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Recombinant invasive *Lactococcus lactis* can transfer DNA vaccines either directly to dendritic cells or across an epithelial cell monolayer

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ABSTRACT

Lactococcus lactis (*L. lactis*), a generally regarded as safe (GRAS) bacterium has recently been investigated as a mucosal delivery vehicle for DNA vaccines. Because of its GRAS status, *L. lactis* represents an attractive alternative to attenuated pathogens. Previous studies showed that eukaryotic expression plasmids could be delivered into intestinal epithelial cells (IECs) by *L. lactis*, or recombinant invasive strains of *L. lactis*, leading to heterologous protein expression. Although expression of antigens in IECs might lead to vaccine responses, it would be of interest to know whether uptake of *L. lactis* DNA vaccines by dendritic cells (DCs) could lead to antigen expression as they are unique in their ability to induce antigen-specific T cell responses. To test this, we incubated mouse bone marrow-derived DCs (BMDCs) with invasive *L. lactis* strains expressing either *Staphylococcus aureus* Fibronectin Binding Protein A (LL-FnBPA+), or *Listeria monocytogenes* mutated Internalin A (LL-mInlA+), both strains carrying a plasmid DNA vaccine (pValac) encoding for the cow milk allergen β-lactoglobulin (BLG). We demonstrated that they can transfect BMDCs, inducing the secretion of the pro-inflammatory cytokine IL-12. We also measured the capacity of strains to invade a polarized monolayer of IECs, mimicking the situation encountered in the gastrointestinal tract. Gentamycin survival assay in these cells showed that LL-mInlA+ is 100 times more invasive than *L. lactis*. The cross-talk between differentiated IECs, BMDCs and bacteria was also evaluated using an *in vitro* transwell co-culture model. Co-incubation of strains in this model showed that DCs incubated with LL-mInlA+ containing pValac:BLG could express significant levels of BLG. These results suggest that DCs could sample bacteria containing the DNA vaccine across the epithelial barrier and express the antigen.

1. Introduction

DNA vaccination has been of great interest since its discovery in the 1990s as it can stimulate both cell-mediated and humoral immune responses [1]. It is known that intramuscular injection of plasmid DNA can induce antibodies, helper and cytotoxic T cell responses, against different viral and bacterial infections in animal models [2]. Although has not yet approved a licensed DNA vaccine for humans, phase I clinical studies have been performed with prototype DNA vaccines for the prevention of many infectious agents [3]. Recently, four DNA vaccines were licensed for veterinary use [4].

One of the concerns about naked DNA immunization is its low immunogenicity, as the antigen is produced in very small amounts *in vivo* due to its non-replicative nature [5]. Also, it is well known that DNA-based vaccines poorly target antigen-presenting cells (APCs) [6]. Therefore, several strategies have been designed to increase the potency of DNA immunization [7]. The use of bacteria as a vehicle for DNA delivery into eukaryotic cells has emerged as a potential approach to enhance its immunogenicity [8]. One attractive feature is their potential for oral administration and the prospect of inducing both mucosal and systemic immune responses [5]. Some attenuated pathogenic species contains an innate tropism

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for specific tissues of the host, directing systemic responses towards the mucosa [8]. Furthermore, bacteria protect the plasmid against degradation [9] before uptake by cells, and serve as immune adjuvant [8]. Another advantage of bacterial vehicles is that they can accommodate large-sized plasmids [10]. Finally, this vaccine platform is considered to be low cost as it does not require preparation of highly purified plasmid DNA [8,9].

Shigella flexneri and *Listeria monocytogenes* have been previously used as experimental delivery systems as they are able to invade intestinal epithelial cells (IECs) and interact with APCs. In order to deliver the DNA, after uptake by the cells most bacteria are rapidly degraded in the phagolysosome, releasing the plasmid that reaches the nucleus where the transcriptional machinery of the host cell activates transcription [10]. Even though they are attenuated for virulence, preexisting immunity and reversion to virulence are major concerns [11]. Recently, the potential use of *Lactococcus lactis* (*L. lactis*), the model Lactic Acid Bacteria (LAB), for the production of biologically useful proteins and for plasmid DNA delivery to eukaryotic cells is being explored [for a review see [11–13]]. *L. lactis* is considered an advantageous vector because it has an established safety profile generated through its long use in the dairy industry as starters for food fermentations, being considered as GRAS (Generally Recognized as Safe) bacterium [11]. Moreover, *L. lactis* does not produce lipopolysaccharides, contains a number of genetic tools developed and does not induce strong host immune responses to its self [11,12,14]. Guimarães and collaborators showed that incubation of *L. lactis* carrying a β-lactoglobulin (BLG) eukaryotic expression plasmid with human intestinal epithelial cell line Caco-2 resulted in expression of BLG [15]. Later, Chatel et al. demonstrated that the same strain could induce expression of BLG in IECs *in vivo* after oral administration in mice [16]. To increase the efficiency of *L. lactis* DNA vaccine delivery, recombinant strains expressing the internalin A (InlA), invasin from *L. monocytogenes* (LL-InlA+) [17] and fibronectin binding protein A (FnBPA) of *Staphylococcus aureus* (LL-FnBPA+) were developed [18]. *In vitro*, they showed a higher ability to invade mammalian cells compared to the wild type (wt) lactococci and resulted in an increase of target DNA expression by Caco-2 cells. Although interesting, these two strategies have some limitations; InlA does not bind to the E-cadherin receptor in mice limiting *in vivo* studies [19], and FnBPA requires an adequate local concentration of fibronectin to bind to integrins [20]. To overcome these drawbacks a recombinant *L. lactis* strain expressing a mutated internalin A (mInlA) that recognizes mouse E-cadherin was developed (LL-mInlA+) [20].

Current knowledge about DNA vaccination using *L. lactis* is mostly based on data obtained in experiments performed with IECs. The aim of this study was to investigate the potential for *L. lactis* to deliver DNA vaccines and obtain antigen expression in dendritic cells (DCs). DCs are major APCs serving as potent inducers of specific cell-mediated immune responses [21], and those in direct contact with the intestinal epithelium have been shown to take up luminal antigens/bacteria [22]. Moreover, DCs are able to secrete interleukins, such as IL-12, that polarizes T cells to the protective T helper 1 (Th1) phenotype [23].

In this study, we measured the ability of noninvasive and invasive *L. lactis* expressing either *S. aureus* FnBPA or *L. monocytogenes* mutated InlA to deliver a plasmid DNA (pValac) [24] encoding for BLG to DCs.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

All bacterial strains and plasmids used are listed in Table 1. *L. lactis* was grown in M17 medium supplemented with 0.5% (w/v) glucose (GM17) at 30 °C without agitation. When required erythromycin (Ery) (10 μg/ml) and/or chloramphenicol (Cm) (10 μg/ml) were added to the medium.

Table 1
Bacterial strains and plasmids used in this work.

Strain/plasmid	Relevant characteristics	Source/reference
Bacterial strains		
MG1363	*L. lactis* MG1363 wild type strain	[25]
NZ9000	A derivative of *L. lactis* MG1363 wild type strain generated by the integration of the *NisRK* genes	[26]
LL	*L. lactis MG1363* strain containing pOri23 plasmid	[27]
LL-mInlA+	*L. lactis* NZ9000 strain containing pOri253:mInlA plasmid	[20]
LL-FnBPA+	*L. lactis* MG1363 strain containing pOri23:FnBPA plasmid	[28]
LL-BLG	*L. lactis* MG1363 strain containing pOri23 and pValac:BLG plasmids	[29]
LlmInlA- BLG	*L. lactis* NZ9000 strain containing pOri253:mInlA and pValac:BLG plasmids	[20]
LLFnBPA-BLG	*L. lactis* MG1363 strain containing pOri23:FnBPA and pValac:BLG plasmids	[29]
Plasmids		
pPL2:mInlA	*E. coli* vector containing mInlA gene, Ery^r	[27]
pOri253IInk	*L. lactis—E. coli* shuttle vector, Ery^r	[20]
pOri23	*L. lactis—E. coli* shuttle vector, Ery^r	[27]
pValac:BLG	*L. lactis—E. coli* shuttle vector carrying the BLG gene under the control of the eukaryotic CMV promoter, Cm^r	[29]
pOri253:mInlA	*L. lactis—E. coli* shuttle vector carrying the mInlA gene under the control of the constitutive PrfA promoter and harboring the native cell wall anchoring signal, Ery^r	[20]
pOri23:FnBPA	*L. lactis—E. coli* shuttle vector carrying the FnBPA gene of *S. aureus*; Ery^r	[28]

Ery^r, Erythromycin resistant; Cm^r, Chloramphenicol resistant.

2.2. Polarized intestinal epithelial cell monolayers

The human intestinal epithelial cell line Caco-2 (ATCC number HTB37) was maintained in DMEM medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS) (Omnilab, Switzerland) and 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium). Trypsin-treated cells were seeded on permeable Transwell filters with 0.4 μm pore PET membranes (Corning Glass Works,) and maintained for 14 days in p-24 plates (Corning Glass Works) (37 °C, 5%CO_2, 95% air atmosphere). Cells formed a confluent polarized cell layer with a distinct apical and basal side [30].

2.3. Bacterial invasion assays

Strains grown to an OD600 of 1.0, were pelleted by centrifugation, washed with PBS and diluted in the tissue culture medium to give a multiplicity of infection (MOI) of around 1000 bacteria per eukaryotic cell. Before incubation, polarized monolayers of Caco-2 cells were treated with 10 mM EDTA buffer (Sigma-Aldrich, St. Louis, MO) to disrupt tight junctions and the transepithelial electrical resistance was monitored as described by Karczewski et al. [31]. Bacterial invasion was measured using the gentamicin survival assay [32].

2.4. Generation of murine bone-marrow derived dendritic cells (BMDCs) and FACS analysis

Bone marrow cells were isolated from the femurs of euthanized BALB/c mice (from Wageningen University animal facility).

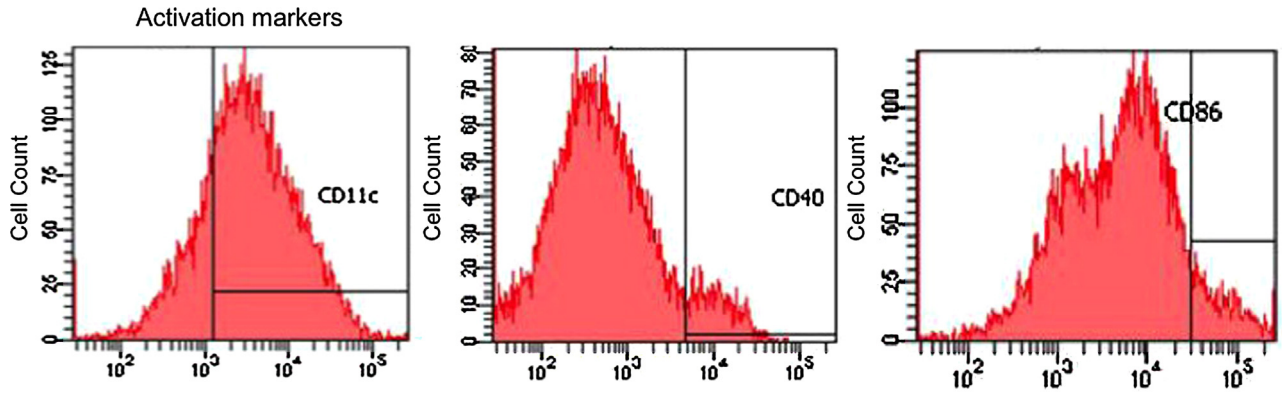

Fig. 1. *Phenotypic maturation of BMDCs confirmed by flow cytometry*. FACS analysis of surface CD11c, CD40 and CD86 receptors in isolated BMDCs. BMDCs cultured during six days were analyzed using a BD Biosciences FACSCanto II flow to measure CD marker expression. Cells were stained with FITC-conjugated anti-mouse CD11c, PE/Cy7-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD40 antibodies and results were analyzed with either BD FACSDiva or Flowjo software. Bone marrow-derived dendritic cells (BMDCs).

Around 2×10^7 of live cells were filtered using a Steriflip® Filter Unit (Millipore) and seeded in a sterile petri dish in complete RPMI-1640, 20 ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating-factor (GM-CSF) (R&D systems) and 0.05 mM of β-mercaptoethanol (Invitrogen). Cells were incubated at 37 °C and on day six, the number and size of BMDCs cells were evaluated by fluorescence activated cell-sorting (FACS) analysis using FITC-conjugated anti-mouse CD11c, PE/Cy7-conjugated anti-mouse CD86 and PE-conjugated anti-mouse CD40 antibodies (all from BD Biosciences). Data was analyzed with either BD FACSDiva or Flowjo software.

2.5. Detection of BLG in BMDCs

The uptake of *L. lactis* strains in BMDCs was determined using a modification of the gentamycin survival assay. Expression and secretion of BLG by DCs was measured in the supernatant after centrifugation at 78.2 g for 10 min; supernatant was stored at −80 °C. Intracellular BLG expression was measured after ultrasonic lysis of cells (3 times, 10 s in 500 μl of PBS containing a cocktail of protease inhibitors—Roche).

2.6. In vitro *transwell co-culture system of differentiated Caco-2 cells and BMDCs*

Epithelial cell monolayers were placed in contact with BMDCs as described previously [22,33]. Caco-2 cells were cultured on the upper face of 3-μm pore Transwell filters (costar, polycarbonate Membrane) for 10–15 days in a 24-well plate (Corning Glass Works) using DMEM medium (Sigma, St. Louis, MO) containing 10% FBS (Omnilab, Switzerland) and 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium). 4×10^5 of isolated BMDCs were seeded on the basolateral side of differentiated Caco-2 and maintained in RPMI-1640 (Zonder Hepes), supplemented with 10% heat-inactivated FCS (Sigma-Aldrich) and 20 ng/ml of GM-CSF (R&D systems) during 18 h at 37 °C in a 5% CO_2/95% air atmosphere.

2.7. BLG detection in both BMDCs and Caco-2 monolayers after bacterial incubation

A co-culture system model described in Section 2.6 was used to evaluate DNA transfer capacity of invasive or noninvasive *L. lactis* across an epithelial cell monolayer. No EDTA treatment was applied to disrupt tight junctions before co-incubation with strains. Bacteria (MOI 1000) were incubated in the apical side of the epithelium for 3 h (37 °C, 5% CO_2). Then, gentamicin (150 μg/ml) was applied for 2 h to avoid bacteria overgrowth. Plate was incubated for 72 h (5% CO_2, 37 °C); 250 μl of PBS containing a cocktail of protease inhibitors (Roche) were added; cells were scraped and harvested using an ultrasonic treatment (3 times, 10 s). Bradford Assay determined protein concentration from each samples. After protein quantification, apical and basolateral supernatant were stored and assayed for BLG production by ELISA with a few modifications. Briefly, ninety-six-well microtiter plates were coated by adding 0,01 mg of total protein (from DCs or Caco-2 extracts) diluted in PBS 1× and plates were incubated overnight at 4 °C. Wells were washed and coated with 3.5 μg/ml of BLG- antibody (Genway Biotech, Inc.) and then maintained at room temperature during 1 h. After this time, plates were washed and second antibody (Rabbit anti-sheep IgG-HRP) diluted 1:1000 in PBS 1X-0.1% BSA was added to the wells. After 1 h at room temperature the following peroxidase substrate and stop solutions were used to yields a measurable green end product, whose absorption were measured at an optical density (OD) of 405 nm on a spectrophotometer.

2.8. BMDCs bacterial stimulation and cytokine quantification in culture supernatants

4×10^5 of isolated BMDCs were plated in 24-well plates (Corning Glass Works) and bacteria were applied (or not) to the cells (MOI 40 bacteria/cell). Plate was maintained at 37 °C in 5% CO_2 for 24 h and supernatant was used to quantify mouse interleukine-10 (IL-10) and interleukine-12 (IL-12) using commercially available ELISA kits (Mabtech, Stockholm, Sweden) following the manufacturer's instructions.

2.9. Statistical analyses

Results are expressed as mean ± SE values. Statistical significance between the groups was calculated using the One Way ANOVA (and nonparametric) test, followed by the "Bonferroni" post-test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. *Invasive* L. lactis *strains can transfer a BLG cDNA directly to BMDCs*

Before measuring the capacity of strains to deliver DNA to DCs, BMDCs maturation was confirmed by FACS analysis (Fig. 1). Next, matured DCs were incubated with non-invasive (LL, LL-BLG) and invasive *L. lactis* carrying pValac:BLG vaccine (LLFnBPA-BLG,

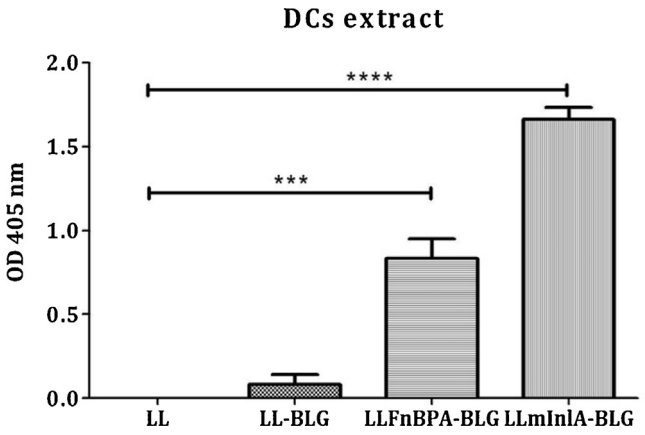

Fig. 2. *BLG detection in BMDCs extracts after co-incubation with L. lactis strains.* BMDCs were isolated from BALB/c mice, cultivated with GM-CSF and incubated with strains (MOI 1:1000) for 1 h. Cells were washed, treated with gentamicin and BLG was assayed in both cells extract and culture supernatants after three days. Data representative from two independent experiments $^{*}p < 0.05$. Bone marrow-derived dendritic cells (BMDCs), *L. lactis* carrying pOri23 plasmid (LL), *L. lactis* harboring both pOri23 and pValac:BLG plasmids (LL-BLG), *L. lactis* expressing *S. aureus* FnBPA and carrying pValac:BLG (LLFnBPA-BLG), *L. lactis* expressing *L. monocytogenes* mInlA and carrying pValac:BLG (LLmInlA-BLG).

LLmInlA-BLG). After three days, BLG was quantified in both culture supernatants and BMDCs extracts. DCs co-incubated with LLFnBPA-BLG were able to express 1.5 times higher amounts of BLG compared to LL-BLG and LL strains, whereas cells incubated with LLmInlA-BLG strain could express almost 2.5 times greater quantities of BLG, compared to the noninvasive strains (Fig. 2). No BLG secretion was observed in culture supernatants (data not shown).

3.2. *Lactococci induces the secretion of IL-12 in BMDCs*

BMDCs were directly challenged by addition (or not) of the bacteria at MOI 40:1. Plate was kept overnight at 37 °C in 5% CO_2/95% air atmosphere and supernatants were assayed for the presence of IL-10 and IL-12. BMDCs co-cultivated with either noninvasive (LL, LL-BLG) or invasive (LLmInlA-BLG, LLFnBPA-BLG) *L. lactis* were able to secrete elevated levels of IL-12 compared to cells that were not incubated with bacteria (using growth medium as a control). Moreover, expression of BLG by DCs as well as the invasive *status* of strains did not affect IL-12 expression (Fig. 3). The induction of IL-10 measured by ELISA was not observed in culture supernatants independently of incubation or not with bacteria (data not shown).

3.3. *Recombinant* L. lactis *expressing invasins are capable to internalize differentiated IECs*

The capacity of invasive (LL-FnBPA+ and LL-mInlA+) or wt (NZ9000 and MG1363) *L. lactis* to internalize a monolayer of Caco-2 cells was investigated. Before co-incubation with bacteria, cell monolayer was treated with 10 mM EDTA solution to disrupt tight junctions in order to expose the mInlA receptor (E-cadherin), which is expressed below the tight junctions on the basolateral side of the cells. A 30 min treatment with EDTA buffer was sufficient to completely disrupt the transepithelial electrical resistance of the monolayer (data not shown). Differentiated Caco-2 cells were incubated with each individual strain for 1 h, non-internalized bacteria were killed by gentamicin, and intracellular bacteria were enumerated after cell lysis. Recombinant LL-mInlA+ and LL-FnBPA+ strains showed 100-fold greater invasion rate compared to the wt strains (NZ9000 and MG1363), as indicated in Fig. 4.

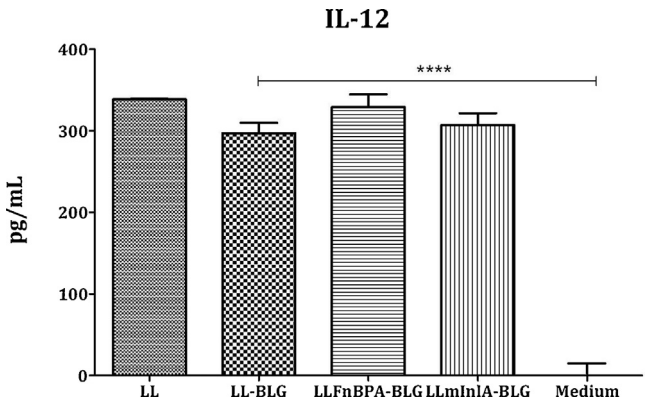

Fig. 3. *IL-12 secretion by BMDCs after co-incubation with non-invasive or invasive L. lactis.* Isolated BMDCs were co-incubated with non-invasive or invasive *L. lactis* (MOI 1:40) during 24 h and culture supernatant was tested for the presence of IL-12 using commercially available ELISA kits. Data representative from two independent experiments. Bone marrow-derived dendritic cells (BMDCs), *L. lactis* carrying pOri23 plasmid (LL), *L. lactis* harboring both pOri23 and pValac:BLG plasmids (LL-BLG), *L. lactis* expressing *S. aureus* FnBPA and carrying pValac:BLG (LLFnBPA-BLG), *L. lactis* expressing *L. monocytogenes* mInlA and carrying pValac:BLG (LLmInlA-BLG).

3.4. *Invasive* L. lactis *are able to deliver pValac:BLG to BMDCs through the IEC monolayer*

In order to evaluate DNA transfer capacity of invasive or non-invasive *L. lactis* through a monolayer of epithelial cells, strains tested were added at the apical surface of the IECs, after which a 3 h gentamicin treatment was applied. For this experiment, no EDTA treatment was applied before co-incubation with strains since we wanted to check if DCs were able to cross the monolayer going through the tight junction between adjacent epithelial cells and sample bacteria present in the apical compartment. After 72 h, cells were lysed, collected, as wells as basal and apical culture supernatants and BLG expression were investigated by ELISA. As demonstrated in Fig. 5, DCs co-cultured with the IEC monolayer which were co-incubated with LLmInlA-BLG strain could

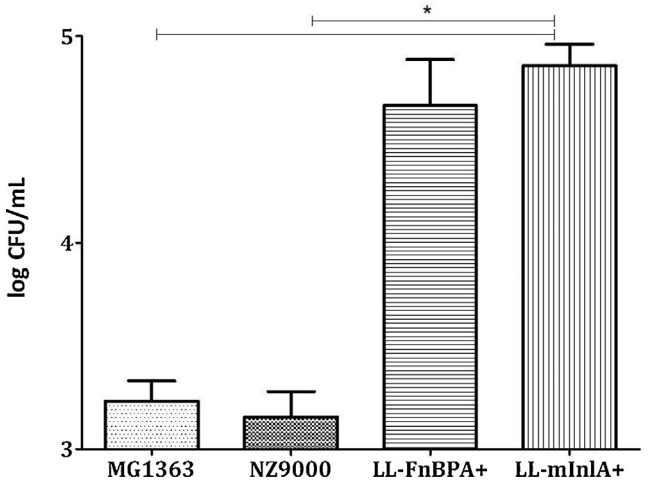

Fig. 4. *Invasiveness scores of wild type or invasive L. lactis into differentiated Caco-2 cells.* Caco-2 cells were cultivated on inserts and after 14 days a monolayer of fully differentiated IECs were obtained. MG1363, NZ9000, LL-mInlA+ and LL-FnBPA+ strains were then co-incubated with cells during 1 h and, after this period, treated with gentamicin for 2 h. Cells were lysed and the number of CFU internalized was measured by plating. **, survival rates were significantly different (One-way ANOVA, Bonferroni's multiple comparison test, $p < 0.05$). Results are means standard deviations of two different experiments, each time done in triplicate. *L. lactis* NZ9000 (NZ9000), *L. lactis* MG1363 (MG1363), *L. lactis* expressing *S. aureus* FnBPA (LL-FnBPA+) and *L. lactis* expressing *L. monocytogenes* mInlA (LL-mInlA+).

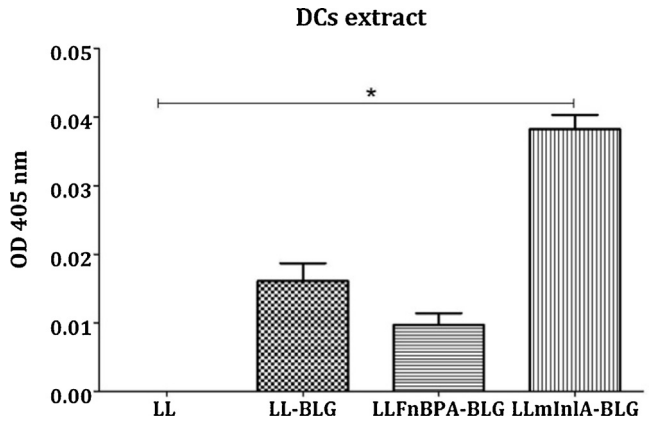

Fig. 5. *BLG expression by BMDCs after incubation with non-invasive or invasive L. lactis.* IECs were apically challenged by addition of bacteria (MOI 1:1000) in the presence BMDCs in the lower compartment. After bacterial stimulation, BLG expression or secretion was measured in extract from IECs, BMDCs, apical or basolateral supernatants by ELISA. Results are expressed as the average between two experiments. $^{*}p < 0.05$. Bone marrow-derived dendritic cells (BMDCs), *L. lactis* carrying pOri23 plasmid (LL), *L. lactis* harboring both pOri23 and pValac:BLG plasmids (LL-BLG), *L. lactis* expressing *S. aureus* FnBPA and carrying pValac:BLG (LLFnBPA-BLG), *L. lactis* expressing *L. monocytogenes* mInlA and carrying pValac:BLG (LLmInlA-BLG).

express significant higher amounts of BLG. No BLG expression by DCs was observed with cells cultured with LL, and low levels were detected for LL-BLG or LLFnBPA-BLG strains. Furthermore, BLG was detected neither in apical or basal culture supernatants nor in Caco-2 extracts (data not shown).

4. Discussion

Several species of LAB, especially *L. lactis*, have proved to be effective mucosal delivery vehicles for both therapeutic proteins and DNA vaccines [11,13,15,16,34]. It was demonstrated that non-invasive or recombinant invasive *L. lactis* is capable to deliver cDNA plasmids either *in vitro* or *in vivo* to IECs [16–18,20]. Here the capacity of these strains to deliver a plasmid DNA to DCs was evaluated either as a purified culture or when these cells formed part of the IEC monolayer. This latter setup allowed investigating whether DCs are able to cross the epithelial membrane and uptake *L. lactis*. We hypothesized that once inside DCs, *L. lactis* would be targeted for degradation releasing pValac:BLG plasmid that would be directed to the nucleus of cells, allowing the expression of BLG. Thus, the production of this protein was measured in IECs and DCs protein extracts in order to qualify this DNA transfer by *L. lactis*. We observed that despite not being able to secrete the allergen, DCs co-incubated with either LLFnBPA-BLG or LLmInlA-BLG were able to produce significant higher amounts of BLG compared to non-invasive (LL, LL-BLG) *L. lactis* (Fig. 2). LLmInlA-BLG strain was the most efficient in transferring pValac:BLG because BMDCs express E-cadherin, receptor of mInlA [35], thus facilitating bacterial adherence. The majority of the works about DNA transfer in DCs is usually based on a technology that uses dendrimers as gene delivery vectors [6,36,37]. However, their application is limited due to its inherent cytotoxicity to mammalian cells [36,38]. Therefore, the use of a GRAS bacterium, such as *L. lactis*, is a promising alternative strategy.

To evaluate DCs immune response against noninvasive and invasive lactococci, isolated BMDCs were co-incubated with strains. The expression of IL-12, a pro-inflammatory interleukin [39] and IL-10, involved in immune-regulation [40], was quantified. BMDCs co-cultivated with either noninvasive or invasive lactococci were able to release elevated levels of IL-12 (300 pg/ml) compared to cells not infected with bacteria; however no IL-10 expression was observed. In a previous study, it was shown that *L. lactis* NZ9000 incubated with BMDCs exhibited pro-inflammatory properties inducing significant levels of IL-1β and IL-12 in DCs as well as high mRNA levels of IL-10 [41]. Others have shown that the type of immune response elicited by LAB is strain dependent as they may favour a Th1 response, a Th2 humoral or tolerogenic, or only an inflammatory response [42–45].

It was vastly demonstrated that the heterologous expression of certain antigens from pathogenic species on *L. lactis* surface can induce a desired type of immune response (Th1 or Th2) (for a review see [11,46]). In this study, the expression of *L. monocytogenes* mInlA or *S. aures* FnBPA in *L. lactis* did not increased IL-12 production by DCs. Expression of BLG, an allergen that usually drives a Th2-mediated immune response by DCs also did not change IL-12 levels in culture supernatants.

Understanding how bacteria interact with the intestinal barrier is a fundamental topic in the development of effective bacterial vaccine vectors [47]. Therefore, in this work the dialog between strains, differentiated IECs and BMDCs was evaluated. The first study performed here was to evaluate if the invasive *L. lactis* strains were able to internalize a monolayer of IECs more efficiently than the wt strains using the gentamicin protection assay [32]. The invasion rate of LL-mInlA+ and LL-FnBPA+ were similar and about 100-fold higher than the invasion rate obtained for wt strains (NZ9000 and MG1363) (Fig. 4). Previous data demonstrated that LL-mInlA+ were 1000 times more invasive than NZ9000 strain in experiments performed with non-confluent Caco-2 cells [20]. The lower invasiveness score obtained after bacterial co-incubation with the IEC monolayer was probably do to the fact that cells were very attached to each other turning E-cadherin receptor, for instance, less accessible.

In order to understand the mechanisms by which *L. lactis* can transfer DNA vaccines *in vivo*, bacteria was co-incubated with a transwell murine co-culture model mimicking the intestinal barrier. DCs were grown on the basolateral side of the inserts while differentiated IECs were cultivated on the apical side. After incubation with strains, BLG production was assayed in apical and basolateral supernatant as well as in Caco-2 and DCs extracts. BLG was only found in DCs incubated with LLmInlA-BLG strain (Fig. 5). This data shows for the first time that DCs can sample an invasive *L. lactis* strain giving new insights on how the DNA transfer process could occur *in vivo*. No BLG secretion by either DCs or Caco-2 cells was observed, differently to what was demonstrated previous in which non-confluent Caco-2 cells co-incubated invasive lactococci could secrete the allergen [20]. Follow-up studies will be performed in near future to evaluate whether DCs could secrete BLG *in vivo* after oral immunization trials.

5. Conclusion

We demonstrated that recombinant invasive *L. lactis* expressing either *S. aureus* FnBPA or *L. monocytogenes* mInlA are capable to transfer DNA directly to BMDCs. This direct contact leaded to the secretion of IL-12 by the cells, independent of the invasive *status*. We also checked that invasive lactococci are able to invade a monolayer of differentiated Caco-2 cells 100 times more efficiently than the wt strains. Using a co-culture model we demonstrated that LL-mInlA+ strain can transfer pValac:BLG to DCs across a monolayer of epithelial cells. Taken together, this study gives new insights on the mechanism of lactococci uptake for delivery of therapeutics.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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