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

Ricardo Calderon-Gonzalez[1], Elisabet Frande-Cabanes[1], Raquel Tobes[2], Eduardo Pareja[2], Lidia Alaez-Alvarez[1] and Carmen Alvarez-Dominguez[1*]

[1]*Grupo de Genómica, Proteómica y Vacunas, Instituto de Investigación Marqués de Valdecilla (IDIVAL), Santander, Spain*

[2]*Era7 Bioinformatics, Granada, Spain*

[*]**Corresponding author:** Carmen Alvarez-Dominguez, Grupo de Genómica, Proteómica y Vacunas. Instituto de Investigación Marqués de Valdecilla (IDIVAL), Laboratorio 124, Edificio IDIVAL, Avda, Cardenal Herrera Oria, s/n. 39011 Santander, Cantabria, Spain, Tel: 34-942315515/74104; Fax: +34-942-315517; E-mail: calvarez@humv.es

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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_{91-99}, DC-$LLO_{189-200}$, DC-$LLO_{190-201}$, DC-$LLO_{189-201}$, DC-$LLO_{296-304}$, DC-$GAPDH_{1-15}$ or DC-$GAPDH_{1-22}$ 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-$GAPDH_{1-22}$ and DC-$GAPDH_{1-15}$ 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_{91-99} vaccine with a strong binding epitope to MHC-I follows protection in both mice strains. DC-$LLO_{296-304}$ vaccine conferred protection only in resistant mice and DC-$LLO_{190-201}$, DC-$LLO_{189-200}$ and DC-$LLO_{189-201}$ 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- T cells in both mice strains.

Discussion: Maximal protection against listeriosis in susceptible and resistant mice is only achieved with DC-$GAPDH_{1-22}$ and DC-$GAPDH_{1-15}$ 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_{91-99} and DC-$LLO_{296-304}$ vaccines that exclusively activate $CD8^+$ T cells in both mice strains conferred significant listeriosis protection. In this regard, DC-$LLO_{189-201}$, DC-$LLO_{189-200}$ or DC-$LLO_{190-201}$ 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-$GAPDH_{1-22}$ and DC-$GAPDH_{1-15}$ vaccines might protect against human listeriosis in high susceptible immune-compromise patients.

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

Abbreviations

CFU: Colony Forming Units; DC: Dendritic Cells; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; i.g: intragastic; i.p: intraperitoneal; 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 pneumonia, 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 $CD4^+$ or $CD8^+$ 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-marrows of 8-12 week-old female Balb/c, with H-2Kd 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 $CD11c^+$ cells isolated with anti-mouse CD11c-coated magnetic beads and MACS separation columns (Miltenyi Biotech Inc., Auburn, CA) [5,11]. DC showed $MHC\text{-}II^+CD11c^+CD40^+CD11b^+CD86^+$F4/80-Gr-1- phenotypes.

Peptides

We used $GAPDH_{1\text{-}15}$, $GAPDH_{1\text{-}22}$, $LLO_{91\text{-}99}$, $LLO_{296\text{-}304}$, $LLO_{189\text{-}201}$, $LLO_{189\text{-}200}$ and $LLO_{190\text{-}201}$ peptides. Peptides were synthesized at CNB facilities (CSIC, Madrid) followed by HPLC and Mass Spectrometry using a MALDI-TOF Reflex™ IV mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide purity was >95% after HPLC. $LLO9_{1\text{-}99}$ peptide is an epitope eluted from H-2Kd MHC class I molecules and $LLO_{296\text{-}304}$ an epitope eluted from H-2Kb MHC class I molecules. $LLO_{189\text{-}200}$ peptide was an epitope eluted from IAd MHC class II molecules and $LLO_{190\text{-}201}$ epitopes from IAb MHC class II molecules [12,15-17] $LLO_{189\text{-}201}$ is a peptide able to bind to IAb and IAd MHC class II molecules [5].

Bacteria

Listeria monocytogenes 10403S strain (LM^{WT}) 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 $GAPDH_{1\text{-}15}$, $GAPDH_{1\text{-}22}$, $LLO_{91\text{-}99}$, $LLO_{296\text{-}304}$, $LLO_{189\text{-}200}$, $LLO_{190\text{-}201}$ or $LLO_{189\text{-}201}$ 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-$LLO_{91\text{-}99}$, DC-$LLO_{189\text{-}200}$, DC-$LLO_{190\text{-}201}$, DC-$LLO_{189\text{-}201}$, DC-$LLO_{296\text{-}304}$, DC-$GAPDH_{1\text{-}15}$, DC-$GAPDH_{1\text{-}22}$ or DC-LM lysate (106 cells/footpad). Homogenates of popliteal lymph nodes were stimulated in vitro with 50 μg/ml of $LLO_{91\text{-}99}$ or $LLO_{296\text{-}304}$ peptides in DC-LLO inoculated vaccines and with $GAPDH_{1\text{-}22}$ peptide in DC-GAPDH inoculated vaccines. For Delayed Type Hypersensitivity (DTH) analysis, Balb/c and C57BL/6 mice immunised i.p with LMWT (5×10^3 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 (1×10^6 cells/mice) or left non-vaccinated (NV) (n=5), all mice were next challenged i.p with 10^3 and 10^4 CFU of LM^{WT}/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+-LLO or GAPDH specific T cells

To confirm the frequency of $LLO_{91\text{–}99}$, $LLO_{296\text{-}304}$, $GAPDH_{1\text{-}15}$ or $GAPDH_{1\text{-}22}$ specific CD8 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. $P \leq 0.05$ 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_{91-99}, $LLO_{189-201}$, $LLO_{189-200}$, $LLO_{190-201}$, $LLO_{296-304}$, $GAPDH_{1-15}$ or $GAPDH_{1-22}$ 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[a]	Early apoptosis[b]	Late apoptosis[c]
NI	$1.59 \times 10^6 \pm 142$	35 ± 0.01	10 ± 0.05
LLO91-99 (µg/ml)			
500	$1.49 \times 10^6 \pm 132$	36 ± 0.02	11 ± 0.02
50	$1.59 \times 10^6 \pm 120$	37 ± 0.03	12 ± 0.08
5	$1.58 \times 10^6 \pm 113$	35 ± 0.02	10 ± 0.09
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.03
LLO189-201 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	36 ± 0.02	10 ± 0.03
50	$1.57 \times 10^6 \pm 120$	37 ± 0.03	12 ± 0.07
5	$1.58 \times 10^6 \pm 113$	35 ± 0.02	12 ± 0.08
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.01
LLO190-201 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	36 ± 0.02	11 ± 0.01
50	$1.56 \times 10^6 \pm 120$	35 ± 0.03	10 ± 0.05
5	$1.58 \times 10^6 \pm 113$	37 ± 0.02	12 ± 0.05
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.07
LLO190-201 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	36 ± 0.02	11 ± 0.10
50	$1.58 \times 10^6 \pm 120$	37 ± 0.03	12 ± 0.15
5	$1.58 \times 10^6 \pm 113$	35 ± 0.02	10 ± 0.05
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.07
LLO296-304 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	36± 0.02	10 ± 0.10
50	$1.57 \times 10^6 \pm 120$	37 ± 0.03	12 ± 0.15
5	$1.58 \times 10^6 \pm 113$	35 ± 0.02	11 ± 0.05
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.07
GAPDH1-15 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	35 ± 0.02	10 ± 0.01
50	$1.58 \times 10^6 \pm 120$	36 ± 0.03	11 ± 0.05
5	$1.58 \times 10^6 \pm 113$	35 ± 0.02	10 ± 0.02
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	12 ± 0.03
GAPDH1-22 (µg/ml)			
500	$1.59 \times 10^6 \pm 132$	36 ± 0.02	11 ± 0.02
50	$1.58 \times 10^6 \pm 120$	37 ± 0.03	12 ± 0.01
5	$1.58 \times 10^6 \pm 113$	37 ± 0.02	12 ± 0.03
0.5	$1.59 \times 10^6 \pm 128$	36 ± 0.01	11 ± 0.01

Table 1: Lack of toxicity of different LLO and GAPDH peptides. [a]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 ($P<0.05$). [b]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) ($P<0.05$). [c]Percentages of early apoptotic cells or programmed cell death (Q4 in Figure S1) (mean ± SD) ($p<0.05$).

Activated DC shows a characteristic $CD11c^+MHC\text{-}II^+CD40^+CD86^+$ 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 $CD11c^+$ cells using MACS magnetic beads (Miltenyi) and loaded ex vivo for 24 hours with 50 µg/ml of LLO_{91-99}, $LLO_{189-201}$, $LLO_{189-200}$, $LLO_{190-201}$, $LLO_{296-304}$, $GAPDH_{1-15}$, $GAPDH_{1-22}$, LPS (10 ng/ml) as a classical DC activator [6,11] or left untreated (DC-CONT). Next, the frequency of $CD11c^+MHC\text{-}II^+$ cells positive for $CD40^+$ or $CD86^+$ DC was assessed as markers of mature and activated DC (Figure 1A). LPS induced the highest frequency of $MHC\text{-}II^{high}$ DC followed by $GAPDH_{1-22}$ and $GAPDH_{1-15}$ peptides. LLO_{91-99} peptide showed an intermediate frequency of $MHC\text{-}II^{high}$ DC. $LLO_{296-304}$ and $LLO_{189-201}$ peptides showed significant $MHC\text{-}II^{high}$ DC frequency, while $LLO_{189-200}$ and $LLO_{190-201}$ peptides showed no frequency (Figure 1A). A similar picture was seen for $CD11c^+MHC\text{-}II^+CD40^+$ or $CD11c^+MHC\text{-}II^+CD86^+$ DC. Differences were even more marked when we analysed the frequency of $CD40^+CD86^+$ 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, $GAPDH_{1-15}$ and $GAPDH_{1-22}$ 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_{91-99} peptide, a strong binder to H-2Kd and a weak binder to H-2Kb MHC-I showed intermediate DC activation. $LLO_{296-304}$ 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, $LLO_{189-201}$ peptides were weak binders to IAb and IAd and showed DC activation comparable to $LLO_{296-304}$ peptide (Figure 1B). However, $LLO_{189-200}$ and $LLO_{190-201}$ were both weak binders to IAb and showed neither binding to IAd nor DC activation, contrary to previous predictions [12].

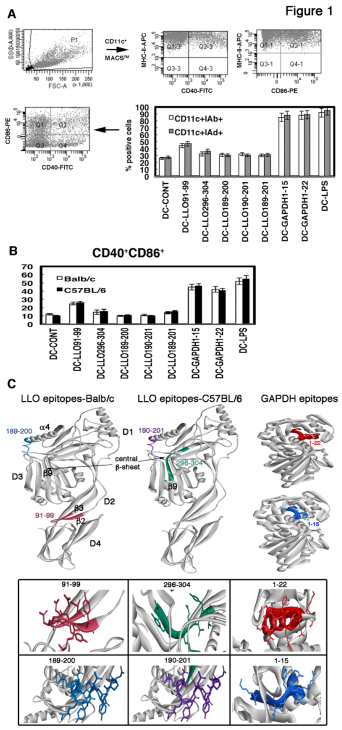

Figure 1: Properties of different LLO and GAPDH peptides as epitopes of DC vaccines.

A, Analysis of DC activation including the strategy applied to DC to select mature and activated $CD11c^+MHC\text{-}II^+CD40^+CD86^+$ 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. $CD11c^+$ DC were loaded ex vivo with different peptides: LLO_{91-99}, $LLO_{189-200}$, $LLO_{190-201}$, $LLO_{189-201}$, $LLO_{296-304}$, $GAPDH_{1-15}$ or $GAPDH_{1-22}$ peptides (50 μg/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 ± SD of triplicate samples ($P<0.05$). B, $CD11c^+MHC\text{-}II^+$ 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, $MHC\text{-}II^+CD40^+CD86^+$ cells. Results are expressed as the mean ± 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 $LLO_{189-200}$ peptide and MHC-II epitope and the pink sequence to LLO_{91-99} peptide and MHC-I epitope. On the centre, the purple sequence corresponds to $LLO_{190-201}$ peptide and MHC-II epitope and the green sequence to $LLO_{296-394}$ and MHC-I epitope. Other structural features of LLO are also shown, as D1, D2, D3 and D4 domains, the central β-sheet and the β-strands or α-helices where serveral epitopes are localized. On the right, the GAPDH predicted 3D structure showing in red the $GAPDH_{1-22}$ peptide and in blue the $GAPDH_{1-15}$ 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 α/β fold with five stranded β-sheets surrounding by six α-helices. D2 domain shows four β-strands forming an antiparallel β-sheet to connect D1 and D4. D3 domain is formed of five-stranded antiparallel β-sheet surrounded by six α-helices. D4 is the domain binding to the phagosomal membranes showing a compact β-sandwich with two four-stranded β-sheets [24]. MHC-II epitopes of both mice strains were localized in same α4-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 β2 and β3-strands in D2 domain (pink tip-loop epitope in Figure 1C). MHC-I epitope of C57BL/6 mice localizes in β9-strand in the central β-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. $GAPDH_{1-22}$ peptide contained six MHC-I and seven MHC-II binding sequences in Balb/c and C57BL/6 mice. While $GAPDH_{1-15}$ peptide contained only three MHC-I binding sequences and two MHC-II binding sequences (Table 2). $GAPDH_{1-15}$ and $GAPDH_{1-22}$ binding sequences to MHC-I molecules cover amino acids 1 to 15 and resembles the loop structure composed of β2 and β3-strands in D2 of LLO. Similar to LLO, $GAPDH_{1-15}$ and $GAPDH_{1-22}$ binding sequences to MHC-II molecules localized also in a large α-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 α-helices as the cases of $GAPDH_{1-15}$, $GAPDH_{1-22}$. Next, it follows the LLO_{91-99} peptides containing a complete loop structure of β-strands that caused intermediate DC activation; while β-strands in loose structures as in $LLO_{296-304}$ peptide only showed low DC activation. Finally, α-helices exclusively caused no DC activation as $LLO_{189-200}$, $LLO_{190-201}$ or $LLO_{189-201}$ peptides. Therefore, combination of binding predictions to MHC-I or MHC-II molecules with structural localizations seems to correlate with DC activation.

MHC-I binding	H-2 allele	Percentile rank	Binding ability	Sequence
LLO_{91-99}	H-2-Kb	67.5	UB	Complete
	H-2-Kd	0.7	GB	Complete
	H-2-Ld	38.3	UB	Complete
$LLO_{296-304}$	H-2Kb	0.2	GB	Complete
	H-2Kd	32	UB	Complete
	H-2Ld	12.5	UB	Complete

$GAPDH_{1-22}$	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
$GAPDH_{1-15}$	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
$LLO_{190-201}$	H-2-IAb	75.1*	WB	Complete
	H-2-IAd	>15000	UB	
$LLO_{189-200}$	H-2-IAb	85	WB	Complete
	H-2-IAd	11364	UB	
$LLO_{189-201}$	H-2-IAb	51.3	WB	Complete
	H-2-IAd	5326	WB-UB	Complete
$GAPDH_{1-22}$	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		2-16
$GAPDH_{1-15}$	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. $GAPDH_{1-22}$ peptide was compared to the predictions of LLO_{91-99} binding to MHC-I molecules and $LLO_{189-201}$ 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_{91-99}, DC-$LLO_{296-304}$, DC-$LLO_{189-200}$, DC-$LLO_{190-201}$, DC-$LLO_{189-201}$, DC-$GAPDH_{1-15}$, DC-$GAPDH_{1-22}$ 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_{91-99} plus $LLO_{296-304}$ peptides for DC-LLO vaccines and with $GAPDH_{1-15}$ plus $GAPDH_{1-22}$ 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_{91-99}, $LLO_{296-304}$, $GAPDH_{1-15}$ and $GAPDH_{1-22}$ peptides together. DC-$GAPDH_{1-22}$ and DC-$GAPDH_{1-15}$ vaccines induced a high level of proliferation equivalent to DC-LM^{WT} lysate vaccines in both mice strains. DC-LLO_{91-99} and DC-$LLO_{296-304}$ vaccines induced the next best T cell proliferations. Mice immunized with DC-$LLO_{189-201}$, DC-$LLO_{189-200}$ and DC-$LLO_{190-201}$ vaccines exhibited only basal levels of T cell proliferations to re-call responses with LLO91-99 or $LLO_{296-304}$, 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 $CD4^+$ T-cell responses (Methods for procedures) since DTH depends mainly on $CD4^+$ T cells [25]. We immunized mice with DC-LLO_{91-99}, DC-$LLO_{189-200}$, DC-$LLO_{190-201}$, DC-$LLO_{189-201}$, DC-$LLO_{296-304}$, DC-$GAPDH_{1-15}$ or DC-$GAPDH_{1-12}$ for 48 hours. DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ generated higher DTH responses than DC-$LLO_{189-201}$ in both mice strains (Figure 2B). DC-$LLO_{189-200}$ 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-$LLO_{190-201}$ 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ 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^{WT} 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ provided the greatest protection in Balb/c and C57BL/6 mice (~97 and 99%, respectively) (Figure 3A).

DC-LLO_{91-99} vaccines conferred also significant protection in Balb/c and C57BL/6 mice (90 and 93%, respectively). However, DC-$LLO_{296-304}$ 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-$LLO_{189-200}$, DC-$LLO_{190-201}$, DC-$LLO_{189-201}$ 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-$GAPDH_{1-15}$ 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-$GAPDH_{1-15}$, DC-$GAPDH_{1-22}$, DC-LLO_{91-99} or DC-$LLO_{296-304}$ showed an increased frequency of $CD11c^+IAb^+CD40^+CD86^+CD8\alpha^+$ mature DC (68% of positive cells), $CD8^+$ 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-$LLO_{189-201}$, DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ immunised mice showed an increased frequency of $CD4^+$ T cells post-challenge (Figure 3B).

Vaccination with DC-$GAPDH_{1-15}$, DC-$GAPDH_{1-22}$, DC-LLO_{91-99}, DC-$LLO_{296-304}$ correlated with high levels of MCP-1, TNF-α and IFN-γ production but only DC-GAPDH1-15 and DC-$GAPDH_{1-22}$ vaccines

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

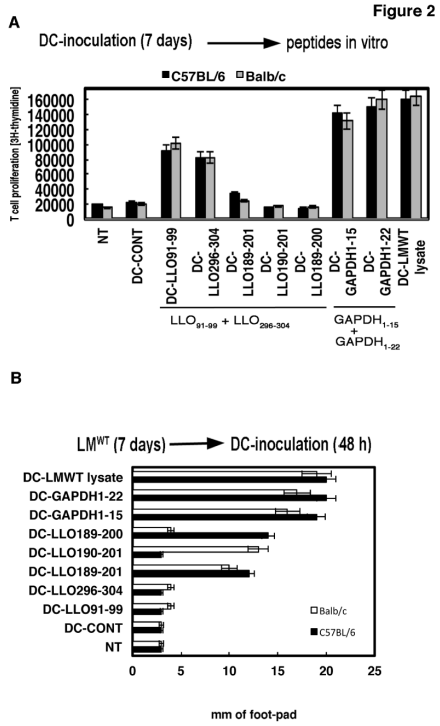

Figure 2: T cell responses induced by DC-LLO and DC-GAPDH vaccines in listeriosis susceptible and resistant mice. A, T cell proliferation in popliteal lymph node homogenates after hind footpad inoculation of C57BL/6 mice (black bars) or Balb/c mice (grey bars) with 1×10^5 of different DC-LLO and DC-GAPDH vaccines prepared as in Figure 1A or saline inoculation (NT) for 7 days. Cells from DC-LLO vaccine inoculations were stimulated in vitro with 50 µg/ml of a mixture of LLO_{91-99} and $LLO_{296-304}$ peptides and cells from DC-GAPDH vaccine inoculations were stimulated with a mixture of $GAPDH_{1-15}$ and $GAPDH_{1-22}$ peptides to analyse CD8 T cell proliferation. DC-controls and NT samples were stimulated in vitro with both peptides mixture. Results show the mean ± SD of [3H]-thymidine incorporation in triplicate samples (P<0.05). B, C57BL/6 (black bars) or Balb/c mice (white bars) were immunised i.p with 5×10^3 CFU of LM/mice for 7 days and next, left hind footpads were inoculated with 1×10^6 cells of different DC-LLO and DC-GAPDH vaccines or saline (NT) for 48 hours, while right hind footpads were not inoculated and served as controls. Footpad swelling was measured with a calliper and expressed as the differences in mm between left and right hind footpads. Results are expressed as the mean ± SD of three different experiments (P<0.05).

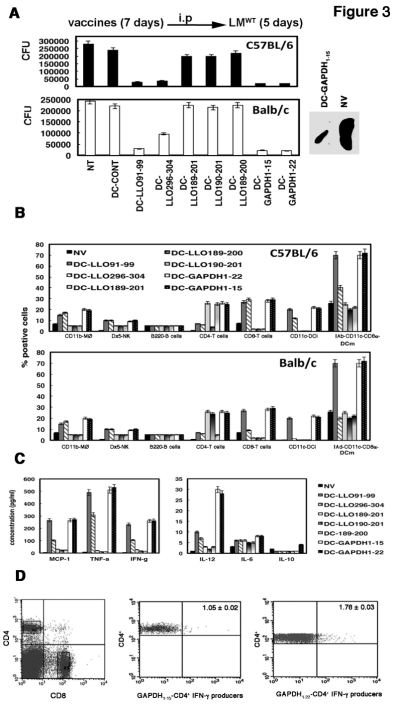

Figure 3: DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ are effective vaccines in listeriosis susceptible and resistant mice. DC vaccines were tested for their ability to protect against listeriosis.

A, C57BL/6 (upper plot) or Balb/c (lower plot) mice were vaccinated i.p for 7 days with different DC vaccine vectors (1×10^6 cells) (DC-CONT, DC-$GAPDH_{1-15}$, DC-$GAPDH_{1-22}$, DC-LLO_{91-99}, DC-$LLO_{296-304}$, DC-$LLO_{189-200}$, DC-$LLO_{189-201}$ or DC-$LLO_{190-201}$) or not vaccinated (NV) (n=5 mice/group) and challenged i.p with 10^3 CFU LM^{WT} (for Balb/c mice) or 10^4 CFU LM^{WT} (for C57BL/6 mice) for 5 days. Results of spleens homogenates are expressed as CFU (mean ± 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-$GAPDH_{1-15}$ vaccinated Balb/c mice that had normal sized spleens, similar results were obtained with C57BL/6 mice and vaccinated with DC-$GAPDH_{1-22}$ or DC-$GAPDH_{1-15}$ vaccines. **B,** MØs ($CD11b^+F4/80^+$), B cells ($CD19^+$), NK ($CD3^+CD49b^+$), CD4 T cells ($CD3^+CD4^+$), CD8 T cells ($CD3^+CD8^+$), DCi ($CD11c^+MHC^-II^+CD40^-CD86^-$), DCm ($CD11c^+MHC^-II^+CD40^+CD86^+CD8\alpha^+$) were quantified in spleen homogenates of C57BL/6 mice (upper plot) or Balb/c mice (lower plot) by FACS. Results expressed the mean ± SD of the percentage of positive cells (P<0.05). **C,** Levels of pro-inflammatory cytokines (MCP-1, TNF-α, IFN-γ, IL-6, IL-10, IL-12) were analysed in mouse sera by CBA. Results expressed as cytokine concentration (pg/ml of mean ± 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^{WT} (103 bc/mice for Balb/c and 104 bc/mice for C57BL/6) for 5 days. Spleen homogenates were stimulated 5 h with $GAPDH_{1-15}$, $GAPDH_{1-22}$, $LLO_{189-200}$, $LLO_{190-201}$ or $LLO_{189-201}$ peptides then intracellular cytokine staining performed. Histograms show the frequency of $GAPDH_{1-15}$ and $GAPDH_{1-22}$-

specific $CD4^+$ T cells and IFN-γ producers in Balb/c spleen homogenates. Frequencies in C57BL/6 mice were similar (1.02 ± 0.01 for $GAPDH_{1-15}$ peptide and 1.70 ± 0.02 for $GAPDH_{1-22}$ peptide). Experiments were performed in triplicate and results are expressed as mean ± SD ($p<0.05$).

We also analysed whether the high efficiency of DC-GAPDH vaccines correlated with the frequencies of IFN-γ producing $CD8^+$ using dimers (Table 3) or $CD4^+$ T cells using intracellular cytokines staining (Figure 3D). DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ vaccines induced high frequencies of IFN-γ producing $CD8^+$ T cells in C57BL/6 and Balb/c mice (4.0 and 4.2%, respectively in Table 3). DC-DC-LLO_{91-99} vaccines show a 3.0% frequency of IFN-γ producing $CD8^+$ 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-$LLO_{296-304}$ vaccines induced only intermediate 0.87% frequencies in C57BL/6 mice and none in Balb/c mice. These results indicated that efficiency of DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ vaccines correlated with high frequencies of IFN-γ producing $CD8^+$ 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 ± 0.01	3.02 ± 0.02
DC-LLO296-304	0.87 ± 0.01	0.03 ± 0.01
DC-GAPDH1-15	4.06 ± 0.01	4.02 ± 0.01
DC-GAPDH1-22	4.26 ± 0.02	4.22 ± 0.03

Table 3: Frequencies of LLO and GAPDH peptides-specific $CD8^+$ 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_{91-99}, $LLO_{296-304}$, $GAPDH_{1-15}$ or $GAPDH_{1-22}$ peptides. The staining cocktail contained the dimeric fusion protein loaded with the peptides, $CD8^+$ and IFN- antibodies. CD8+ cells were gated for anti-IFN- staining (% Gated dimer-CD8) to calculate the frequencies of CD8+-LLO_{91-99}, $CD8^+$ $LLO_{296-304}$, CD8+-$GAPDH_{1-15}$ or CD8+-$GAPDH_{1-22}$ restricted cells and IFN- producers.

We also analysed the percentages of peptide specific $CD4^+$ T cells and observed that DC-GAPDH1-15 and DC-GAPDH1-22 vaccines also induced detectable numbers of peptide-specific $CD4^+$ T cells (1.05 and 1.76% percentages, respectively) (Figure 3D), suggesting that vaccine efficiencies correlate with simultaneous and significant frequencies of IFN-γ producing $CD4^+$ and $CD8^+$ 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 $CD8^+$ T-cell immunity while $CD4^+$ T-cells played a less relevant but necessary role (Pamer, 2004). Only two Listeria antigens, LLO and GAPDH contained epitopes that inducing $CD4^+$ and $CD8^+$ T-cells, confered 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ based in a previous study showing $GAPDH_{1-22}$ peptide binding to MHC-I and MHC-II molecules. We also prepared five DC-LLO vaccines DC-LLO_{91-99}, DC-$LLO_{296-304}$, DC-$LLO_{189-200}$, DC-$LLO_{190-201}$ and DC-$LLO_{189-201}$, 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ vaccines containing a combination of several MHC-I and MHC-II binding epitopes in a single peptide with a loop structure combined with α-helices, showed the highest DC activation capacities. As a consequence, these DC-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ vaccines induced high frequencies of peptide-specific IFN-γ producing $CD8^+$ and $CD4^+$ 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-$GAPDH_{1-15}$ and DC-$GAPDH_{1-22}$ vaccines associated with increased frequency in the spleens of NK cells and activated CD8α+ DC that produced significant levels of IL-12. $CD8\alpha^+$ DC have been reported to play a critical role in Listeria protection producing IL-12 and activating IFN-γ producing $CD4^+$ and $CD8^+$ T cells [29,30].

The most effective DC-LLO vaccine, DC-LLO_{91-99} presented a good 90% protection in both mice strains, but with less prominent frequencies of activated $CD8\alpha^+$ DC as well as peptide-specific IFN-γ producing $CD8^+$ T cells and no induction of $CD4^+$ T cells. To add that LLO_{91-99} peptide contained only one MHC-I binding epitope and showed only intermediate DC activation abilities. DC-$LLO_{296-304}$ 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-$LLO_{189-200}$, DC-$LLO_{190-201}$ or DC-$LLO_{189-201}$ vaccines showed DC activation or protection abilities.

$GAPDH_{1-15}$ and $GADPH_{1-22}$ 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. $GAPDH_{1-15}$ and $GADPH_{1-22}$ seemed also appealing epitopes to include in conventional, cell-free vaccines or chimeric constructs with LLO_{91-99} epitopes due to their potential to protect low and high susceptible individuals.

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