

## A Dendritic Cell-Targeted Vaccine Loaded with Glyceraldehyde-3-Phosphate Dehydrogenase Peptides Proposed for Individuals at High Risk of Listeriosis

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### Abstract

**Objective:** Individuals at risk of *Listeria* infection lacked vaccines to prevent the development of severe listeriosis symptoms. Here, we identify the best epitopes from *Listeria* proteins GAPDH and LLO for an optimal DC vaccine candidate to protect against individuals high susceptible to listeriosis.

**Methods:** We vaccinated high susceptible Balb/c and low susceptible C57BL/6 mice with a single dose of DC-LLO<sub>91-99</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-201</sub>, DC-LLO<sub>296-304</sub>, DC-GAPDH<sub>1-15</sub> or DC-GAPDH<sub>1-22</sub> vaccines. CFU in spleens, predictions of DC-peptide binding to MHC-I and MHC-II and analysis of Th1 immune responses were used to assess vaccine efficiency.

**Results:** DC-GAPDH<sub>1-22</sub> and DC-GAPDH<sub>1-15</sub> vaccines with intermediate binding epitopes to MHC-I and weak binders to MHC-II, provided maximal protection in *Listeria* susceptible and resistant mice. DC-LLO<sub>91-99</sub> vaccine with a strong binding epitope to MHC-I follows protection in both mice strains. DC-LLO<sub>296-304</sub> vaccine conferred protection only in resistant mice and DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-200</sub> and DC-LLO<sub>189-201</sub> vaccines with binding epitopes to MHC-II, lacked protection properties. Maximal protection in listeriosis correlated with increased splenic CD8 $\alpha^+$  DC, enhanced IL-12 production and high frequencies of CD8 $^+$  and CD4 $^+$  producing IFN- $\gamma$  T cells in both mice strains.

**Discussion:** Maximal protection against listeriosis in susceptible and resistant mice is only achieved with DC-GAPDH<sub>1-22</sub> and DC-GAPDH<sub>1-15</sub> vaccines, able to activate simultaneously CD4 $^+$  and CD8 $^+$  T cells as they include binding epitopes to both, MHC-I and MHC-II. CD8 $^+$  T cell activation seemed predominant as DC-LLO<sub>91-99</sub> and DC-LLO<sub>296-304</sub> vaccines that exclusively activate CD8 $^+$  T cells in both mice strains conferred significant listeriosis protection. In this regard, DC-LLO<sub>189-201</sub>, DC-LLO<sub>189-200</sub> or DC-LLO<sub>190-201</sub> vaccines that activated CD4 $^+$  T cells caused no protection at all. We observed that a combination of several MHC-I and at least one MHC-II binding epitopes in a single peptide provided the highest listeriosis protection.

**Conclusion:** DC-GAPDH<sub>1-22</sub> and DC-GAPDH<sub>1-15</sub> vaccines might protect against human listeriosis in high susceptible immune-compromise patients.

**Keywords:** Listeriosis; Dendritic cells; Vaccines; Listeriolysin O; Glyceraldehyde-3-phosphate-dehydrogenase

### Abbreviations

CFU: Colony Forming Units; DC: Dendritic Cells; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; i.g: intragastric; i.p: intra-peritoneal; i.v: intravenous; LLO: Listeriolysin O; LM: *Listeria monocytogenes*; MHC: Major Histocompatibility Complex

### Introduction

Pregnant women, neonates and the elderly are the individuals suffering human listeriosis but also chronic autoimmune, hepatic transplanted and oncologic patients become infected. While no vaccine is available for human listeriosis, this pathogen has increased 5-fold its annual incidence in our country health institutions [1-3]

and, therefore, there is an urgency to develop a safe and wide vaccine that covers the diversity of all human groups at risk of listeriosis. The reasons for this increase in listeriosis referred to the aging of the population and to the significant increase on the half-life of oncological and transplanted patients with the new biological therapies. In this regard, the recent availability of *Listeria*-based therapies for certain tumours using attenuated strains of *Listeria* [4], also predicted the convenience to offer these patients at high risk of listeriosis with alternative immune-therapies to develop immune protection before applying the tumour therapies. Low doses of this pathogen are sufficient to cause severe listeriosis complications in pregnant women and newborns [3], but also to confer significant protection in healthy individuals [5]. However, the *Listeria*-based therapies for tumour patients used a bacterial burden 500-fold higher than the classical doses used for listeriosis vaccination, requiring longer and aggressive antibiotic treatments to eliminate the bacteria.

To add that tumour therapies with attenuated strains lacking listeriolysin O (LLO) require other *Listeria* antigens for vaccination purposes.

In the search for a listeriosis vaccine for individuals at high risk of listeriosis that might consider antigens different than LLO and enhance the clinical outcomes, dendritic cells (DC) vaccines loaded with glyceraldehyde-3-phosphate dehydrogenase peptides are interesting approaches [5]. The ability of DC-vaccines to induce T cells against intracellular infections such as HIV, *Streptococcus pneumoniae*, *Chlamydia trachomatis* or *Mycobacterium* [6-10] and their potency to confer protection with a single dose of vaccine [5,11] are two interesting features for proposing a DC-vaccine for listeriosis.

The diversity of the potential recipients of a listeriosis vaccine might be classified as groups at low and high risk of listeriosis. The group at low risk of listeriosis might correspond to healthy individuals, while the group at high risk correspond to immune-compromise patients. These two groups justify the use of inbred mouse strains with different susceptibility to this pathogen [12]. A low susceptibility is regulated by the Hc locus on chromosome 2. The C57BL/6 mouse strain contain the resistant allele at the Hc locus and are significantly less susceptible to intravenous, i.v., intragastric, i.g. or intraperitoneal, i.p., challenge with *Listeria* than the more susceptible mouse strain Balb/c [13]. We hypothesized that C57BL/6 mice might mimic the group at low risk of listeriosis, while Balb/c mice, the group at high risk of listeriosis.

In this study, we addressed the potential of different DC-GAPDH vaccines for listeriosis compare to DC-LLO vaccines in mouse strains with varied susceptibility to *Listeria* infection as a valid approach to evaluate candidates for human trials, characterized for wide diversity. We also examine different parameters as indicators of effective vaccines such as epitopes binding and structural analysis to MHC-I and MHC-II molecules, immune populations, frequencies of specific CD4<sup>+</sup> or CD8<sup>+</sup> T-cells and cytokine production.

The significance of DC-GAPDH vaccines implies the use of GAPDH peptides in future conventional, cell-free vaccines. This is especially important since human listeriosis is not very well characterized and antigens other than LLO that induced cellular immunity have not been reported [14].

## Methods

### Cells

DC were obtained from femur bone-marrow of 8-12 week-old female Balb/c, with H-2Kb MHC class I and IAd class II genotypes, or C57BL/6 mice with H-2Kb MHC class I and IAb class II genotypes [15]. DC were differentiated with GM-CSF, and CD11c<sup>+</sup> cells isolated with anti-mouse CD11c-coated magnetic beads and MACS separation columns (Miltenyi Biotech Inc., Auburn, CA) [5,11]. DC showed MHC-II<sup>+</sup>CD11c<sup>+</sup>CD40<sup>+</sup>CD11b<sup>+</sup>CD86<sup>+</sup>F4/80-Gr-1- phenotypes.

### Peptides

We used GAPDH<sub>1-15</sub>, GAPDH<sub>1-22</sub>, LLO<sub>91-99</sub>, LLO<sub>296-304</sub>, LLO<sub>189-201</sub>, LLO<sub>189-200</sub> and LLO<sub>190-201</sub> peptides. Peptides were synthesized at CNB facilities (CSIC, Madrid) followed by HPLC and Mass Spectrometry using a MALDI-TOF Reflex<sup>™</sup> IV mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide purity was >95% after HPLC. LLO<sub>91-99</sub> peptide is an epitope eluted from H-2Kd MHC class I molecules and LLO<sub>296-304</sub> an epitope eluted from

H-2Kb MHC class I molecules. LLO<sub>189-200</sub> peptide was an epitope eluted from IAd MHC class II molecules and LLO<sub>190-201</sub> epitopes from IAb MHC class II molecules [12,15-17] LLO<sub>189-201</sub> is a peptide able to bind to IAb and IAd MHC class II molecules [5].

### Bacteria

*Listeria monocytogenes* 10403S strain (LM<sup>WT</sup>) was obtained from D.A. Portnoy (University of California, Berkeley, CA, USA).

### DC vaccines

Cultured DC were ex vivo loaded with 50 µg/ml of GAPDH<sub>1-15</sub>, GAPDH<sub>1-22</sub>, LLO<sub>91-99</sub>, LLO<sub>296-304</sub>, LLO<sub>189-200</sub>, LLO<sub>190-201</sub> or LLO<sub>189-201</sub> peptides for 24 h to prepare the different DC-vaccines of the study and analysed for cell surface markers by FACS to assure quality and phenotypes.

### T-cell responses elicited by DC-LLO or DC-GAPDH vaccines

For Cytotoxic T Lymphocyte (CTL) analysis, mice were inoculated in the hind footpads of Balb/c and C57BL/6 mice with DC prepared vaccines, empty DC (DC-CONT), DC-LLO<sub>91-99</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-201</sub>, DC-LLO<sub>296-304</sub>, DC-GAPDH<sub>1-15</sub>, DC-GAPDH<sub>1-22</sub> or DC-LM lysate (106 cells/footpad). Homogenates of popliteal lymph nodes were stimulated in vitro with 50 µg/ml of LLO<sub>91-99</sub> or LLO<sub>296-304</sub> peptides in DC-LLO inoculated vaccines and with GAPDH<sub>1-22</sub> peptide in DC-GAPDH inoculated vaccines. For Delayed Type Hypersensitivity (DTH) analysis, Balb/c and C57BL/6 mice immunised i.p with LM<sup>WT</sup> (5×10<sup>3</sup> CFU) were inoculated 7 days later in left hind footpads with DC vaccines. Non-inoculated right hind footpads served as negative controls. The measurements of footpad thickness with a calliper quantified the DTH response. DTH results are expressed in millimetres ± SD of three different experiments.

### Vaccinations

Seven days after intraperitoneal (i.p) immunization of Balb/c and C57BL/6 mice with the different DC-vaccines (1×10<sup>6</sup> cells/mice) or left non-vaccinated (NV) (n=5), all mice were next challenged i.p with 10<sup>3</sup> and 10<sup>4</sup> CFU of LM<sup>WT</sup>/mice, respectively, and five days later all mice were bled before termination [18,19]. Sera were stored at -80°C to measure cytokines by FACS analysis. At termination spleens and livers were photographed, homogenized and CFUs counted in blood agar plates.

### Frequencies of CD8<sup>+</sup>-LLO or GAPDH specific T cells

To confirm the frequency of LLO<sub>91-99</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub> or GAPDH<sub>1-22</sub> specific CD8<sup>+</sup> T cells producing IFN-γ, we used recombinant soluble dimeric mouse H-2Kb:Ig (for C57BL/6 mice) or H-2Ld:Ig (for Balb/c mice) fusion proteins following the manufacturer's instructions (DimerX I; BD Bioscience) and as previously described [5,20].

### Statistical analysis

For statistical analysis, Student's t test was applied. ANOVA analysis was applied to cytokine measurements. P ≤ 0.05 was considered significant. GraphPad software was used for generation of graphs.

## Ethics statement

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the Spanish Ministry of Science, Research and Innovation. The Committee on the Ethics of Animal Experiments of the University of Cantabria approved the protocol (Permit Number: 2012/06) that follows the Spanish legislation (RD 1201/2005). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts were made to minimize suffering.

## Results

### Selection of LLO and GAPDH peptides for DC vaccines

First, we examined the effect of LLO and GAPDH peptides on DC toxicity and activation [6,11]. Toxicity was examined with Trypan-blue staining and annexin-V fluorescence after DC loading for 24 hours with 50 µg/ml of LLO<sub>91-99</sub>, LLO<sub>189-201</sub>, LLO<sub>189-200</sub>, LLO<sub>190-201</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub> or GAPDH<sub>1-22</sub> peptides. As it is shown in Table 1, none of the peptides tested affected DC viability (left column in Table 1) or induce apoptosis (medium and right columns in Table 1 and Figure S1 in Supplemental file). Therefore, we consider that 50 µg/ml of each peptide presented no toxicity and was adequate for preparation of DC vaccines.

Reagent	DC viability <sup>a</sup>	Early apoptosis <sup>b</sup>	Late apoptosis <sup>c</sup>
NI	1.59 × 10 <sup>6</sup> ± 142	35 ± 0.01	10 ± 0.05
LLO91-99 (µg/ml)			
500	1.49 × 10 <sup>6</sup> ± 132	36 ± 0.02	11 ± 0.02
50	1.59 × 10 <sup>6</sup> ± 120	37 ± 0.03	12 ± 0.08
5	1.58 × 10 <sup>6</sup> ± 113	35 ± 0.02	10 ± 0.09
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.03
LLO189-201 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	36 ± 0.02	10 ± 0.03
50	1.57 × 10 <sup>6</sup> ± 120	37 ± 0.03	12 ± 0.07
5	1.58 × 10 <sup>6</sup> ± 113	35 ± 0.02	12 ± 0.08
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.01
LLO190-201 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	36 ± 0.02	11 ± 0.01
50	1.56 × 10 <sup>6</sup> ± 120	35 ± 0.03	10 ± 0.05
5	1.58 × 10 <sup>6</sup> ± 113	37 ± 0.02	12 ± 0.05
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.07
LLO190-201 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	36 ± 0.02	11 ± 0.10
50	1.58 × 10 <sup>6</sup> ± 120	37 ± 0.03	12 ± 0.15
5	1.58 × 10 <sup>6</sup> ± 113	35 ± 0.02	10 ± 0.05
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.07
LLO296-304 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	36 ± 0.02	10 ± 0.10
50	1.57 × 10 <sup>6</sup> ± 120	37 ± 0.03	12 ± 0.15

5	1.58 × 10 <sup>6</sup> ± 113	35 ± 0.02	11 ± 0.05
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.07
GAPDH1-15 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	35 ± 0.02	10 ± 0.01
50	1.58 × 10 <sup>6</sup> ± 120	36 ± 0.03	11 ± 0.05
5	1.58 × 10 <sup>6</sup> ± 113	35 ± 0.02	10 ± 0.02
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	12 ± 0.03
GAPDH1-22 (µg/ml)			
500	1.59 × 10 <sup>6</sup> ± 132	36 ± 0.02	11 ± 0.02
50	1.58 × 10 <sup>6</sup> ± 120	37 ± 0.03	12 ± 0.01
5	1.58 × 10 <sup>6</sup> ± 113	37 ± 0.02	12 ± 0.03
0.5	1.59 × 10 <sup>6</sup> ± 128	36 ± 0.01	11 ± 0.01

**Table 1:** Lack of toxicity of different LLO and GAPDH peptides. <sup>a</sup>In vitro toxicity assayed into DC incubated with peptides for 24 hours and next stained with Trypan blue. Results are expressed as the number of cells ± SD and experiments were performed in triplicates (P<0.05). <sup>b</sup>DC were incubated with peptides for 24 hours and next incubated with 7-ADD and annexin V to analyse fluorescence by FACS. Results are expressed as the percentages of late apoptotic cells or necrotic death, (Q2 in Figure S1) (P<0.05). <sup>c</sup>Percentages of early apoptotic cells or programmed cell death (Q4 in Figure S1) (mean ± SD) (p<0.05).

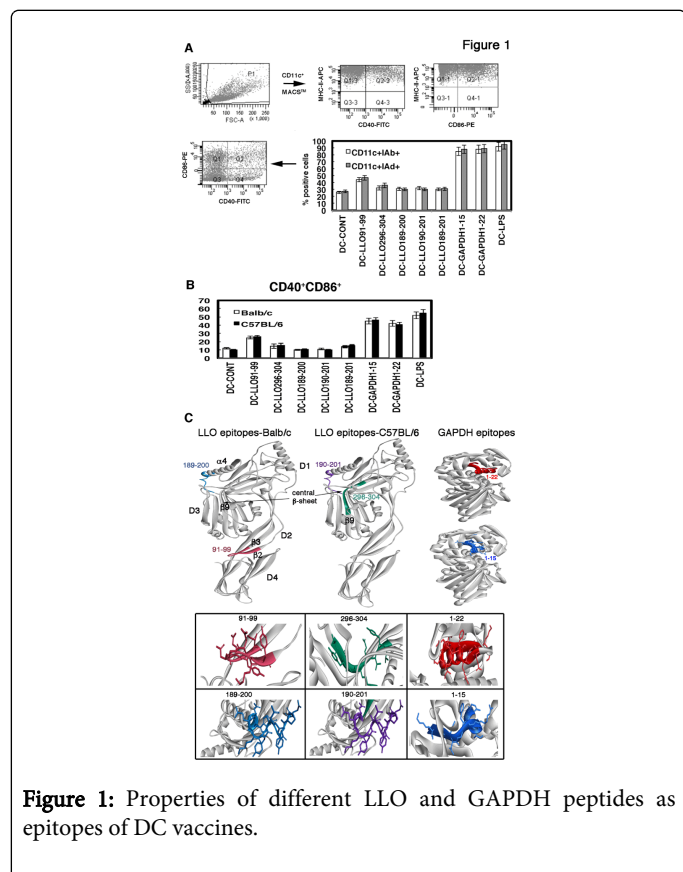
Activated DC shows a characteristic CD11c<sup>+</sup>MHC-II<sup>+</sup>CD40<sup>+</sup>CD86<sup>+</sup> phenotype that predicted vaccine efficiency [6,11,19]. To examine DC activation by LLO or GAPDH peptides at day 5 after GM-CSF differentiation, DC were purified as CD11c<sup>+</sup> cells using MACS magnetic beads (Miltenyi) and loaded ex vivo for 24 hours with 50 µg/ml of LLO<sub>91-99</sub>, LLO<sub>189-201</sub>, LLO<sub>189-200</sub>, LLO<sub>190-201</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub>, GAPDH<sub>1-22</sub>, LPS (10 ng/ml) as a classical DC activator [6,11] or left untreated (DC-CONT). Next, the frequency of CD11c<sup>+</sup>MHC-II<sup>+</sup> cells positive for CD40<sup>+</sup> or CD86<sup>+</sup> DC was assessed as markers of mature and activated DC (Figure 1A). LPS induced the highest frequency of MHC-II<sup>high</sup> DC followed by GAPDH<sub>1-22</sub> and GAPDH<sub>1-15</sub> peptides. LLO<sub>91-99</sub> peptide showed an intermediate frequency of MHC-II<sup>high</sup> DC. LLO<sub>296-304</sub> and LLO<sub>189-201</sub> peptides showed significant MHC-II<sup>high</sup> DC frequency, while LLO<sub>189-200</sub> and LLO<sub>190-201</sub> peptides showed no frequency (Figure 1A). A similar picture was seen for CD11c<sup>+</sup>MHC-II<sup>+</sup>CD40<sup>+</sup> or CD11c<sup>+</sup>MHC-II<sup>+</sup>CD86<sup>+</sup> DC. Differences were even more marked when we analysed the frequency of CD40<sup>+</sup>CD86<sup>+</sup> double positive DC (Figure 1B).

Variability in the binding properties of LLO and GAPDH epitopes to MHC-I or MHC-II molecules can explain differences on DC activation. Moreover, epitope-binding predictions to either MHC-I or MHC-II molecules could prognosticate the best antigen epitopes of pathogens for vaccine formulations. Therefore, we compared the binding abilities of LLO and GAPDH peptides to MHC class I or class II molecules using a binding prediction approach, the IEDB analysis resource Consensus tool that combines predictions from ANN, SMM and Comblib [20-23]. IEDB analysis predicted that good binders showed percentile ranks <10 and weak binders percentile ranks <100. We observed that predictions of peptide binding to MHC-I and MHC-II molecules correlated with DC activation, being MHC-I binders predominant. In this regard, GAPDH<sub>1-15</sub> and GAPDH<sub>1-22</sub> peptides were strong binders to H-2Kb and H-2Ld MHC-I and weak binders to

IAb and IAd MHC-II (Table 2) and presented the highest DC-activation (Figure 1B). Next, LLO<sub>91-99</sub> peptide, a strong binder to H-2Kd and a weak binder to H-2Kb MHC-I showed intermediate DC activation. LLO<sub>296-304</sub> peptide, a strong binder to H-2Kb and a weak binder to H-2Kd and H-2Ld MHC-I molecules showed significant DC activation. Interestingly, LLO<sub>189-201</sub> peptides were weak binders to IAb and IAd and showed DC activation comparable to LLO<sub>296-304</sub> peptide (Figure 1B). However, LLO<sub>189-200</sub> and LLO<sub>190-201</sub> were both weak binders to IAb and showed neither binding to IAd nor DC activation, contrary to previous predictions [12].

sequence to LLO<sub>91-99</sub> peptide and MHC-I epitope. On the centre, the purple sequence corresponds to LLO<sub>190-201</sub> peptide and MHC-II epitope and the green sequence to LLO<sub>296-304</sub> and MHC-I epitope. Other structural features of LLO are also shown, as D1, D2, D3 and D4 domains, the central  $\beta$ -sheet and the  $\beta$ -strands or  $\alpha$ -helices where serveral epitopes are localized. On the right, the GAPDH predicted 3D structure showing in red the GAPDH<sub>1-22</sub> peptide and in blue the GAPDH<sub>1-15</sub> peptide. Lower images correspond to enlargement of LLO and GAPDH epitopes for a more detailed structural analysis.

Next, we localized LLO and GAPDH binding epitopes to MHC-I and MHC-II molecules of Balb/c and C57BL/6 mice in the predicted 3D structures obtained from the recently reported LLO crystal structure [24]. We applied the LLO epitopes to the bioinformatics modelling of the PBDI: 4CDB (upper images in Figure 1C) that shows 4 domains (D1, D2, D3 and D4). D1 domain contained a  $\alpha/\beta$  fold with five stranded  $\beta$ -sheets surrounding by six  $\alpha$ -helices. D2 domain shows four  $\beta$ -strands forming an antiparallel  $\beta$ -sheet to connect D1 and D4. D3 domain is formed of five-stranded antiparallel  $\beta$ -sheet surrounded by six  $\alpha$ -helices. D4 is the domain binding to the phagosomal membranes showing a compact  $\beta$ -sandwich with two four-stranded  $\beta$ -sheets [24]. MHC-II epitopes of both mice strains were localized in same  $\alpha$ -helix in D1 domain (blue and purple epitopes in Figure 1C). We observed that MHC-I epitope of Balb/c mice localizes in a tip or loop form by  $\beta$ 2 and  $\beta$ 3-strands in D2 domain (pink tip-loop epitope in Figure 1C). MHC-I epitope of C57BL/6 mice localizes in  $\beta$ 9-strand in the central  $\beta$ -sheet of D3 in a loose structure connecting with D1 (green loop epitope of Figure 1C). We used the Automated Comparative Protein Modelling Server SWISS-MODEL available at: (<http://www.expasy-ch/swissmod/SWISS-MODEL.html>) to produce the theoretical 3D predictive model for Listeria monocytogenes GAPDH. GAPDH<sub>1-22</sub> peptide contained six MHC-I and seven MHC-II binding sequences in Balb/c and C57BL/6 mice. While GAPDH<sub>1-15</sub> peptide contained only three MHC-I binding sequences and two MHC-II binding sequences (Table 2). GAPDH<sub>1-15</sub> and GAPDH<sub>1-22</sub> binding sequences to MHC-I molecules cover amino acids 1 to 15 and resembles the loop structure composed of  $\beta$ 2 and  $\beta$ 3-strands in D2 of LLO. Similar to LLO, GAPDH<sub>1-15</sub> and GAPDH<sub>1-22</sub> binding sequences to MHC-II molecules localized also in a large  $\alpha$ -helix structure containing at least two putative epitopes, amino acids 4 to 15 and amino acids 8 to 22 (enlarged images in Figure 1C and Table 2). These structural predictions suggested that epitopes causing the highest DC activation were in loop structures combined to  $\alpha$ -helices as the cases of GAPDH<sub>1-15</sub>, GAPDH<sub>1-22</sub>. Next, it follows the LLO<sub>91-99</sub> peptides containing a complete loop structure of  $\beta$ -strands that caused intermediate DC activation; while  $\beta$ -strands in loose structures as in LLO<sub>296-304</sub> peptide only showed low DC activation. Finally,  $\alpha$ -helices exclusively caused no DC activation as LLO<sub>189-200</sub>, LLO<sub>190-201</sub> or LLO<sub>189-201</sub> peptides. Therefore, combination of binding predictions to MHC-I or MHC-II molecules with structural localizations seems to correlate with DC activation.



A, Analysis of DC activation including the strategy applied to DC to select mature and activated CD11c<sup>+</sup>MHC-II<sup>+</sup>CD40<sup>+</sup>CD86<sup>+</sup> cells. DC from Balb/c or C57BL/6 mice were differentiated in vitro with 30 ng/ml of GM-CSF for 5 days, detached and positive selected using anti-mouse CD11c-coated magnetic beads and MACSTM separation columns. CD11c<sup>+</sup> DC were loaded ex vivo with different peptides: LLO<sub>91-99</sub>, LLO<sub>189-200</sub>, LLO<sub>190-201</sub>, LLO<sub>189-201</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub> or GAPDH<sub>1-22</sub> peptides (50  $\mu$ g/ml) for 24 hours or left unloaded (DC-CONT). Cells were washed and samples acquired using a FACSCanto flow cytometer and percentages of double or triple positive cells for each type of marker combination are shown. Results are expressed as the mean  $\pm$  SD of triplicate samples ( $P < 0.05$ ). B, CD11c<sup>+</sup>MHC-II<sup>+</sup> DC from C57BL/6 (black bars) or Balb/c mice (white bars) were stained for CD40-FITC or CD86-PE to select triple positive cells, MHC-II<sup>+</sup>CD40<sup>+</sup>CD86<sup>+</sup> cells. Results are expressed as the mean  $\pm$  SD of triplicate samples ( $P < 0.05$ ). C, 3D structure of LLO based in the crystal structure recently reported showing the MHC-I and MHC-II epitopes in Balb/c and C57BL/6 mice. On the left, the blue sequence corresponds to LLO<sub>189-200</sub> peptide and MHC-II epitope and the pink

MHC-I binding	H-2 allele	Percentile rank	Binding ability	Sequence
LLO <sub>91-99</sub>	H-2-Kb	67.5	UB	Complete
	H-2-Kd	0.7	GB	Complete
	H-2-Ld	38.3	UB	Complete
LLO <sub>296-304</sub>	H-2Kb	0.2	GB	Complete
	H-2Kd	32	UB	Complete
	H-2Ld	12.5	UB	Complete

GAPDH <sub>1-22</sub>	H-2-Kb	1.6	GB	5-15*
	H-2-Kb	3.3	WB	5-13*
	H-2-Kd	4.7	WB	8-16
	H-2Kd	5.1	WB	2-13
	H-2-Ld	2.3	IB	8-18
	H-2Ld	3.4	WB	1-10
GAPDH <sub>1-15</sub>	H-2-Kb	3.3	WB	5-13*
	H-2Kd	5.1	WB	2-13
	H-2Ld	3.4	WB	1-10
MHC-II binding	H-2 allele	Binding threshold	Binding ability	Sequence
LLO <sub>190-201</sub>	H-2-IAb	75.1*	WB	Complete
	H-2-IAd	>15000	UB	
LLO <sub>189-200</sub>	H-2-IAb	85	WB	Complete
	H-2-IAd	11364	UB	
LLO <sub>189-201</sub>	H-2-IAb	51.3	WB	Complete
	H-2-IAd	5326	WB-UB	Complete
GAPDH <sub>1-22</sub>	H-2-IAb	69.15*	WB	4-18*
	H-2-IAb	70.23	WB	5-19
	H-2-IAb	70.91	WB	2-16
	H-2-IAb	71.11	WB	3-17
	H-2-IAb	74.95*	WB	8-22*
	H-2-IAb	75.61	WB-UB	7-21
	H-2-IAd	3840	WB-UB	2-16
GAPDH <sub>1-15</sub>	H-2-IAb	75.72	WB	Complete
	H-2-IAd	3240	WB-UB	Complete

**Table 2:** H-2 binding force predictions for LLO and GAPDH epitopes. Predictions of binding of peptides to MHC molecules performed with the IEDB analysis source Consensus tool. The lower the percentile ranks obtained, the better the binders. GAPDH<sub>1-22</sub> peptide was compared to the predictions of LLO<sub>91-99</sub> binding to MHC-I molecules and LLO<sub>189-201</sub> binding to MHC-II molecules. \*Comparative peptide sequence with similar binding percentiles as LLO peptides. Binding abilities: GB, good binder; WB, weak binder; UB, unable to bind. H-2 binders percentile ranks, <1.0: GB, <2.5: IB, <5: WB and >10:UB (correspond to IC values of <50 nM: high affinity, <500: intermediate affinity, <5000: low affinity). MHC-II binding thresholds, <50: GB, <500: WB, >5000: UB.

T-cell responses induced by DC-vaccines loaded with LLO or GAPDH peptides. Next, we checked whether DC vaccines loaded with LLO or GAPDH peptides elicited T cell responses. CTL responses were evaluated in susceptible Balb/c and resistant C57BL/6 mice after immunization of the hind footpads with DC-LLO<sub>91-99</sub>, DC-LLO<sub>296-304</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-201</sub>, DC-GAPDH<sub>1-15</sub>, DC-GAPDH<sub>1-22</sub> peptides or DC-LMWT lysate. Seven days post-immunization, popliteal lymph nodes were isolated and stimulated *in vitro* for re-call immune responses with 50 µg/ml of LLO<sub>91-99</sub> plus LLO<sub>296-304</sub> peptides for DC-LLO vaccines and with GAPDH<sub>1-15</sub> plus GAPDH<sub>1-22</sub> peptides for DC-GAPDH vaccines and T-cell responses measured by thymidine proliferation assays. DC-control, DC-LMWT lysate vaccines and Non-Treated mice (NT) were stimulated *in vitro* with 50 µg/ml of LLO<sub>91-99</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub> and GAPDH<sub>1-22</sub>

peptides together. DC-GAPDH<sub>1-22</sub> and DC-GAPDH<sub>1-15</sub> vaccines induced a high level of proliferation equivalent to DC-LM<sup>WT</sup> lysate vaccines in both mice strains. DC-LLO<sub>91-99</sub> and DC-LLO<sub>296-304</sub> vaccines induced the next best T cell proliferations. Mice immunized with DC-LLO<sub>189-201</sub>, DC-LLO<sub>189-200</sub> and DC-LLO<sub>190-201</sub> vaccines exhibited only basal levels of T cell proliferations to re-call responses with LLO<sub>91-99</sub> or LLO<sub>296-304</sub>, as expected (Figure 2A). These data suggested that cytotoxic T cell responses generated by DC-vaccines were peptide specific.

Next, we examined the DTH response in LMWT-immunised mice to evaluate whether DC loaded *ex vivo* with different LLO or GAPDH peptides also induced CD4<sup>+</sup> T-cell responses (Methods for procedures) since DTH depends mainly on CD4<sup>+</sup> T cells [25]. We immunized mice with DC-LLO<sub>91-99</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-201</sub>, DC-LLO<sub>296-304</sub>, DC-GAPDH<sub>1-15</sub> or DC-GAPDH<sub>1-12</sub> for 48 hours. DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> generated higher DTH responses than DC-LLO<sub>189-201</sub> in both mice strains (Figure 2B). DC-LLO<sub>189-200</sub> was the next best DTH response in Balb/c mice (white bars in Figure 2B) and only basal responses in C57BL/6 mice (black bars in Figure 2B). DTH responses generated by DC-LLO<sub>190-201</sub> vaccines were significant in C57BL/6 mice and basal levels in Balb/c mice. These data indicated that CD4 responses elicited by DC-vaccines were significant and specific for each peptide.

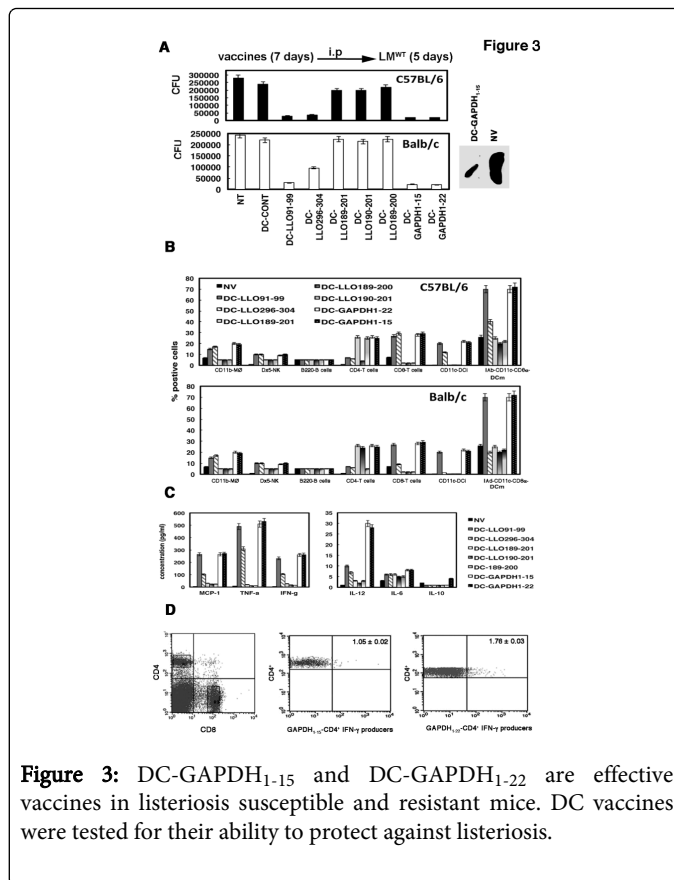
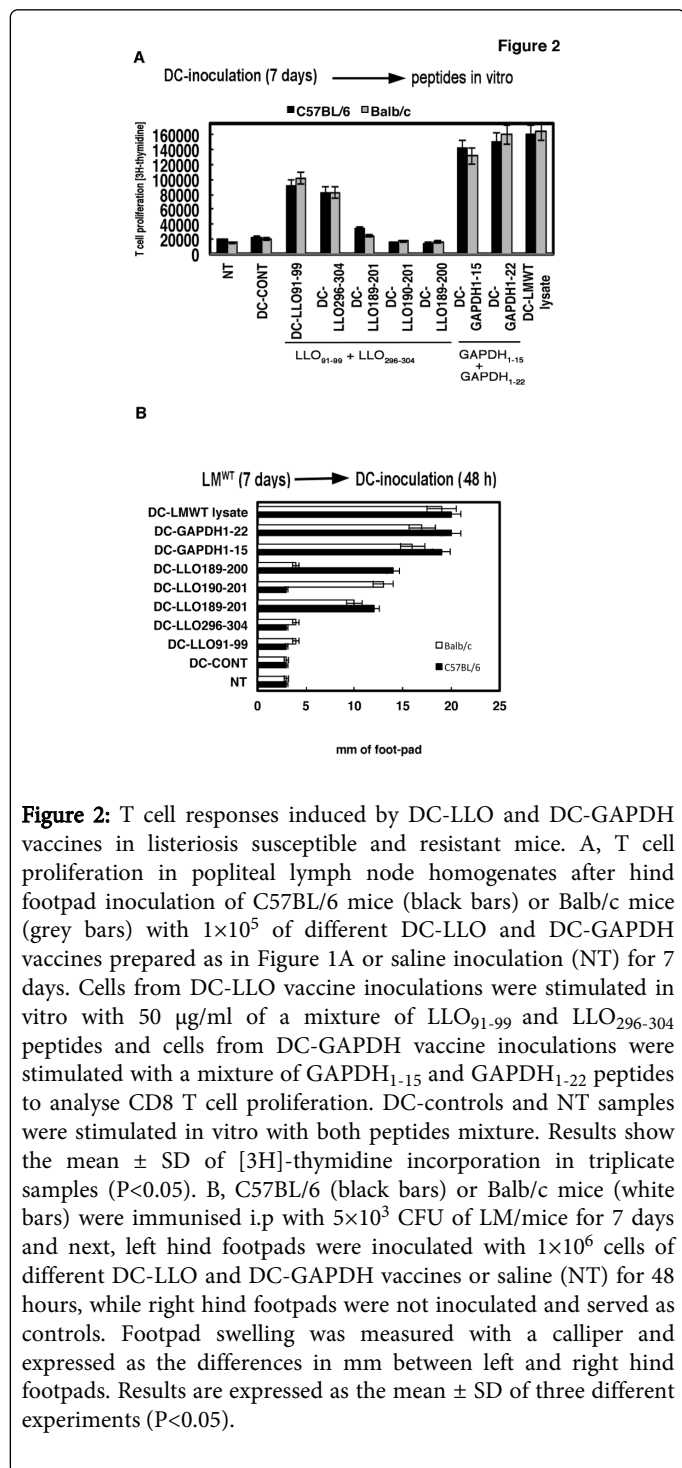
DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> are effective vaccines in mice with different susceptibilities to Listeria infection. We next compared protection against listeriosis of different DC-LLO and DC-GAPDH vaccines in Balb/c and C57BL/6 with different susceptibilities to Listeria infection [13,26]. Mice were immunized *i.p* with a single dose of vaccines and 7 days later administered a LM<sup>WT</sup> challenge. Five days post-challenge, all mice were bled, sacrificed and livers and spleens recovered to count CFU and analyse cell populations in both mice strains. DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> provided the greatest protection in Balb/c and C57BL/6 mice (~97 and 99%, respectively) (Figure 3A).

DC-LLO<sub>91-99</sub> vaccines conferred also significant protection in Balb/c and C57BL/6 mice (90 and 93%, respectively). However, DC-LLO<sub>296-304</sub> vaccines only conferred significant protection in C57BL/6 mice (~90%) (Figure 3A) and intermediate levels of protection in Balb/c mice (~60%) (white bars in Figure 3A). DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub>, DC-LLO<sub>189-201</sub> vaccines conferred no significant protection either in Balb/c or C57BL/6 mice (2 and 5%, respectively). Vaccination efficiency was associated with normal spleen size (images of DC-GAPDH<sub>1-15</sub> vaccines in Figure 3A) compared with enlarged spleens (images of NV mice in Figure 3A) and granulomatous livers of non-vaccinated mice (NV) (data not shown).

To analyse the immune responses elicited by DC-vaccines, we examined cell populations of spleens of vaccinated and non-vaccinated mice. Animals vaccinated with DC-GAPDH<sub>1-15</sub>, DC-GAPDH<sub>1-22</sub>, DC-LLO<sub>91-99</sub> or DC-LLO<sub>296-304</sub> showed an increased frequency of CD11c<sup>+</sup>IAb<sup>+</sup>CD40<sup>+</sup>CD86<sup>+</sup>CD8α<sup>+</sup> mature DC (68% of positive cells), CD8<sup>+</sup> T cells (~30% of positive cells), MØ (20% of positive cells) and NK (11% of positive cells) in their spleens post-challenge. However, only DC-LLO<sub>189-201</sub>, DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> immunised mice showed an increased frequency of CD4<sup>+</sup> T cells post-challenge (Figure 3B).

Vaccination with DC-GAPDH<sub>1-15</sub>, DC-GAPDH<sub>1-22</sub>, DC-LLO<sub>91-99</sub>, DC-LLO<sub>296-304</sub> correlated with high levels of MCP-1, TNF-α and IFN-γ production but only DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines

also produced high IL-12 levels post-challenge in both mouse strains (Figure 3C shows results of C57BL/6 mice) [5,11].



**Figure 3:** DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> are effective vaccines in listeriosis susceptible and resistant mice. DC vaccines were tested for their ability to protect against listeriosis. **A**, C57BL/6 (upper plot) or Balb/c (lower plot) mice were vaccinated i.p for 7 days with different DC vaccine vectors ( $1 \times 10^6$  cells) (DC-CONT, DC-GAPDH<sub>1-15</sub>, DC-GAPDH<sub>1-22</sub>, DC-LLO<sub>91-99</sub>, DC-LLO<sub>296-304</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>189-201</sub> or DC-LLO<sub>190-201</sub>) or not vaccinated (NV) ( $n=5$  mice/group) and challenged i.p with  $10^3$  CFU LM<sup>WT</sup> (for Balb/c mice) or  $10^4$  CFU LM<sup>WT</sup> (for C57BL/6 mice) for 5 days. Results of spleens homogenates are expressed as CFU (mean  $\pm$  SD) and obtained from triplicate samples of three independent experiments ( $P < 0.01$ ). Images correspond to spleens of non-vaccinated mice (NV) showing splenomegaly versus DC-GAPDH<sub>1-15</sub> vaccinated Balb/c mice that had normal sized spleens, similar results were obtained with C57BL/6 mice and vaccinated with DC-GAPDH<sub>1-22</sub> or DC-GAPDH<sub>1-15</sub> vaccines. **B**, M $\phi$ s (CD11b<sup>+</sup>F4/80<sup>+</sup>), B cells (CD19<sup>+</sup>), NK (CD3<sup>+</sup>CD49b<sup>+</sup>), CD4 T cells (CD3<sup>+</sup>CD4<sup>+</sup>), CD8 T cells (CD3<sup>+</sup>CD8<sup>+</sup>), DCi (CD11c<sup>+</sup>MHC-II<sup>+</sup>CD40<sup>-</sup>CD86<sup>-</sup>), DCm (CD11c<sup>+</sup>MHC-II<sup>+</sup>CD40<sup>+</sup>CD86<sup>+</sup>CD8 $\alpha$ <sup>+</sup>) were quantified in spleen homogenates of C57BL/6 mice (upper plot) or Balb/c mice (lower plot) by FACS. Results expressed the mean  $\pm$  SD of the percentage of positive cells ( $P < 0.05$ ). **C**, Levels of pro-inflammatory cytokines (MCP-1, TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-10, IL-12) were analysed in mouse sera by CBA. Results expressed as cytokine concentration (pg/ml of mean  $\pm$  SD,  $P < 0.05$ ) and correspond to Balb/c vaccinations, results were similar after vaccination of C57BL/6 mice (data not shown). **D**, C57BL/6 or Balb/c mice were vaccinated with same vaccines as in A and challenged with LM<sup>WT</sup> (103 bc/mice for Balb/c and 104 bc/mice for C57BL/6) for 5 days. Spleen homogenates were stimulated 5 h with GAPDH<sub>1-15</sub>, GAPDH<sub>1-22</sub>, LLO<sub>189-200</sub>, LLO<sub>190-201</sub> or LLO<sub>189-201</sub> peptides then intracellular cytokine staining performed. Histograms show the frequency of GAPDH<sub>1-15</sub> and GAPDH<sub>1-22</sub>

specific CD4<sup>+</sup> T cells and IFN- $\gamma$  producers in Balb/c spleen homogenates. Frequencies in C57BL/6 mice were similar ( $1.02 \pm 0.01$  for GAPDH<sub>1-15</sub> peptide and  $1.70 \pm 0.02$  for GAPDH<sub>1-22</sub> peptide). Experiments were performed in triplicate and results are expressed as mean  $\pm$  SD ( $p < 0.05$ ).

We also analysed whether the high efficiency of DC-GAPDH vaccines correlated with the frequencies of IFN- $\gamma$  producing CD8<sup>+</sup> using dimers (Table 3) or CD4<sup>+</sup> T cells using intracellular cytokines staining (Figure 3D). DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines induced high frequencies of IFN- $\gamma$  producing CD8<sup>+</sup> T cells in C57BL/6 and Balb/c mice (4.0 and 4.2%, respectively in Table 3). DC-DC-LLO<sub>91-99</sub> vaccines show a 3.0% frequency of IFN- $\gamma$  producing CD8<sup>+</sup> T cells in C57BL/6 mice (H2-Kb:Ig-peptide dimers data in Table 3) and a 2.16% frequency in Balb/c mice (H2-Ld:Ig-peptide dimers data in Table 3). DC-LLO<sub>296-304</sub> vaccines induced only intermediate 0.87% frequencies in C57BL/6 mice and none in Balb/c mice. These results indicated that efficiency of DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines correlated with high frequencies of IFN- $\gamma$  producing CD8<sup>+</sup> T-cells in mice with high (Balb/c) or low (C57BL/6) susceptibility to listeriosis.

Vaccination type	% Gated dimer-CD8/ peptide (H-2Kb: Ig dimer)	% Gated dimer-CD8/ peptide (H-2Ld: Ig dimer)
DC-LLO91-99	2.16 $\pm$ 0.01	3.02 $\pm$ 0.02
DC-LLO296-304	0.87 $\pm$ 0.01	0.03 $\pm$ 0.01
DC-GAPDH1-15	4.06 $\pm$ 0.01	4.02 $\pm$ 0.01
DC-GAPDH1-22	4.26 $\pm$ 0.02	4.22 $\pm$ 0.03

**Table 3:** Frequencies of LLO and GAPDH peptides-specific CD8<sup>+</sup> T cells induced by DC vaccines. Splenocytes from vaccinated mice were incubated with recombinant dimeric H-2Kb: Ig or H-2Ld: Ig fusion proteins loaded with LLO<sub>91-99</sub>, LLO<sub>296-304</sub>, GAPDH<sub>1-15</sub> or GAPDH<sub>1-22</sub> peptides. The staining cocktail contained the dimeric fusion protein loaded with the peptides, CD8<sup>+</sup> and IFN- antibodies. CD8<sup>+</sup> cells were gated for anti-IFN- staining (% Gated dimer-CD8) to calculate the frequencies of CD8<sup>+</sup>-LLO<sub>91-99</sub>, CD8<sup>+</sup> LLO<sub>296-304</sub>, CD8<sup>+</sup>-GAPDH<sub>1-15</sub> or CD8<sup>+</sup>-GAPDH<sub>1-22</sub> restricted cells and IFN- producers.

We also analysed the percentages of peptide specific CD4<sup>+</sup> T cells and observed that DC-GAPDH1-15 and DC-GAPDH1-22 vaccines also induced detectable numbers of peptide-specific CD4<sup>+</sup> T cells (1.05 and 1.76% percentages, respectively) (Figure 3D), suggesting that vaccine efficiencies correlate with simultaneous and significant frequencies of IFN- $\gamma$  producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

## Discussion

The objective of this study was to identify the best epitopes from Listeria proteins GAPDH and LLO for an optimal DC vaccine candidate to protect against individuals high susceptible to listeriosis. Human listeriosis is a rare infectious disease with no available vaccine that has increased significantly its annual incidence in Spain from a mean incidence rate of 0.16 cases per 100.000 inhabitants per year to a mean incidence rate of 0.56 in last decade. The causes for the increase in listeriosis incidence associate in part with the aging of the population, an increase in the life expectancy of immune-compromise patients, genetic changes in certain Listeria serotypes responsible for

outbreaks and new biological therapies offered to chronic autoimmune and oncologic patients such as Listeria-based therapies using attenuated strains [1-5]. Listeria-based therapies use a bacterial burden 500-fold higher than the doses used with pathogenic bacteria that might exhaust these patients immune system. Moreover, the high bacterial doses would need longer and inflated antibiotic treatments, promoting undesirable resistances. In this context, a cost-effective measure to reinforce these patients immune system, enhance overall clinical outcomes and prevent unnecessary infections might be vaccination with DC loaded *ex vivo* with peptides. Vaccine formulations with cellular DC are safe alternatives for induction of cellular immunity against different infectious diseases such as HIV, Chlamydia trachomatis, Streptococcus pneumoniae and even tuberculosis [6-10]. Listeriosis recipients of vaccines might classify in two groups, healthy individuals but at risk such as pregnant women, newborns or the elderly and immune-compromised patients. We refer to these groups as listeriosis low and high susceptible individuals and C57BL/6 and Balb/c inbred mouse strains are representative of these groups, respectively. Listeria protection is mainly dependent on CD8<sup>+</sup> T-cell immunity while CD4<sup>+</sup> T-cells played a less relevant but necessary role (Pamer, 2004). Only two Listeria antigens, LLO and GAPDH contained epitopes that inducing CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, conferred significant protection in high and low susceptible mice to Listeria infection, Balb/c and C57BL/6 mice, respectively [5,11,16,18,27,28]. We designed two DC-GAPDH vaccines, DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> based in a previous study showing GAPDH<sub>1-22</sub> peptide binding to MHC-I and MHC-II molecules. We also prepared five DC-LLO vaccines DC-LLO<sub>91-99</sub>, DC-LLO<sub>296-304</sub>, DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub> and DC-LLO<sub>189-201</sub>, based in the epitopes eluted from MHC-I and MHC-II molecules from high Balb/c, and low C57BL/6 susceptible mice to Listeria infection [16]. Only DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines showed a high efficiency of DC loading and activation in both mouse strains. Epitope binding predictions to MHC-I and MHC-II molecules and 3D structural studies explained the higher efficiency of DC-GAPDH vaccines. DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines containing a combination of several MHC-I and MHC-II binding epitopes in a single peptide with a loop structure combined with  $\alpha$ -helices, showed the highest DC activation capacities. As a consequence, these DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines induced high frequencies of peptide-specific IFN- $\gamma$  producing CD8<sup>+</sup> and CD4<sup>+</sup> T-cells that translated into protection against Listeria challenge in low and high susceptible mice. We also observed that the high 98% protection with DC-GAPDH<sub>1-15</sub> and DC-GAPDH<sub>1-22</sub> vaccines associated with increased frequency in the spleens of NK cells and activated CD8 $\alpha$ <sup>+</sup> DC that produced significant levels of IL-12. CD8 $\alpha$ <sup>+</sup> DC have been reported to play a critical role in Listeria protection producing IL-12 and activating IFN- $\gamma$  producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells [29,30].

The most effective DC-LLO vaccine, DC-LLO<sub>91-99</sub> presented a good 90% protection in both mice strains, but with less prominent frequencies of activated CD8 $\alpha$ <sup>+</sup> DC as well as peptide-specific IFN- $\gamma$  producing CD8<sup>+</sup> T cells and no induction of CD4<sup>+</sup> T cells. To add that LLO<sub>91-99</sub> peptide contained only one MHC-I binding epitope and showed only intermediate DC activation abilities. DC-LLO<sub>296-304</sub> vaccines showed high 90% protection values in low susceptible mice but only intermediate levels of protection in high susceptible mice, predicting a lower efficiency in listeriosis patients at high risk such as chronic autoimmune or oncologic patients [1,4]. No other DC-LLO<sub>189-200</sub>, DC-LLO<sub>190-201</sub> or DC-LLO<sub>189-201</sub> vaccines showed DC activation or protection abilities.

GAPDH<sub>1-15</sub> and GADPH<sub>1-22</sub> peptides showed 98% homology with *Streptococcus pneumoniae* or *Streptococcus pyogenes* [27], appearing as interesting epitopes to include in DC vaccines for the group of patients highly susceptible to *Listeria* as immune-compromise patients in treatment for cancer or chronic autoimmune diseases. These patients are also susceptible to other opportunistic infections such as those caused by *Streptococcus* genus, therefore DC-GAPDH vaccines will be a cost-effective immune-therapy preventing several infections of the elderly. GAPDH<sub>1-15</sub> and GADPH<sub>1-22</sub> seemed also appealing epitopes to include in conventional, cell-free vaccines or chimeric constructs with LLO<sub>91-99</sub> epitopes due to their potential to protect low and high susceptible individuals.

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