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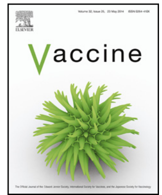

# Recombinant invasive *Lactococcus lactis* can transfer DNA vaccines either directly to dendritic cells or across an epithelial cell monolayer

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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.

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## 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). Around $2 \times 10^7$ of live cells were filtered using a Steriflip® Filter

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