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A gold glyco-nanoparticle carrying a listeriolysin O peptide and formulated with Advax™ delta inulin adjuvant induces robust T-cell protection against listeria infection

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ABSTRACT

In the search for an effective vaccine against the human pathogen, *Listeria monocytogenes* (*Listeria*), gold glyconanoparticles (GNP) loaded with a listeriolysin O peptide LLO_{91-99} (GNP-LLO) were used to immunise mice, initially using a dendritic cell (DC) vaccine approach, but subsequently using a standard parenteral immunisation approach. To enhance vaccine immunogenicity a novel polysaccharide adjuvant based on delta inulin (Advax™) was also co-formulated with the GNP vaccine. Confirming previous results, DC loaded *in vitro* with GNP-LLO provided better protection against listeriosis than DC loaded *in vitro* using free LLO peptide. The immunogenicity of GNP-LLO loaded DC vaccines was further increased by addition of Advax™ adjuvant. However, as DC vaccines are expensive and impracticable for prophylactic use, we next asked whether the same GNP-LLO antigen could be used to directly target DC *in vivo*. Immunisation of mice with GNP-LLO plus Advax™ adjuvant induced LLO-specific T-cell immunity and protection against *Listeria* challenge. Protection correlated with an increased frequency of splenic $CD4^+$ and $CD8^+$ T cells, NK cells and $CD8\alpha^+$ DC, and Th1 cytokine production (IL-12, IFN-γ, TNF-α, and MCP-1), post-challenge. Enhanced T-cell epitope recruitment post-challenge was seen in the groups that received Advax™ adjuvant. Immunisation with GNP-LLO_{91-99} plus Advax™ adjuvant provided equally robust *Listeria* protection as the best DC vaccine strategy but without the complexity and cost, making this a highly promising strategy for development of a prophylactic vaccine against listeriosis.

1. Introduction

Candidate prophylactic vaccines against *Listeria monocytogenes* (*Listeria*) include live attenuated pathogens [1–4], live vector-based approaches [5–7] and subunit vaccines [8,9]. However, the use of live vaccines in at-risk immunocompromised individuals including pregnant women poses major safety risks, making a non-living subunit vaccine a more preferable approach. Unfortunately, subunit vaccines by themselves are generally ineffective due to overall poor immunogenicity and inability, in particular, to generate the T-cell immunity required for protection against intracellular organisms such as *Listeria*.

Dendritic cell (DC) vaccines are produced by isolation of DC from a subject, *in vitro* loading of the DC with the relevant vaccine antigen, and then intravenous or subcutaneous injection of the live DC back into the subject. Based on their improved ability to induce T-cell immunity, DC vaccines have been proposed as solutions for induction of T-cell protection against intracellular infections, such as HIV [10]. Our group previously reported a DC vaccine loaded with a peptide from the *Listeria* protein, glyceraldehyde 3-phosphate

Abbreviations: APC, antigen-presenting cells; CFU, colony forming units; Ctsd, cathepsin-D; DC, dendritic cells; GNP, gold glyconanoparticles; *i.p*, intra-peritoneal; *i.v*, intra-venous; LLO, listeriolysin O; MHC, major histocompatibility complex; MIIC, MHC-class II antigen loading compartments; MØ, macrophages; LM, Listeria monocytogenes.

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dehydrogenase (GAPDH), induced generation of GAPDH-specific $CD8^+$ and $CD4^+$ T cells and conferred protection against *Listeria* [11], consistent with the importance of T-cell responses against GAPDH in *Listeria* protection [12–14]. Other *Listeria* antigens that contain $CD4^+$ and $CD8^+$ T-cell epitopes and that might therefore enhance the efficacy of a DC-based *Listeria* vaccine include listeriolysin (LLO), ActA, and p60 [11,15,16]. To date, only T-cell immunity against LLO has been reported in humans recovering from listeriosis [17], suggesting a potential role of in protection. We therefore wished to test whether a T-cell vaccine targeting LLO could protect against listeriosis. In particular, we sought to test whether a nanoparticle-conjugated LLO peptide could increase DC vaccine efficacy [10,18] and also whether a novel T-cell adjuvant could enhance LLO vaccine immunogenicity [19]. Gold glyconanoparticles (GNPs) constitute a nanoscale metallic core to which self-assembled monolayers of carbohydrate ligands are covalently linked by means of thiol chemistry [20]. Due to their water dispersibility, biocompatibility, resistance to enzymatic degradation, ease of preparation, and ability to incorporate different ligands, GNPs are highly versatile [21,22]. In this study, therefore, we sought to test the potential of a glucose-labelled GNP presenting the LLO_{91-99} peptide (GNP-LLO) to protect against listeriosis in a murine model. In the first approach, we sought to apply the previous approach of loading DC *in vitro* using the GNP-LLO carrier prior to injection of the DC back into the animals. However, given the logistical difficulties of the DC vaccine approach, we also wished to test whether, rather than being used for DC vaccine loading *in vitro*, the GNP-LLO antigen could be used for parenteral immunisation along the lines of a traditional vaccine.

Given the poor T-cell responses induced by subunit vaccines, we sought to further enhance GNP-LLO immunogenicity by use of Advax™, a novel polysaccharide adjuvant derived from microparticles of delta β-D-[2-1]poly(fructo-furanosyl)α-D-glucose (delta inulin) [25]. In animal models Advax™ has been shown to enhance cellular plus humoral immunity against a broad spectrum of antigens including influenza [26], Japanese encephalitis [27], West Nile virus [28], hepatitis B [29], and HIV [30] vaccines. Advax™ has also been shown effective and safe in human trials of pandemic influenza [31] and hepatitis B [29], vaccines.

2. Methods

2.1.1. Peptides and adjuvants

We used as immunogens, peptides representing known *Listeria* T cell epitopes, namely $GAPDH_{1-22}$, LLO_{91-99} and $LLO_{189-201}$ [6,11,15]. Immunisation with free $LLO_{189-200}$ and $LLO_{190-201}$ peptides has previously been shown to induce $CD4^+$ T cell responses in mice but did not confer *Listeria* protection [11,32]. All peptides were synthesised at CNB, CSIC, Madrid followed by high performance liquid chromatography (HPLC) and mass spectrometry using a MALDI-TOF Reflex™ IV mass spectrometer (Bruker Daltonics, Bremen, Germany). Peptide purity was >95% after HPLC. Advax™ adjuvant (Adv1) was supplied by Vaxine (Adelaide, Australia) [31,33–35], and was mixed with antigen by simple admixture immediately before immunisation of mice.

2.1.2. Bacteria

L. monocytogenes 10403S strain (LM^{WT}) was obtained from D.A. Portnoy (University of California, Berkeley, CA, USA).

2.1.3. Preparation of gold glyconanoparticles bearing LLO_{91-99} peptides (GNP-LLO_{91-99})

LLO_{91-99} peptide with a C-terminal cysteamide ($LLO_{91-99}C(O)NHCH_2CH_2SH$, 14 mg, purity 95%) was purchased from GenScript and 5-(mercapto)pentyl-β-D-glucopyranoside ($GlcC_5SH$) was prepared as reported in the literature [36]. GNP preparation is included in *Supplemental information*. Average gold diameter 1.5 ± 0.5 nm (the size histogram in *Fig. S1, panel A* was obtained by counting 600 particles in the TEM image).

2.1.4. Preparation of DC vaccines

Bone-marrow 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) [6,11] from 8 to 12 week-old female CD1, Balb/c or C57BL/6 mice. Bone marrow macrophages (DM) were obtained from same femurs and differentiated with M-CSF, as described [11]. DC used were $MHC\text{-}II^+CD11c^+CD40^+CD11b^{+/-}CD86^{+/-}F4/80^-Gr\text{-}1^-$ and of DM were $CD11b^+MHC\text{-}II^+F4/80^{+/-}CD11c^-Gr\text{-}1^-CD40^-CD86^-$. Cultured DC were *ex vivo* loaded with 50 μg/ml $GAPDH_{1-22}$, LLO_{91-99} or $LLO_{189-201}$ peptides or with 5 μg/ml of GNP-LLO_{91-99} for 24 h. Cells were analysed for apoptosis by FACS analysis using annexin-V-APC and H-1770 (BD-Biosciences), for cell viability with Trypan blue staining, and cell surface markers by FACS.

2.1.5. T-cell assays

T-cell analysis, cytotoxic T lymphocytes responses and delayed type hypersensitivity (DTH) analysis are described in *Supplemental information*.

2.1.6. Preparation of peptide-coupled GNP vaccines

GNP carrying approximately 90% glucose and 10% LLO_{91-99} (*Fig. S1, panel A*) were prepared by reduction *in situ* of Au(III) salt with sodium borohydride, as described [21,37]. The ligands (LLO_{91-99} peptide and glucose) were derivatised with thiol-ending linkers to attach them to the gold core (*Fig. S1, panel A*). 5-Mercaptopentyl β-D-glucopyranoside ($GlcC_5SH$) with a short aliphatic linker, as previously reported [36] was used as a ligand partner to control the density of LLO_{91-99} peptide on the GNPs. Glucose is ideal to confer water dispersibility and biocompatibility to the GNPs without interfering with the antigenic properties of LLO_{91-99} peptide [24]. LLO peptides and GNP-LLO_{91-99} did not adversely affect DC viability or promote apoptosis (*Fig. S1, panel B* and DC column in *Table S1*). They were also well tolerated at 50 μg/ml concentrations in C57BL/6, CD-1 or Balb/c mice strains, (C57BL/6 column in *Table S1*), while at 500 μg/ml concentrations they caused decreased DC viability and increased IL-1 concentration suggesting potential toxicity (*Table S1*) [38].

2.1.7. GNP immunogenicity assessment

We immunised mouse footpads with 0.1 μg of recombinant LLO (LLO_{rec}) and then evaluated T-cell proliferative recall responses to *in vitro* stimulation with LLO_{91-99}, $LLO_{189-201}$, GNP-LLO_{91-99}, LM^{WT} lysate (LM-lysate) or LLO_{rec} at doses ranging from 0 to 500 μg/ml. T-cell proliferation was dose dependent peaking at 50 μg/ml and then declining for all antigens (*Fig. S1, panel C*). GNP-LLO_{91-99} induced a high level of T-cell proliferation equivalent to LM-lysate (*Fig. S1, panel C*). LLO_{91-99} and LLO_{rec} induced the next best T-cell proliferation, while $LLO_{189-201}$ was lowest. To test whether the higher T-cell recall responses with GNP-LLO_{91-99} might reflect improved MHC-I antigen processing by lymph node APC, we compared MHC-I peptide presentation after *ex vivo* loading of DC or DM with LLO_{91-99} or GNP-LLO_{91-99}. MHC-I molecules were immuno-precipitated and levels of LLO peptides bound to MHC-I assessed by western-blot using rabbit anti-LLO antibodies, as previously reported for MHC-II molecules [5,11]. GNP-LLO_{91-99} resulted in higher efficiency loading than free LLO_{91-99} peptides of MHC-I molecules on either DC or

DM (*Fig. S1, panel D*), with far higher presentation by DC than DM (*Fig. S1, panel D*).

2.1.8. Ex vivo activation of DC vaccines

We examined the effect of LLO peptide carriers on DC vaccine activation [6,10]. Activated DC show a characteristic $CD11c^+MHC\text{-}II^+CD40^+CD86^+$ phenotype [10,18]. To examine DC activation by LLO carriers, at day 5 after GM-CSF differentiation, DC were purified as $CD11c^+$ cells using MACS magnetic beads (Miltenyi) and loaded *ex vivo* for 24 h with 5 μg/ml of GNP-LLO_{91-99} in presence or absence of Advax™ adjuvant (50 μg/ml) or with 50 μg/ml of LLO_{91-99}, $LLO_{189-201}$, LM-lysate 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 (*Fig. S2, panel A*). DC-*Listeria* lysate induced the highest frequency of $MHC\text{-}II^{high}$ DC followed by GNP-LLO_{91-99} with or without Advax™, which were both significantly higher than $LLO_{189-201}$ (*Fig. S2, panel B*). A similar picture was seen for $CD11c^+MHC\text{-}II^+CD40^+$ or $CD11c^+MHC\text{-}II^+CD86^+$ DC. Differences between GNP loaded and free peptide were even more marked when we analysed the frequency of $CD40^+CD86^+$ double positive DC (*Fig. S2, panel C*).

2.1.9. Immunisation

DC vaccines loaded *in vitro* using either free peptide or GNP-conjugated peptides are indicated by placement of "DC" in front of the specific antigen. If the name of the vaccine does not contain "GNP" then this means DC loading or immunisation was done with free peptide. Vaccines used were either DC vaccines (DC-LLO_{91-99}, DC-$LLO_{189-201}$, DC-$GAPDH_{1-22}$, DC-GNP-LLO_{91-99}, all at doses of 1×10^6 DC per immunisation), or standard vaccines (GNP-LLO_{91-99} at 5 μg per immunisation). Standard vaccines were administered with or without Advax™ adjuvant (250 μg per immunisation). Mice were immunised intra-peritoneally (*i.p*) ($n = 5$), intravenously in the tail vein (*i.v*) ($n = 5$) or left non-vaccinated (NV). Seven days after immunisation mice were challenged *i.p* or *i.v* with 5×10^3 CFU of *Listeria* microorganisms/mouse [2]. Sera were stored at −80 °C to measure cytokines by FACS analysis. At termination spleens and livers were homogenised and *Listeria* colony forming units (CFUs) counted in blood agar plates.

2.1.10. Frequencies of $CD8^+$-LLO_{91-99} specific T cells

To confirm the frequency of IFN-γ producing LLO_{91-99} specific CD8T cells we used recombinant soluble dimeric mouse H-$2K^b$:Ig (for C57BL/6 mice) or H-$2K^d$:Ig (for Balb/c mice) fusion proteins following the manufacturer's instructions (DimerX I; BD Bioscience) and as previously described [2,11].

2.1.11. 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 minimise suffering.

2.1.12. 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.

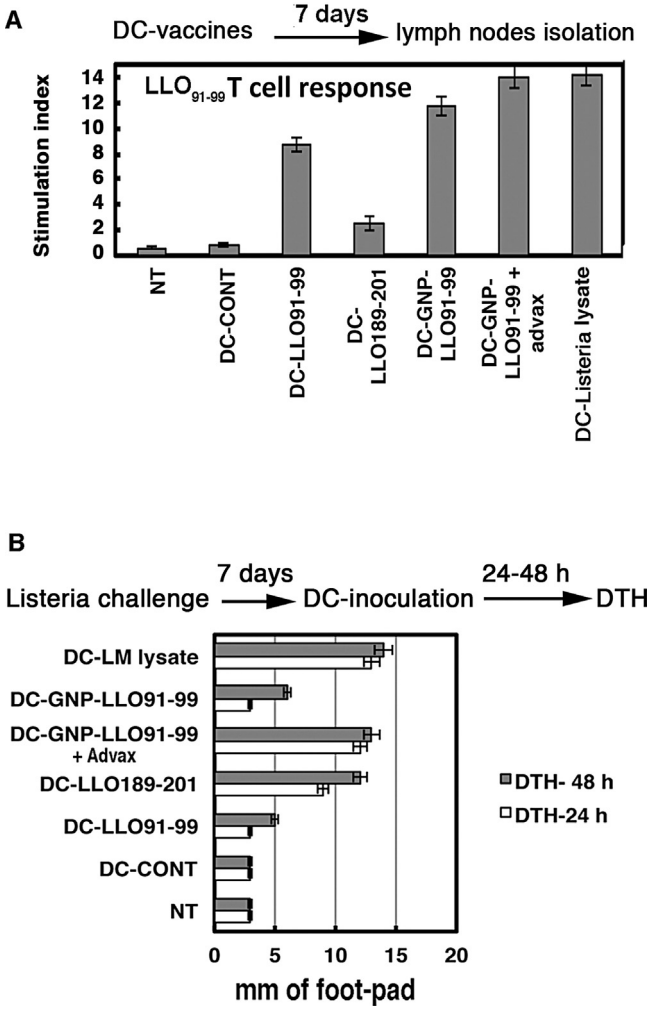

Fig. 1. *Ex vivo loading of DC vaccines and T cell responses.* (A) T-cell proliferation in response to stimulation *in vitro* with 50 μg/ml of LLO_{91-99} in popliteal lymph node homogenates 7 days after footpad inoculation with 1×10^5 cells of DC vaccines or saline (NT) [14]. Results show the mean ± SD of the stimulation index calculated as ratio of proliferation in active wells/proliferation of control samples in triplicate ($P < 0.05$). (B) Mice were immunised *i.p* with 5×10^3 CFU of *Listeria*/mice and 7 days later DTH assays performed. Results are expressed as the mean difference± SD of footpad swelling at 24 and 48 h inactive and control limbs, representative example of three independent experiments ($P < 0.05$).

3. Results

3.1. DC vaccines loaded using GNP-peptide carriers induce T-cell proliferative responses against Listeria

Dendritic cells stimulate both $CD4^+$ and $CD8^+$ T cells in listeriosis [14]. To evaluate whether dendritic cell (DC) vaccines loaded using GNP-peptide carriers might be more efficient in inducing T-cell responses than DC loaded using free peptides, mice were immunised with DC that had been loaded in different ways. Seven days post-immunisation, popliteal lymph nodes were stimulated *in vitro* with LLO_{91-99} peptide and antigen-specific T-cell proliferative responses measured. Immunisation with DC loaded *in vitro* with GNP-LLO_{91-99} induced higher T-cell proliferation responses than immunisation with DC loaded using free LLO_{91-99} peptide, indicating the importance for induction of T-cell memory of attachment of the LLO_{91-99} peptide to the GNP to enable efficient loading of DC vaccines. DC loaded *in vitro* with GNP-LLO_{91-99} induced

an equivalent T-cell proliferative response to a whole *Listeria* lysate, consistent with efficient DC loading and induction of T-cell responses by the GNP-peptide carrier (Fig. 1A). DC loaded with a $CD4^+$ restricted epitope ($LLO_{189–201}$) [11], failed to induce a T-cell proliferative response, suggesting the DC vaccines preferentially induce a $CD8^+$ T-cell response.

3.2. DC vaccines loaded using GNP-peptide carriers induce DTH responses in Listeria-sensitised mice

Next, we examined the delayed type hypersensitivity (DTH) response to the DC vaccines in previously *Listeria*-sensitised mice as an *in vivo* measure of the ability of the different DC vaccines to induce T-cell activation. The aim was to evaluate whether DC loaded *in vitro* with GNP-$LLO_{91–99}$ when injected into the footpad of previously sensitised mice would trigger a typical Th1-type T-cell response as measured by DTH [7]. We injected *Listeria* sensitised mice in the footpad with DC loaded *in vitro* with $LLO_{91–99}$ or $LLO_{189–201}$ free peptides or with GNP-$LLO_{91–99}$. In fact, DC loaded with $LLO_{189–201}$ generated greater DTH responses in the sensitised mice than DC loaded with $LLO_{91–99}$ or GNP-$LLO_{91–99}$, suggesting that $LLO_{189–201}$ is normally a dominant T-cell epitope after *Listeria* infection (Fig. 1B). Interestingly, the DTH response to DC loaded with GNP-$LLO_{91–99}$ in the presence of Advax™ adjuvant was similar in size to the DC-$LLO_{189–201}$ response suggesting the $LLO_{91–99}$ epitope could be turned into a dominant epitope, providing it was presented by DC at a sufficiently high level together with sufficient co-stimulatory molecule expression induced by the Advax™ adjuvant (Fig. 1B).

3.3. Immunisation with GNP-loaded DC vaccines provides protection of mice against listeriosis

We next assessed protection against listeriosis using the different DC vaccine vectors, DC-$LLO_{91–99}$, DC-$LLO_{189–201}$, DC-$GAPDH_{1–22}$, DC-*Listeria* lysate, DC-GNP-$LLO_{91–99}$, and empty DCs (DC-CONTROL) when compared to immunisation with free GNP-$LLO_{91–99}$. DC-$GAPDH_{1–22}$ was included as a positive control since it previously showed good *Listeria* protection [11]. Mice were immunised *i.v* with a single dose of vaccine. Although *i.v* is an unusual immunisation route for standard vaccines, it is a typical route for administration of cellular DC vaccines [6]. Seven days post-immunisation the mice were administered a live *Listeria* challenge. Five days post-challenge, all mice were sacrificed and livers and spleens recovered to count *Listeria* colony forming units (CFU) and analyse the immune cell populations. DC-$GAPDH_{1–22}$ and DC-GNP-$LLO_{91–99}$ provided the greatest *Listeria* protection (~97 and 99% CFU reduction, respectively), followed by DC-$LLO_{91–99}$ (~94%) (Fig. 2A). DC-$LLO_{189–201}$ conferred no significant protection (5%). Notably, immunisation of mice with free GNP-$LLO_{91–99}$ (as opposed to DC pre-loaded *in vitro* with GNP-$LLO_{91–99}$) conferred intermediate protection (~60% CFU reduction). Control non-vaccinated mice (NV) were not protected and as expected had enlarged spleens and granulomatous livers post-challenge (Fig. 2A, right hand figure).

3.4. Immunisation with GNP-loaded DCs increases the frequency of DC and CD8T cells in the spleen post-challenge

Protection in listeriosis is typically reflected by increases in the frequency of splenic macrophages (MØ), natural killer cells (NK), mature DC and activated T cells post *Listeria* challenge [14,39]. We therefore examined the cell populations in the spleens of immunised mice 5 days post-challenge. Mice immunised with DC-$LLO_{91–99}$, DC-$GAPDH_{1–22}$, DC-GNP-$LLO_{91–99}$ or free GNP-$LLO_{91–99}$ particles showed an increased frequency of mature DC (70% of positive cells), $CD8^+$ T cells (~32% of positive cells), MØ (18–19% of positive cells) and NK (10% of positive cells) in their spleens, post-challenge. Only DC-$LLO_{189–201}$ and DC-$GAPDH_{1–22}$ immunised mice showed an increased frequency of $CD4^+$ T cells post-challenge (Fig. 2B).

Table 1
Frequencies of $LLO_{91–99}$-$CD8^+$ T cells induced by DC vaccines. Splenocytes of vaccinated mice incubated with recombinant dimeric H-$2K^b$:Ig fusion protein loaded with $LLO_{91–99}$ peptide. Frequencies of $CD8^+$-$LLO_{91–99}$ and IFN-γ producers were performed as described in Section 2.1.10. Results are expressed as the mean ± SD ($P < 0.05$).

Vaccination type	% Total dimer-CD8/$LLO_{91–99}$	% Gated dimer-CD8/$LLO_{91–99}$
DC-LM-lysate	0.05 ± 0.01	0.48 ± 0.01
DC-$LLO_{91–99}$	0.06 ± 0.01	1.22 ± 0.02
DC-GNP-$LLO_{91–99}$	0.17 ± 0.02	3.42 ± 0.02
GNP-$LLO_{91–99}$	0.05 ± 0.01	0.35 ± 0.01
GNP-$LLO_{91–99}$ + Advax	0.20 ± 0.01	3.21 ± 0.02

3.5. Listeria protection by GNP-loaded DC is associated with an enhanced Th1 T-cell response

We next evaluated whether *Listeria* protection of immunised mice was associated with enhanced Th1 cytokine production, as would be predicted for protection against an intracellular bacterium. Immunisation with DC-$LLO_{91–99}$, DC-$GAPDH_{1–22}$, DC-GNP-$LLO_{1–99}$ and, to a lesser extent, free GNP-$LLO_{91–99}$ was associated with high levels of MCP-1, TNF-α and IFN-γ production, consistent with a strong Th1 response (Fig. 2C). However, only DC-$GAPDH_{1–22}$ and DC-GNP-$LLO_{1–99}$ immunisation was associated with measurable IL-12 production, post-challenge, (Fig. 2C), which correlated with these groups having the greatest *Listeria* protection (Fig. 2A) [6,11]. Next, we compared the ability of the different vaccines to induce LLO-specific T-cell immunity. DC-GNP-$LLO_{91–99}$ induced a higher frequency of LLO-specific IFN-γ^+ $CD8^+$ T cells (3.42%), than induced by DC-$LLO_{91–99}$ (1.22%), (Table 1). The response induced by DC-GNP-$LLO_{91–99}$ thereby compared favourably to the 4.2% frequency of LLO-specific $CD8^+$ T cells obtained with the most protective *Listeria* vaccine we had identified to date, DC-$GAPDH_{1–22}$, [11].

3.6. Advax™ adjuvant enhances Listeria protection by GNP-$LLO_{91–99}$

DC vaccines are expensive and impracticable. However, standard immunisation with free GNP-$LLO_{91–99}$ vaccine only provided intermediate levels of *Listeria* protection, (~60% CFU reduction, Fig. 2), that was inferior to the protection obtained with the DC vaccines. We wished to test, therefore, whether formulation of GNP-$LLO_{91–99}$ with Advax™ adjuvant might enhance its ability to protect when administered through a traditional vaccine approach. Mice immunised *i.p* with a single dose of GNP-$LLO_{91–99}$ plus Advax™ did indeed exhibit enhanced protection (84%) when compared to mice immunised with GNP-$LLO_{91–99}$ alone (60%) (Fig. 3A). This thereby achieved comparable levels of *Listeria* protection to those previously only achieved with the DC vaccine approach.

Analysis of spleen populations 5 days post-challenge confirmed that *i.p* immunisation with GNP-$LLO_{91–99}$ plus Advax™ adjuvant, induced a similar frequency of mature DC, $CD8^+$ T cells and NK cells and a higher frequency of $CD4^+$ T cells when compared to the DC vaccine, DC-GNP-$LLO_{91–99}$ (Fig. 3B). This suggests that providing it was formulated with Advax™ adjuvant, standard immunisation with GNP-$LLO_{91–99}$ was as good or better at inducing T-cell responses and protection against *Listeria* than the much more complex DC vaccine approach.

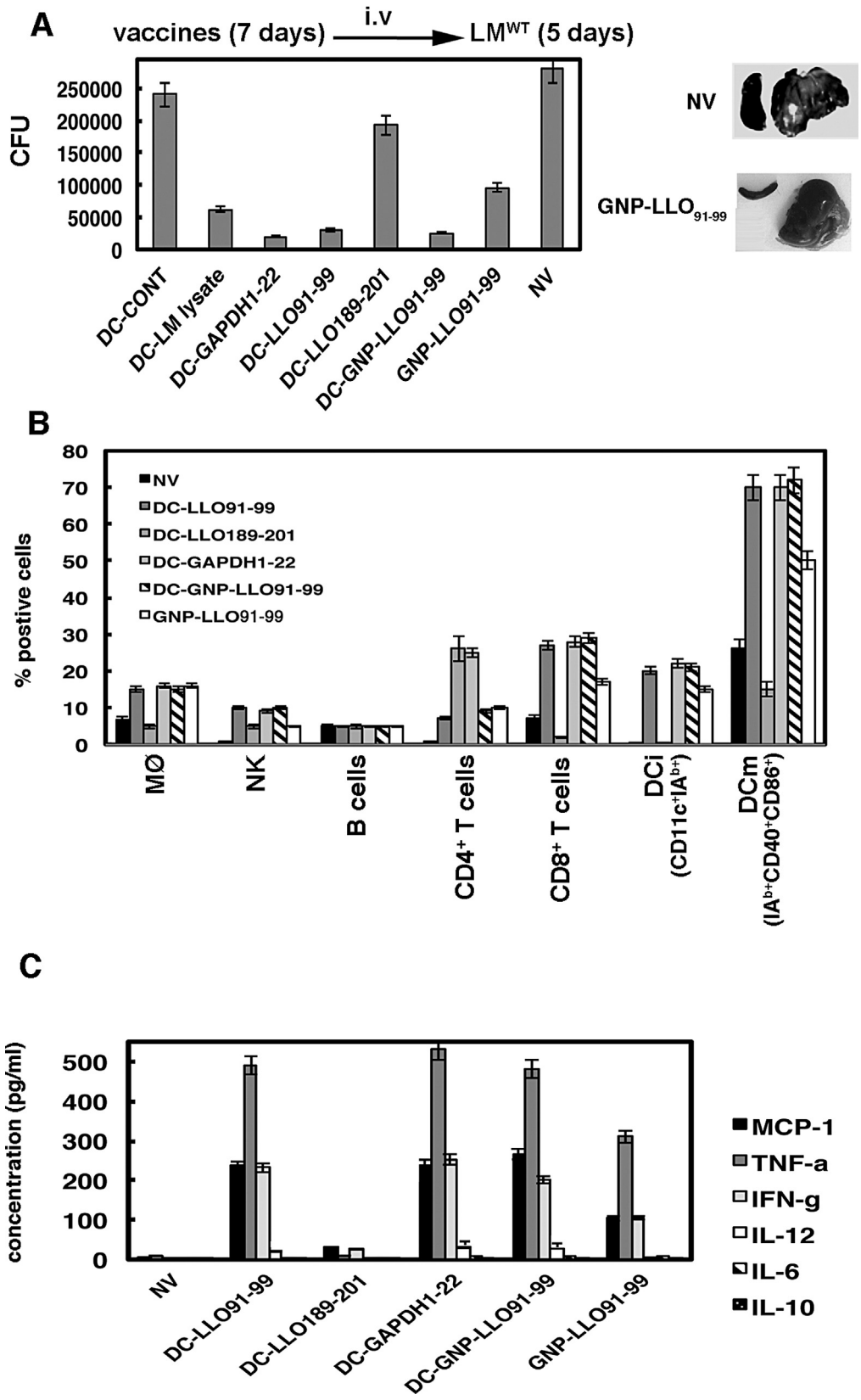

Fig. 2. *GNP-LLO$_{91-99}$ vaccine protects against listeriosis.* DC vaccines were tested for ability to protect against listeriosis. (A) C57BL/6 mice were vaccinated once *i.v* with various DC vaccine vectors (1×10^6 cells) (DC-CONT, DC-LM lysate, DC-GAPDH$_{1-22}$, DC-LLO$_{91-99}$, DC-LLO$_{189-201}$, DC-GNP-LLO$_{91-99}$), soluble GNP-LLO$_{91-99}$ (5 μg/ml) or not vaccinated (NV) ($n = 5$ mice/group) and 7 days later challenged *i.v* with 5×10^3 CFU of *Listeria*. *Listeria* counts in spleen homogenates expressed as CFU (mean ± SD) obtained from triplicate samples of three independent experiments ($P < 0.01$). Images corresponding to spleens and livers of non-vaccinated (NV) *versus* GNP-LLO$_{91-99}$ vaccinated mice (GNP-LLO$_{91-99}$). (B) Percentages of different spleen cell populations are shown: MØs, B cells, NK, CD4T cells, CD8T cells, DCi, DCm quantified in spleen homogenates by FACS. Results expressed as 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, $P < 0.05$.

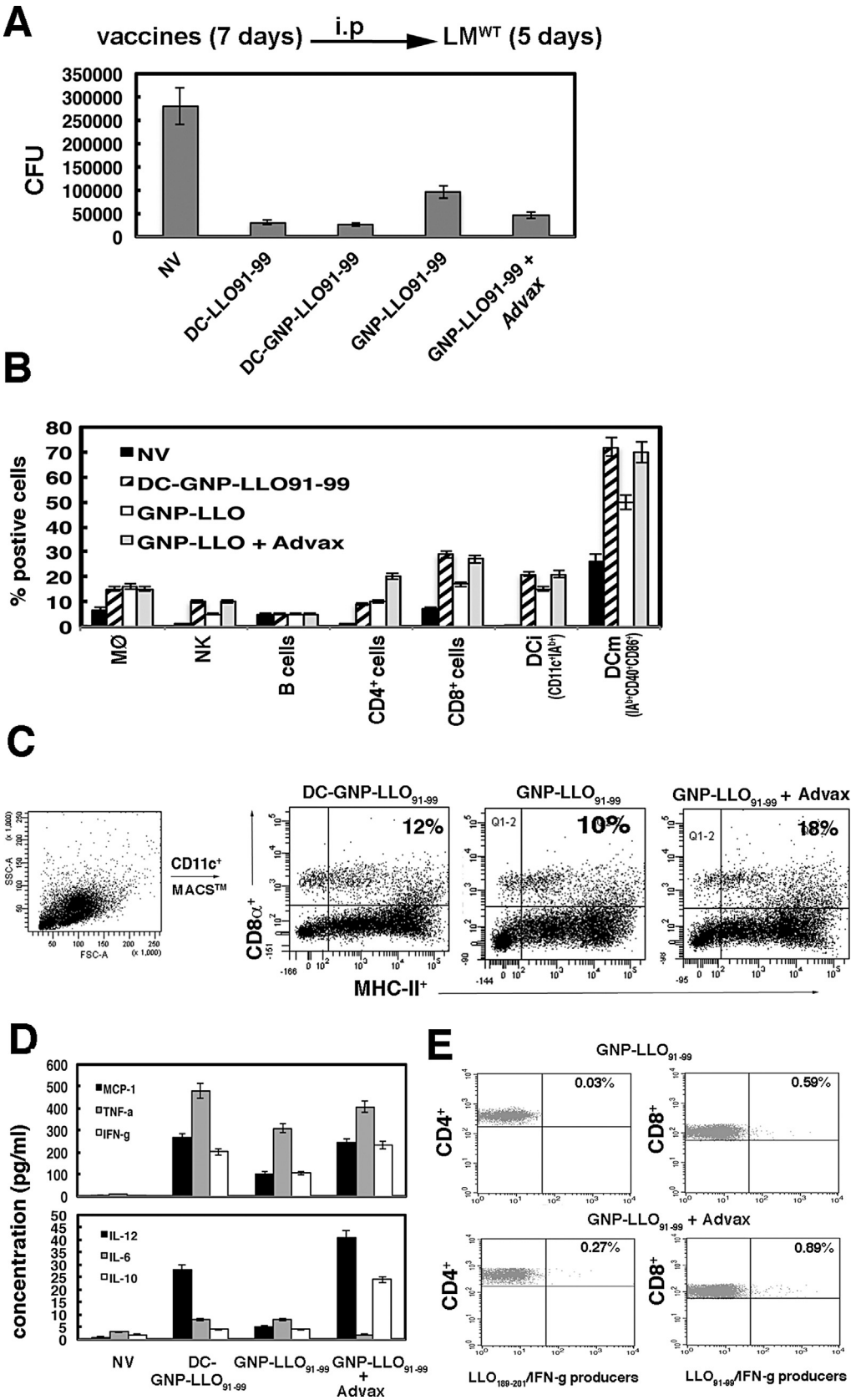

Fig. 3. *Effect of Advax*™ *adjuvant on GNP-LLO*$_{91-99}$ *vaccine protection.* (A) C57BL/6 mice were vaccinated *i.p* with DC-LLO$_{91-99}$ or DC-GNP-LLO$_{91-99}$ (1×10^6 cells), or soluble GNP-LLO$_{91-99}$ (5 μg/ml) with or without Advax adjuvant (1 μg/ml) or not vaccinated (NV) (n = 5 mice/vaccine vector). Seven days post-immunisation they were challenged *i.p* with 5×10^3 of *Listeria* (LM^{WT}). *Listeria* counts in spleen homogenates 5 days post-challenge (CFU mean ± SD) of three independent experiments ($P < 0.01$). (B) Spleen homogenates 5 days post-challenge were analysed by FACS (mean± SD of percentages of positive cells, $P < 0.05$). (C) Splenic DC from mice vaccinated as in ***A*** were analysed to quantitate CD11c$^+$MHC-II$^+$CD8α$^+$ DC (percentages of MHC-II$^+$CD8α$^+$ positive cells (mean ± SD) of three different experiments, $P < 0.05$). (D) Serum cytokine levels 5 days post-challenge in immunised mice (mean ± SD, $P < 0.05$). (E) Mice vaccinated as in (A) and challenged with 5×10^3 *Listeria* (LM^{WT}). Spleen homogenates 5 days post-challenge

3.7. Advax-adjuvanted GNP-LLO$_{91-99}$ increases splenic IL-12$^+$ CD8α$^+$ DC post-challenge

CD8α$^+$ DCs play a critical role in presentation of *Listeria* antigens to protective CD4$^+$ and CD8$^+$ T cells and, in particular, in IL-12 production that correlates with protection [39,40]. We therefore analysed splenic DC content, post-challenge. The frequency of splenic CD8α$^+$ DC was almost doubled from 10% to 18% when GNP-LLO$_{91-99}$ was formulated with Advax^{TM} adjuvant (Fig. 3C). This contrasted with a low frequency (2–3%) of CD8α$^+$ DC in the spleen of non-vaccinated mice post-challenge. Notably, immunisation with free GNP-LLO$_{91-99}$ plus Advax^{TM} adjuvant generated a higher frequency of splenic CD8α$^+$ DC than the DC vaccines (DC-GNP-LLO$_{91-99}$ or DC-LLO$_{91-99}$) (10%) (Fig. 3C). Hence, when formulated with Advax^{TM} adjuvant, standard immunisation with GNP-LLO$_{91-99}$ was as good or better than the more complex DC vaccine approach at inducing splenic CD8α$^+$ DC that play a critical role in listeria protection

3.8. Formulation of GNP-LLO$_{91-99}$ vaccine with Advax^{TM} adjuvant enhances Th1 cytokine responses

Since formulation of vaccines with Advax^{TM} increased the frequency of both CD8$^+$ T cells and CD8α$^+$ DC in the spleen post-*Listeria* challenge, we next examined serum cytokine levels post-challenge in splenocytes of mice immunised with or without Advax^{TM} adjuvant. Mice immunised with GNP-LLO$_{91-99}$ plus Advax^{TM} exhibited significantly greater MCP-1, TNF-α and IFN-γ production when compared to mice immunised with GNP-LLO$_{91-99}$ alone, consistent with Advax^{TM} driving an enhanced Th1-response against *Listeria*. Cytokine levels in mice immunised with GNP-LLO$_{91-99}$ plus Advax^{TM} were equivalent to those seen with the DC vaccine, DC-GNP-LLO$_{91-99}$ (Fig. 3D, upper panel). Immunisation with GNP-LLO$_{91-99}$ plus Advax^{TM} was also associated with significantly greater splenocyte IL-12 and IL-10 production and reduced IL-6 production when compared to immunisation with GNP-LLO$_{91-99}$ alone (Fig. 3D, lower panel), consistent with the Advax^{TM} adjuvant enhancing the Th1 response against *Listeria*.

3.9. Formulation of GNP-LLO$_{91-99}$ with Advax^{TM} adjuvant enhances IFNγ-producing Listeria-specific CD4$^+$ and CD8$^+$ T cells

Next, we evaluated the effect of Advax^{TM} on the ability of standard immunisations with GNP-LLO$_{91-99}$ to induce LLO-specific CD8$^+$ and CD4$^+$ T cells using IFN-γ as a Th1 subset marker. Five days post *Listeria* challenge, mice immunised with GNP-LLO$_{91-99}$ alone had a detectable frequency (0.59%) of LLO$_{91-99}$ specific IFN-γ+ CD8$^+$ T cells, but as expected had no responses against the CD4$^+$ T cell epitope not contained in the vaccine (Fig. 3E, upper panel). Immunisation with GNP-LLO$_{91-99}$ plus Advax^{TM} not only resulted in a significant increase in the frequency of LLO$_{91-99}$ specific IFN-γ+ CD8$^+$ T cells (0.89%) but also resulted in the appearance of a significant number of IFN-γ+ CD4$^+$ T cells recognising the CD4 epitope, LLO$_{189-201}$, not present in the vaccine (Fig. 3E, lower panel). Thus the Advax adjuvant enhanced the ability of the *Listeria* vaccine to prime for a process of T-cell epitope spreading in response to *Listeria* infection.

To confirm the above findings, the ability of Advax^{TM} when added to either DC vaccines or free GNP-LLO$_{91-99}$ vaccine to induce LLO$_{91-99}$-specific CD8$^+$ cells was tested using H2-K^b-LLO$_{91-99}$ dimers to label and identify the antigen-specific T cells (H2-K^d-LLO$_{91-99}$ dimers were used with similar results, data not shown). Addition of Advax^{TM} to GNP-LLO$_{91-99}$ resulted in a 4-fold increase in the frequency of LLO$_{91-99}$-specific CD8$^+$ cells *versus* GNP-LLO$_{91-99}$ vaccine alone, and was superior to all other vaccine formulations including the DC vaccine, DC-GNP-LLO$_{91-99}$ (Table 1, left hand column). The effects of Advax^{TM} were even more pronounced when H2-K^b-LLO$_{91-99}$ positive cells were gated for IFN-γ, with ~10-fold higher frequency of IFN-γ expressing H2-K^b-LLO$_{91-99}$ positive cells in mice vaccinated with GNP-LLO$_{91-99}$ plus Advax *versus* mice vaccinated with free GNP-LLO$_{91-99}$ alone (Table 1, right hand column). This confirmed that providing the vaccine included Advax^{TM} adjuvant, standard immunisation with GNP-LLO$_{91-99}$ was superior to all other vaccine approaches, including use of the best GNP-loaded DC vaccine, at inducing *Listeria*-specific IFN-γ$^+$ CD8$^+$ T cells, the critical cells involved in protection.

4. Discussion

The objective of this study was to identify an optimal vaccine formulation to protect against listerosis. *Listeria* protection is largely dependent on CD4$^+$ and CD8$^+$ T cells rather than antibody-mediated immunity [14,17]. Live vector vaccines are inherently unsuitable for *Listeria* vaccines given their risk of causing serious infection in immune-compromised individuals. As an alternative, cellular DC-based vaccines where DC are loaded with *Listeria* antigens *ex vivo* [6,11,19] and injected back into the host have been proposed for induction of protective cellular immunity. We initially designed a DC vaccine based on loading of DC with LLO$_{91-99}$ peptide using conjugated GNP (GNP-LLO$_{91-99}$) and showed that this provided a high efficiency of DC loading, *in vitro* [22, 23]. DC-GNP-LLO$_{91-99}$ induced CD8$^+$ T cell immunity in immunised mice that translated into protection against *Listeria* challenge. However DC vaccines are impractical and expensive to make, necessitating that we find an alternative strategy for delivery of our GNP-based vaccine. To overcome these problems, we next tested whether standard immunisation with GNP-LLO$_{91-99}$ rather than using it to load DC *in vitro*, could target and load DC *in vivo*. However, only partial protection was obtained by standard immunisation with free GNP-LLO$_{91-99}$ vaccine, inferior to that obtained with the DC vaccine. To overcome this problem, we next formulated the free GNP-LLO$_{91-99}$ antigen with Advax^{TM}, a polysaccharide adjuvant shown effective and safe in a broad range of animal models and human trials [25–31,33–35]. In addition to a strong human safety record, Advax^{TM} was selected based on its ability to induce robust CD4$^+$ and CD8$^+$ T cell responses [30,35]. As a proof of principle we first showed that Advax^{TM} enhanced the immunogenicity of the DC vaccine, GNP-LLO$_{91-99}$. This finding suggests Advax adjuvant may offer benefits for DC vaccines for other indications such as cancer, where the cost and complexity of DC vaccines is less an issue. However, given our desire to move away from the DC vaccine approach for *Listeria*, the most important finding was that Advax^{TM} significantly enhanced the immunogenicity of GNP-LLO$_{91-99}$ when administered as a standard vaccine. Notably, this translated into improved *Listeria* protection to a level we had previously only achieved using DC vaccines. The enhanced protection obtained with the Advax-adjuvanted GNP-LLO$_{91-99}$ vaccine was associated with an increased frequency of splenic CD4$^+$ and CD8$^+$ T cells, NK cells and CD8α$^+$ DC. The latter have been reported to play a critical role in *Listeria* protection *via* ability to produce IL-12 and activate IFN-γ producing CD4$^+$ and CD8$^+$ T cells [39,40]. Notably, immunisation with Advax-adjuvanted GNP-LLO$_{91-99}$ enhanced production of the Th1 cytokines, IL-12, IFN-γ, TNF-α, and MCP-1, while reducing the Th2 cytokine, IL-6. Interestingly, immunisation with Advax-adjuvanted

were stimulated for 5 h with LLO$_{91-99}$ or LLO$_{189-201}$ peptides then intracellular cytokine staining performed. Histograms show the frequency of LLO$_{189-201}$-specific CD4$^+$ T cells and frequency of these making IFN-γ producers (left plots) and the frequency of LLO$_{91-99}$-specific CD8$^+$ T cells and frequency of these making IFN-γ (right plots). Experiments were performed in triplicate and results are expressed as mean ± SD ($p < 0.05$).

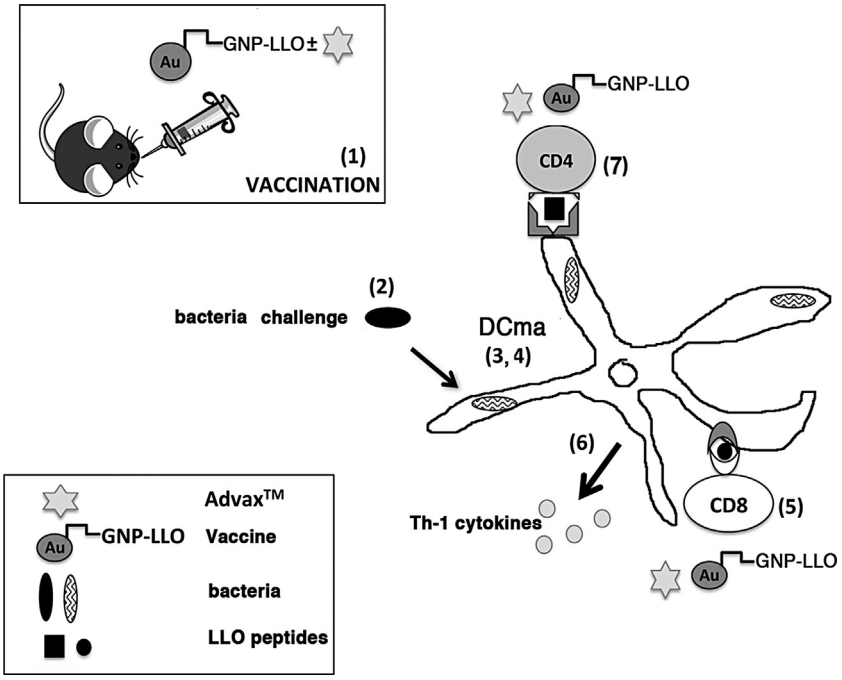

Fig. 4. *Hypothetical model to explain the effect of Advax™ adjuvant on GNP-LLO$_{91-99}$ protection against listeriosis.* Diagram depicting predicted events when mice are vaccinated with soluble GNP-LLO$_{91-99}$ carriers in the presence or absence of Advax™ adjuvant (step 1) and then challenged with live *Listeria* (step 2). Explanation of the model is described in text of the Section 4.

GNP-LLO$_{91-99}$ was also associated with increased production of the anti-inflammatory cytokine, IL-10, but unlike other studies this increased IL-10 was not associated with enhanced *Listeria* susceptibility [32]. IL-10 may normally act as an inverse marker of IFN-γ production as IL-10 is downregulated by IL-12 and IFN-γ and *vice versa*. Because IL-12 and IFN-γ are dominant in determining *Listeria* protection and were both markedly increased by Advax-adjuvanted GNP-LLO$_{91-99}$, the IL-10 even although increased would not be expected to block IFN-γ-mediated protection in this situation, but may actually have been beneficial by reducing inflammatory damage in response to *Listeria* infection. Immunisation with Advax-adjuvanted GNP-LLO$_{91-99}$ resulted in an increased frequency of LLO$_{91-99}$ specific IFN-γ$^+$ CD8$^+$ T cells, post-challenge. More surprising was the increased frequency of LLO$_{189-201}$ specific IFN-γ$^+$ CD4$^+$ T cells, as this CD4$^+$ epitope was not present in the vaccine the mice received so these CD4$^+$ T cells must have been generated in response to the *Listeria* itself. This suggests that mice immunised with Advax™ adjuvant have a greater capacity for T-cell epitope spreading after *Listeria* challenge, thereby explaining the broader T-cell recognition of *Listeria* epitopes in the mice that received the Advax-adjuvanted GNP-LLO$_{91-99}$ vaccine.

Our model for how vaccination with Advax-adjuvanted GNP-LLO$_{91-99}$ provides enhanced protection against listeriosis is depicted in Fig. 4. In brief, injection of soluble GNP-LLO$_{91-99}$ carriers (step 1) targets the conjugated peptide to conventional splenic DC that are stimulated to a mature phenotype and can then prime LLO$_{91-99}$-specific CD8$^+$ T cells (step 3). Upon exposure to *Listeria* (step 2), these CD8$^+$ T cells become activated and produce IFN-γ that in turn enhances DC IL-12 production and consequent NK cell activation. A Th1 feedback loop is generated, which assists the induction of inflammatory and activated DC and MØ that phagocytose and kill the bacteria (step 4). Advax™ adjuvant recruits and interacts with DC resulting in an increase in mature DC and, in particular, CD8α$^+$ DC, which after loading with GNP-conjugated LLO$_{91-99}$, migrate to the splenic white zones where they become activated and induce expansion of *LLO*-specific CD4$^+$ and CD8$^+$ T cells (step 5). Upon exposure to *Listeria*, these LLO-specific CD8$^+$ T cells become activated and induce high levels of IFN-γ, IL-12, MCP-1, and TNF-α. These Th1 cytokines enhance *Listeria* phagocytosis and destruction, while increased IL-10 may help prevent excess inflammation (step 6). CD8$^+$ T cells generated by the Advax-adjuvanted vaccine enhance epitope spreading by inducing DC to express additional *Listeria* epitopes, thereby allowing recruitment of additional CD4$^+$ T cells able in response to these MHC-II epitopes. This promotes further expansion and activation of *LLO*-specific T cells including IFN-γ producing LLO$_{189-201}$-specific CD4$^+$ T cells (step 7) that synergise with IFN-γ-producing CD8$^+$ T cells in *Listeria* control.

Overall, these results support further development of GNP-peptide carriers together with Advax™ adjuvant as a vaccine platform with potential applicability to prophylactic immunisation against *Listeria*, plus other broader uses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.01.062.

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