in the non-invasive *L. lactis* MG1363 (pValac:*ESAT*-6) group. The same difference was observed regarding the cellular immune response, with the IFN-γ stimulus. These results may suggest that the invasive status provides greater ability to deliver the pValac:*ESAT*-6 vector in a mucosal surface, resulting in a better induction of mucosal and cellular immune responses.

Thus, the mechanism of action of this DNA vaccine, *L. lactis* FnBPA+ (pValac:*ESAT*-6), most likely mimics a viral infection. *L. lactis* FnBPA carrying a DNA plasmid is internalized by intestinal cells more efficiently, and the plasmid can reach the nucleus leading to ESAT-6 expression. Thus, the major histocompatibility complex I (MHC-I) antigen presentation pathway enables the immune system to detect cells with peptides from the pValac:*ESAT*-6 plasmid, such as infected enterocytes and dendritic cells (DCs). DCs are able to engulf bacteria from the intestinal lumen and through M cells and then express ESAT-6 endogenously. If not directly infected, DCs can acquire exogenous antigens from producing ESAT-6 enterocytes and present them to the immune system via the MHC-II pathway. In this context, specific CD4+ T cells become Th1 IFN-γ-producing cells, B lymphocytes are activated in sIgA (anti-ESAT-6)-producing plasma cells, and specific CD8+ T cells become cytotoxic T lymphocytes (CTLs) (Fig. 6).

In summary, these results show that the oral administration of *L. lactis* FnBPA+ (pValac:*ESAT*-6) is a good alternative for the delivery of DNA plasmids in mucous membranes and generates a specific immune response to the tuberculosis ESAT-6 antigen, suggesting the applicability and effectiveness of this novel DNA delivery-based strategy.

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Conflict of interest The authors declare that they have no conflict of interest.

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