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Original Article

The intact structural form of LLO in endosomes cannot protect against listeriosis

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Abstract: LLO is the major immuno-dominant antigen in listeriosis and is also required for protective immunity. Two forms of LLO can be observed in endosomal membranes, a LLO intact form and a Ctsd-processed $LLO_{1\text{-}491}$ form. Endosomes obtained from resting macrophages contained only LLO intact forms, while endosomes obtained from IFN-activated macrophages contained both forms. Both types of endosomes elicited $LLO_{90\text{-}91}/CD8^+$ and $LLO_{189\text{-}201}/CD4^+$ specific immune responses. However, only endosomes containing the Ctsd-processed $LLO_{1\text{-}491}$ form showed significant $CD4^+$ and $CD8^+$ T cell responses similar to LM infected bone marrow derived macrophages and characteristic of protective *Listeria* immunity. Moreover, endosomes with intact LLO could not confer protection as vaccine carriers against murine listeriosis. While endosomes with Ctsd-processed $LLO_{1\text{-}491}$ form showed a moderate ability, slightly lower than high efficiency vaccine vectors as MØ infected with LM. These studies argue that all cell-free membrane vesicles might serve as valid vaccine carriers against infectious agents. Exclusively those cell-free vesicles MIIC competent for LLO processing are protective vaccines vectors since they recruit significant numbers of mature dendritic cells to the vaccination sites and contain a $LLO_{1\text{-}491}$ form that might be accessible for MHC class I and class II antigen presentation.

Keywords: LLO structural forms, listeriosis, endosomes, vaccines

Introduction

Listeria monocytogenes (LM) elicits a potent and multifactor innate and adaptive immune response that makes this pathogen an attractive vector for vaccine designs [1]. LM immune response and protection ability depends on the main virulence factor listeriolysin O (LLO) that triggers a Th1 based immune response. In this regard, LLO is the major immunodominant antigen able to elicit both $CD4^+$ and $CD8^+$ T cells restricted immune responses [2]. High dose inoculation of mice with LLO-deficient bacterial mutants might prime $CD8^+$ T cells and protect against listeriosis, while wild type LM need only low doses to confer protection [3]. Safety concerns are critical when considering LM vaccines as a clinical alternative, even when using bacterial mutants with attenuated virulence. First, listeriosis is a serious and potentially fatal disease, especially in immunocompromised fellows. Second, the potential cytotoxicity of LLO decreases the chances to be used as a direct DNA-vaccine or protein-vaccine. However, efficiency of LM vectors in treating neoplastic diseases or other infectious diseases in which a Th1-based immune response is required, suggest that new *Listeria*-based vaccines should be prepared [4].

Cellular based vaccines such as dendritic cells (DC) or macrophages (MØ) have showed good vaccination efficiencies against infectious agents [5, 6]. Moreover, MØ seem to co-operate with DC and transfer them processed antigens, a mechanism known as cross-presentation [7, 8]. However, for ethical reasons the use of cell lines in immunization protocols in humans is not feasible either. Therefore, cell-free membrane vesicles are a good alternative as vaccine

vehicles.

In listeriosis a major issue is the priming of listericidal $CD8^+$ or $CD4^+$ T cells by antigen-presenting cells (APC) as a requirement to develop protective immunity [9]. Recently, DC emerged as the APC responsible for priming $CD8^+$ T cells [10]. On the other hand, MØ prime $CD4^+$ T cells directly and $CD8^+$ T cells by cross-presentation to DC. The LLO elicited response after LM infection revealed that LLO_{91-99} is the dominant MHC-class I restricted epitope activating $CD8^+$ T cells and $LLO_{189-201}$ is the MHC-class II restricted epitope that generates the strongest $CD4^+$ T cell response [11, 12].

LLO antigen processing should precede the formation of these immunodominant epitopes. In this regard, MØ antigen processing is performed in endosomes as they contain proteases to degrade antigens into peptides that load MHC class I and class II molecules. We have recently reported that endosomes were able to digest LLO by the action of the protease cathepsin-D [13]. We localized two LLO forms in these endosomal membranes, an intact LLO and a Ctsd-processed LLO_{1-491} form. Here we have studied the possibility that endosomes containing either one of these LLO forms might mimic the immune response elicited by the pathogen and confer protection against listeriosis. We compare the efficiencies as vaccines of different endosomes as cell-free vesicles with vaccination protocols using LM infected MØ.

Materials and methods

Cells and cytokine treatment

Bone-marrow derived macrophages (BM-DM) were obtained from femurs of 8-12 week old female CBA/J mice (Taconic Farm. Denmark). Bone-marrow derived cells were cultured in DMEM-20%FCS-1mM glutamine-1 mM NEAA-25 ng/ml M-CSF-50 µg/ml gentamicin-30µg/ml vancomycin (D20) in bacteriological dishes for 7 -days to differentiate into MØ (BM-DM) [14]. Murine recombinant IFN- (IFN-) was obtained from Sigma and cells were treated 24 hours with 10 ng/ml of this cytokine before endosome isolation.

FACS analysis of spleens, measurement of IFN- intracellular and peritoneal exudate cells (PEC)

We used FACS analysis for cell surface labelling of splenocytes or PEC (100.000 cells), using monoclonal antibodies FITC or PE-labelled (BD. Biosciences) against the following markers: CD11b, CD11c, Ly6C, Ly6G, Dx5, IA^k, F4/80, CD4 or CD8. Peptides used were LLO_{1-99} and $LLO_{189-201}$. For in vitro culture, splenocytes were plated into 96-well round-bottom plates (5 x 10^6 cells/ml) and stimulated with the indicated peptides (10^{-6} M) for 5 h (intracellular cytokine staining) as described [15]. Stimulated cells were surface stained for CD4 and CD8 and then fixed and permeabilized using a cytofix/ cytoperm kit (BD Biosciences, San Jose, CA). Cells were stained for IFN- with anti-body anti-IFN-PE-labelled. Samples were acquired using a FACSCanto flow cytometer (BD. Biosciencies). Data were gated to incluye exclusively $CD4^+$ or $CD8^+$ events, and the percentages of these cells expressing IFN- was determined according with the manufacturers recommendations (BD. Biosciences). Data were analyzed using FlowJo software (Treestar, Ashland, OR).

Bacteria

Listeria monocytogenes 10403S strain (LM) was obtained from D.A. Portnoy (UCLA. La Jolla. CA) and Escherichia coli expressing recombinant N-terminal-tagged His-LLO_{529} full length protein (LLO) was also kindly provided by D.A. Portnoy and cultured in LB medium containing 30 mg/ml kanamycin [16].

Recombinant LLO preparation and LLO peptides

Recombinant, full-length LLO (LLO) was expressed in large quantities as His-fusion protein in *E. coli* strain BL21 upon induction with 1 mM IPTG for 5 h at 37°C. His-tagged recombinant proteins were purified with TALON resin, according to the manufacturer's instructions (Clontech). LLO_{1-99} and $LLO_{189-201}$ were synthesized by *F. Roncal* (CNB. CSIC. Madrid) with a purity higher than 95% after HPLC and Mass Spectrometry.

Endosome isolation

Isolation of LLO loaded endosomes from BM-DM was previously reported [13]. In brief, BM-DM pre-treated or not with mIFN- for 24 h (2 x 10^8 cells) were incubated with 300 µg/ml recombinant LLO for 15 min for endosome isolation. Cells were homogenized in homogenization buffer-EDTA (HBE) (250 mM sucrose/0.5 mM

EGTA/20 mM HEPES-KOH, pH 7.2) to obtain post-nuclear fractions (PNS), quickly frozen in liquid nitrogen and stored at -80°C. To obtain endosomal fractions, thawed PNS were quickly diluted 1/20 in HBE, centrifuged at 37000 *g* for 1 min and the resulting supernatants were centrifuged at 50.000 *g* for 5 min. To prepare extracted endosomal membranes (M-Endo), endosomes were permeabilized with 0.5% Triton X -100 for 15 min on ice, centrifuged for 25 min (50.000 *g* for endosomes). The recovered supernatants contained the endosomal membranes as previously described [13]. The purity of the organelles was monitored by biochemical analysis and protein concentrations were analysed using BCA reagent. All BM-DM preparations comprised less than 2% plasma membrane, 0.2% Golgi and 0.25% endosomal contamination, and contained protein at a concentration of approximately 100 mg/ml.

Haemolytic assay

The haemolytic activity of different vaccine types was determined by the level of haemoglobin released from 0.5% cultures of sheep red blood cells (SRBC) and compared with the activity obtained with 300 μg/ml recombinant LLO as previously described [13]. In brief, 30 μg of BM-DM treated or not with IFN- and incubated with the following reagents: LLO (300 μg/ml) for 15 min at 4ºC (bound), LLO (300 μg/ml) for 15 min at 37ºC (internalized), LM infected as in the infection protocols or 30 μg of lysates from endosomal preparations containing LLO from BM-DM treated or not with IFN-. Meanwhile, SRBC (0.5%) were prepared by washing five times with saline solution until the absorbance measured at OD_{450} was less than 0.1. Thereafter, the different vaccine types prepared and the recombinant LLO were added to 0.5% SRBC solution and incubated in 96-well plates in a total volume of 200 ml for 30 min at room temperature. After centrifugation at 750 *g* for 10 min, 50 ml of the supernatant was collected and the absorbance measured as described above. Haemolytic units are defined as the dilution of the sample that causes 50% of haemoglobin release from 200 ml of 0.5% SRBC. Controls included were 0% haemolysis using the cell culture medium and for 100% haemolysis by incubating SRBC with distilled water.

Western and immune-precipitation assays

30 μg of isolated endosomes were loaded per lane onto SDS-PAGE. Gels were transferred onto NC membranes. Primary antibodies were incubated o/n at 4ºC: rabbit anti-MHC II cytoplasmic chain, rabbit polyclonal anti-LLO specific antibody (Diatheva), 4F11 (mouse monoclonal anti-Rab5a), rabbit anti-cathepsin-D and rabbit anti-Rac2 (kindly provided by G. Bockoch. UCLA. CA. USA). Thereafter, secondary antibodies horseradish peroxidase conjugated (Jackson Laboratories) and developed by ECL (Amersham). To detect LLO bound to MHC class II molecules, endosomal lysates were immunoprecipitated with mouse anti-IA^k antibody (10. 3. 62). Immunoprecipitates were run onto SDS-PAGE and transferred to NC membranes. Primary antibody (rabbit anti-LLO) was incubated o/n at 4ºC, followed by incubation with secondary antibody horse-radish peroxidase (HRP) conjugated. Western blots were developed by ECL.

Vaccination protocol

30 μg of LLO loaded endosomes from IFN-treated (ENDO-IFN/LLO) or not BM-DM (ENDO/LLO) or LM infected BM-DM (1 x 10^6 cells) pretreated or not for 24 h with mIFN-, were intraperitoneal (*i.p)* inoculated into 8-12 weeks old female CBA/J mice (n = 5 per condition) for 7 days or non-vaccinated (NV). Next, all mice were inoculated with 5 x 10^3 LM for 3 additional days. Peritoneal cells (PEC) were obtained after Hank´s wash of the peritoneal cavitiy and labelled with antibodies against the following cell surface markes: CD11b, CD11c, Ly6C, Ly6G, Dx5, IA^k and F4/80 and analyzed by FACS. Spleens were homogeneized and cell populations analyzed by FACS analysis and CFU counted in homogenates. Results represent the mean ± SD of three different experiments. Statistics were applied.

Statistical analysis

For statistical analysis, the Student's *t* test was applied and *p* values calculated as significance.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Ministry of Science, Research and Innovation. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Cantabria (Permit Number: 2009/12) following the spanish legislation (RD

Table 1. Cytotoxicity activity of different vaccine carriers.

Vaccine type	% Haemolytic activity[a]
LLO	100 ± 03
[b]BM-DM/LLO^{bound}	80 ± 02
BM-DM-IFN /LLO^{bound}	80 ± 05
BM-DM/LLO^{int}	51 ± 02
BM-DM-IFN/LLO^{int}	50 ± 04
BM-DM/LM	2 ± 005
BM-DM-IFN/LM	3 ± 003
ENDO/LLO	1 ± 005
ENDO-IFN/LLO	2 ± 002

[a]SRBC haemolytic analysis after treatment with 300 μg/ml of recombinant LLO or the different vaccine types and performed as in *Materials and Methods*. Results are expressed as percentage of haemolytic activity compared with 100% haemolysis ± SD. Results are the mean ± SD of triplicate samples ($p < 0.05$). [b]The different vaccine types to be tested for their haemolytic activity were the following: BM-DM/LLO^{bound} corresponds with untreated BM-DM incubated for 15 min with 300 μg/ml of LLO at 4°C, BM-DM-IFN/LLO^{bound} corresponds with IFN-γ treated BM-DM incubated for 15 min with 300 μg/ml of LLO at 4°C, BM-DM/LLO^{int} corresponds with untreated BM-DM incubated for 15 min with 300 μg/ml of LLO at 37°C, BM-DM-IFN/LLO^{int} corresponds with IFN-γ treated BM-DM incubated for 15 min with 300 μg/ml of LLO at 37°C, BM-DM/LM corresponds with untreated BM-DM infected with LM, BM-DM-IFN/LM corresponds with IFN-γ treated BM-DM infected with LM, ENDO/LLO corresponds to 30 μg of isolated endosomes from untreated BM-DM loaded with LLO and ENDO-IFN/LLO corresponds to 30 μg of isolated endosomes from IFN-γ treated BM-DM loaded with LLO.

1201/2005). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Results

The LLO structural forms of endosomes show no citotoxicity but different immune features to be used as vaccines

BM-DM infected with LM or incubated with LLO may be toxic to be used as vaccine vehicles since LLO is a pore-forming protein able to lyse membranes and cause tissue damage. Therefore, we first check the haemolytic activity onto sheep red blood cells (SRBC) of different LM related vectors that may be used as vaccines. We tested the following vaccine vectors: BM-DM treated or not with IFN- and either infected with LM or surface loaded with LLO (300 μg/ml of LLO, 15 min at 4°C) or internalized with LLO (300 μg/ml of LLO, 15 min at 37°C) or endosomes prepared from BM-DM treated or not with IFN- and loaded with LLO (300 μg/ml of LLO, 15 min at 37°C and then endosomes isolated) [13]. We also used recombinant LLO as a positive control of 100% haemolysis. We observed that while BM-DM infected with LM (BM-DM/LM or BM-DM-IFN/LLO rows in **Table 1**) or endosomes loaded with LLO (ENDO/LLO or ENDO-IFN/LLO rows in **Table 1**) caused very low haemolysis, barely 2-3%, BM-DM surface loaded with LLO (BM-DM/LLO^{bound} or BM-DM-IFN/LLO^{bound} rows in **Table 1**) showed 80% haemolysis and BM-DM that internalized LLO for 15 min (BM-DM/LLO^{int} or BM-DM-IFN/LLO^{int} rows in **Table 1**) presented 50-51 % haemolysis. Since LLO can be ingested by receptor-mediated endocytosis as the case of Toll-like receptors or by pinocytosis, the above-mentioned results suggested that surface bound LLO retains the haemolytic activity. Interestingly, LLO loaded in endosomes caused no haemolysis at all, suggesting that the haemolytic activity of LLO after 15 min of internalization observed in BM-DM with internalized LLO, might correspond with a percentage of LLO remaining as surface bound. The lack of haemolysis observed in BM-DM infected with LM indicated that the production of active LLO after 1 hour of infection should be minimal. These overall data focussed our interest to the following vaccine vectors, endosomes loaded with LLO and BM-DM infected with LM due to their safer properties for tissue integrity, as they cause almost no haemolysis.

Vaccine designs containing pathogenic LM are highly questioned for human use, therefore, endosomes are cell-free vesicles, non-toxic and with appealing vaccine features as antigen presenting compartments (MIIC) that might be postulated for human vaccine designs. Among the MIIC characteristics of endosomes, we can highlight the following ones, MHC II molecules loaded with peptides, proteases participating in antigen degradation such as cathepsin-D (Ctsd) and markers of MIIC compartments such as the endosomal regulator Rab5a [17]. In this regard, endosomes loaded with LLO and obtained from IFN- treated or not BM-DM represented two con-

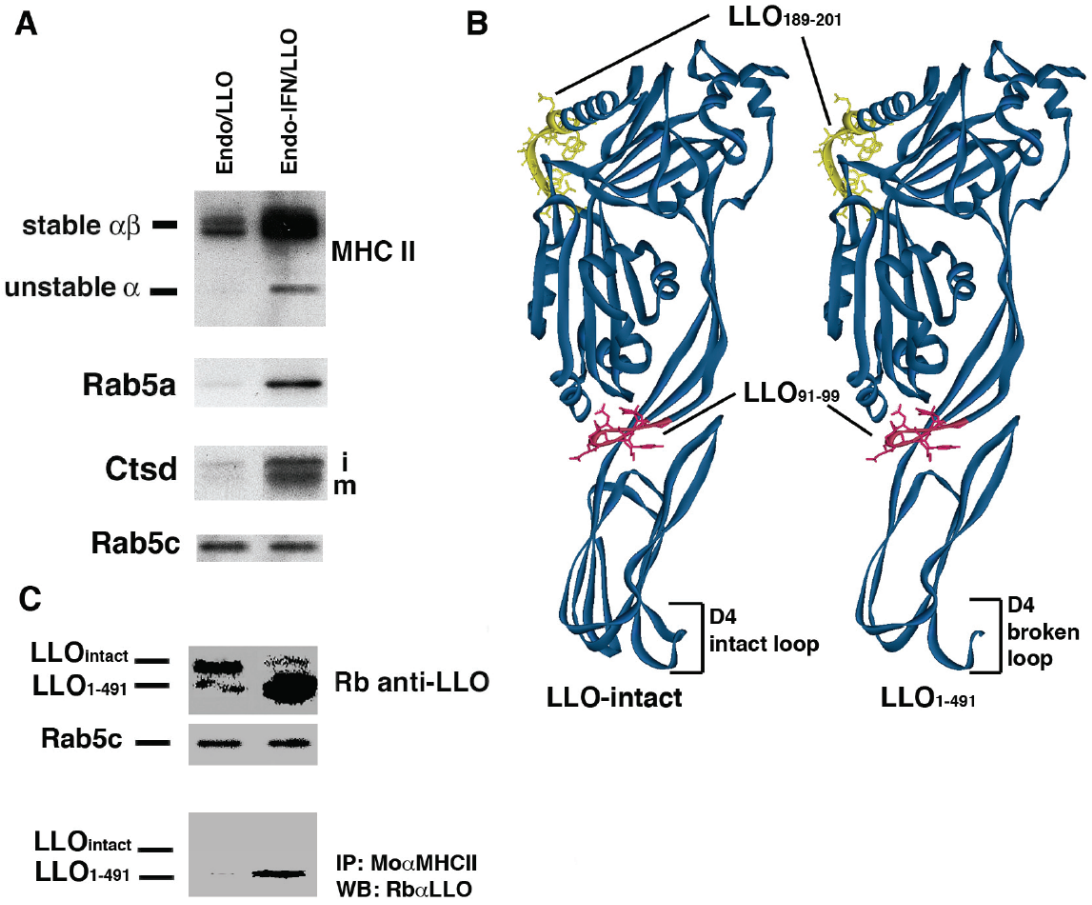

Figure 1. Features of the two LLO structural forms in endosomes. Endosomes were obtained from BM-DM (2 x 10^8 cells) pre-treated (Endo-IFN/LLO) or not (Endo/LLO) with mIFN-γ followed by incubation with 300 μg/ml of LLO. **A**, endosomal membranes (30 μg) were prepared as described in Materials and Methods and loaded on SDS-PAGE gels, transferred and western-blots developed with rabbit anti-MHC II α chain, rabbit anti-Rab5c, 4F11 monoclonal anti-Rab5a or rabbit anti-Ctsd antibodies. Rab5c is used as control for protein loading. **B**, 3D model structures represent LLO intact (right structure) and Ctsd-degraded $LLO_{1\text{-}491}$ from (left structure). MHC-I immuno-dominant epitope $LLO_{91\text{-}99}$ is shown in red and MHC-II immuno-dominant epitope $LLO_{189\text{-}201}$ in yellow. **C**, Upper lane labelled as Rb anti-LLO shows a western-blot of Endo/LLO and Endo-IFN/LLO endosomes that evaluate the levels of LLO intact and Ctsd-degraded $LLO_{1\text{-}491}$ forms in these vesicles. As loading control of these lysates we include Rab5c as in Figure 1A. Lower lanes correspond to immuno-precipates of Endo/LLO and Endo-IFN/LLO endosomes with mouse anti-MHC-II (IA^k) (10.3.62 antibody) and western-blot developed with Rb anti-LLO antibody.

ditions of high (ENDO-IFN/LLO) and low expression (ENDO/LLO) of MIIC markers, respectively. The analysis of SDS-stable MHC II dimers is a valid measurement of MHC II molecules loaded with peptides [18, 19], we observed (**Figure 1A**) that LLO loaded endosomes from resting BM-DM contained low levels of SDS-stable MHC II dimers (Endo/LLO). However, LLO loaded endosomes from IFN- activated BM-DM (Endo-IFN/LLO) contained significant levels of SDS-stable MHC II dimers. These results strongly argue that only LLO loaded endosomes from IFN- activated BM-DM are MIIC competent, while LLO loaded endosomes from resting BM-DM are not. We also confirmed that LLO loaded endosomes from IFN- activated BM-DM contained high levels of other markers of MIIC compartments such as Rab5a or matured forms of active Ctsd [13]. These results were specific since the expression of Rab5c was unmodified in both vesicles and served as protein loading control. Next, we confirmed that these vesicles were competent for antigen processing following the levels of two LLO structural forms after Ctsd degradation.

This approach was previously reported by our group to distinguish vesicles with adequate LLO processing function or low processing capability depending on the levels of LLO_{1-491} Ctsd-degraded forms [13]. Ctsd-mediated LLO degradation occurs between residues 491-WW-492. This LLO structural form lacks the loop in D4 domain responsible for membrane binding and the characteristic cytotoxicity of this pore-forming protein (**Figure 1B**, left 3D model). The LLO intact structural form (**Figure 1B**, right 3D model) conserves the integrity of this loop in D4 domain and retains membrane binding and cytotoxicity. In this regard, LLO loaded endosomes from resting BM-DM present mainly LLO intact forms (~60 kDa), while LLO loaded endosomes from IFN- activated BM-DM contain mainly the LLO_{1-491} Ctsd-degraded forms (~52 kDa) (**Figure 1C**, Rb anti-LLO lanes). These results were not explained by differences in LLO uptake by BM-DM treated or not with IFN- since BM-DM lysates from both cell types showed similar amounts of LLO intact and LLO degraded forms (*data not shown*). We next examined which LLO form was co-precipitated with MHC class II molecules by performing an immunoprecipitation with a monoclonal anti-MHC class II antibody, following by western blot with the specific rabbit anti-LLO antibody. Only the LLO_{1-491} molecular form (~52 kDa) co-precipitated with MHC class II molecules in LLO loaded endosomes from IFN- activated BM-DM (**Figure 1C**, IP: Mo MHCII and WB: Rb LLO lane). While LLO loaded endosomes from resting BM-DM contains LLO intact forms that do not co-precipitate with MHC class II molecules. These results suggest that LLO loaded endosomes from IFN- activated BM-DM are good LLO processing compartments containing the Ctsd-degraded LLO_{1-491} form able to bind to MHC class II molecules. On the contrary, LLO loaded endosomes from resting BM-DM were poor LLO processing compartments containing a LLO intact form unable to bind to MHC class II molecules.

3D structural modelling of both LLO forms allowed us to hypothesize that Ctsd-degraded LLO forms lacking the membrane loop in D4 domain, appeared with higher accessibility to the LLO immuno-dominant epitope LLO_{91-99} specific for MHC class I molecules (**Figure 1B**, right 3D model labelled as pink amino acids). In fact, LLO_{91-99} located in D2 domain is in close proximity to the broken loop of D4 domain and suggest that presentation of LLO_{91-99} epitope to MHC class I molecules is favoured in LLO loaded endosomes from IFN- activated BM-DM. This hypothesis supports that LLO immunodominant epitope of MHC class II molecules, $LLO_{189-201}$, might show similar accessibility in LLO intact and Ctsd-degraded LLO forms (**Figure 1B**, 3D models labelled as yellow amino acids). The poor LLO processing abilities and lack of LLO co-precipitation with MHC class II molecules observed in LLO loaded endosomes from resting BM-DM, suggested that $LLO_{189-201}$-MHC class II dimers have not formed in these vesicles. LLO loaded endosomes from IFN- activated BM-DM appeared to show all features for the generation of $LLO_{189-201}$-MHC class II dimers, meaning good abilities for LLO processing and LLO_{1-491} co-precipitation with MHC class II molecules. Therefore, we hypothesized that LLO loaded endosomes from IFN- activated BM-DM are efficient for LLO presentation to MHC class I and class II molecules. However, LLO loaded endosomes from resting BM-DM are inefficient for LLO presentation to either MHC class I or class II molecules.

Vaccination against murine listeriosis using different cell-free vesicles compared to vaccination with LM infected BM-DM

Next, we used these two types of endosomes with different LLO processing and presentation abilities to MHC class I and class II molecules as vaccine carriers against a sub-lethal dose of LM infection. We also used as positive control resting or IFN- activated BM-DM and infected with pathogenic LM, since similar vaccination protocols have been reported as efficient vaccines against different infectious agents [5]. In this regard, vaccination of mice with LM infected resting or IFN- activated BM-DM conferred a 25-35 fold higher protection (**Figure 2**, BM-DM/LM and BM-DM-IFN/LM bars, respectively) than non-vaccinated mice (NV). Therefore, BM-DM infected with LM seems good vaccine vehicles able to protect against LM infection. Vaccination with LLO loaded endosomes from IFN- activated BM-DM showed a moderate efficiency with 3.5 fold values of protection (**Figure 2,** ENDO-IFN/LLO bars). However, LLO loaded endosomes from resting BM-DM were inefficient vaccine vectors as they barely confer protection against LM infection (**Figure 2**, ENDO/LLO bars). We confirmed the endosomal requirement since lysis of the endosomes before mice inoculation caused no protection at all (data in legend, **Fig-**

ure 2). Significance of these findings was obtained after applying statistics with the *t* student's tests to three different experiments and calculations of *p* values ($p < 0.05$).

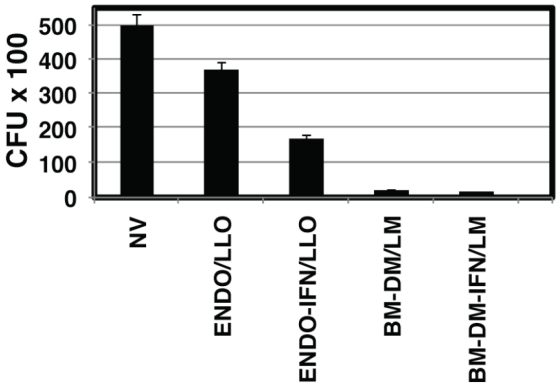

Figure 2. Vaccination against murine listeriosis using endosomes compared to vaccination with LM infected BM-DM. CBA/J mice were vaccinated *i.p* or not (NV) with 30 µg of LLO loaded endosomes from resting BM-DM (ENDO/LLO), LLO loaded endosomes from IFN-γ activated BM-DM (ENDO-IFN/LLO), LM infected resting BM-DM (1 x 10^6 cells) or LM infected and IFN-γ activated BM-DM (1 x 10^6 cells) for 7 days (n = 5/vaccination type). Next, mice were inoculated with LM (5 x 10^3 bc/mice) for 3 days. Spleens were homogenized and CFU counted in blood agar plates. The results are the mean ± SD of three different experiments ($p < 0.01$).

These results suggested that cell-free vesicles are competent protective vectors, only if they show LLO processing abilities and competence for MHC class I and class II antigen presentations.

Specific immune responses elicited by the different vaccine vectors

To explain the failure of LLO loaded endosomes from resting BM-DM as protective vectors, we examined the LLO specific immune response elicited in vaccinated mice. Listericidal $CD4^+$ and $CD8^+$ T cells that produce IFN- mediate protective T cell immunity against LM. The most immunodominant $CD8^+$ specific LLO epitope corresponds to $LLO_{91\text{-}99}$ and the $CD4^+$ immunodominant LLO epitope corresponds to $LLO_{189\text{-}201}$ [7, 8]. Therefore, we analyzed the percentage of $CD8^+$ T cells specific for the epitope $LLO_{91\text{-}99}$ and the percentage of $CD4^+$ T cells specific for the epitope $LLO_{189\text{-}201}$ in the spleens of vaccinated mice. The approach measures the IFN- intracellular of $CD4^+$ or $CD8^+$ T cells specific for each LLO peptide as a read out of LLO specific T cell responses [15]. The procedure is detailed in **Figure 3** showing the ENDO/LLO data as example. First, $CD4^+$ and $CD8^+$ cells are selected and from each set (upper right flow histogram in **Figure 3**), next, the $CD4^+/LLO_{189\text{-}201}$ IFN- producers and $CD8^+/LLO_{91\text{-}99}$ IFN- producers are

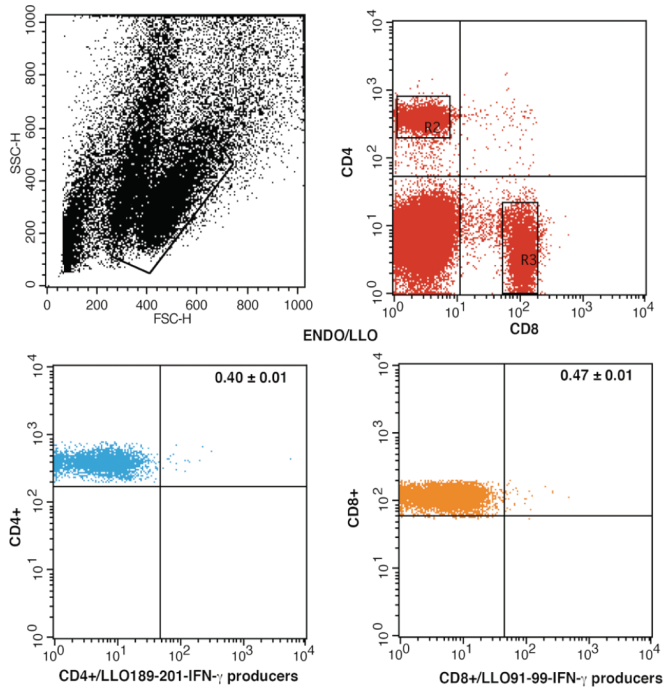

Figure 3. Percentages of $CD4^+LLO_{189\text{-}201}$ and $CD8^+LLO_{91\text{-}99}$ after vaccination with ENDO/LLO. CBA/J mice vaccinated *i.p* with 30 µg of LLO loaded endosomes from resting BM-DM (ENDO/LLO) for 7 days (n = 5/vaccination type). Next, mice were inoculated with LM (5 x 10^3 bc/mice) for 3 days. Splenocytes obtained from homogenized spleens after the vaccination protocol were stimulated for 5 hours with $LLO_{91\text{-}99}$ or $LLO_{189\text{-}201}$ peptides in the presence of brefeldin A for intracellular cytokine staining. LLO peptide stimulated splenocytes surface stained for CD4 or CD8 were fixed and permeabilized using a cytofix/cytoperm kit (BD. Biosciences). Stimulated cells were surface stained for $CD4^+$ FITC-labelled or $CD8^+$ APC-labelled and data gated to include exclusively $CD4^+$ or $CD8^+$ events, R2 and R3 gates, respectively (upper right flow histogram). Lower flow histograms shows the percentages of CD4+/$LLO_{189\text{-}201}$ IFN-γ producers from the R2 gate (lower left) and $CD8^+/LLO_{91\text{-}99}$ IFN-γ producers from the R3 gate (lower right), respectively. Samples are performed in triplicates and results are expressed ad the mean ± SD ($p < 0.05$).

Table 2. Specific LLO immune response in spleens of vaccinated mice.

Peptide LLO_{91-99}	$CD4^+$ T cells[b]	$CD8^+$ T cells	$CD4^{+c}$-$IFN_{intracell}$	$CD8^+$-$IFN_{intracell}$
NV[a]	25 ± 0.02	18 ± 0.01	0.03 ± 0.001	0.1 ± 0.001
ENDO/LLO	33 ± 0.02	22 ± 0.03	0.02 ± 0.002	0.47 ± 0.002
ENDO-IFN/LLO	29 ± 0.01	23 ± 0.01	0.05 ± 0.002	0.71 ± 0.004
BM-DM/LM	35 ± 0.03	25 ± 0.02	0.04 ± 0.001	0.99 ± 0.007
BM-DM-IFN/LM	30 ± 0.02	26 ± 0.03	0.02 ± 0.002	1.4 ± 0.02
Peptide $LLO_{189-201}$				
NV	28 ± 0.01	19 ± 0.02	0.19 ± 0.005	0.01 ± 0.0
ENDO/LLO	31 ± 0.03	21 ± 0.03	0.40 ± 0.02	0.03 ± 0.001
ENDO-IFN/LLO	29 ± 0.02	23 ± 0.03	0.56 ± 0.04	0.03 ± 0.002
BM-DM/LM	34 ± 0.01	27 ± 0.02	0.68 ± 0.06	0.01 ± 0.001
BM-DM-IFN/LM	32 ± 0.03	28 ± 0.03	0.78 ± 0.05	0.0 ± 0.0

[a]CBA/J mice were vaccinated *i.p* or not (NV) with 30 µg of LLO loaded endosomes from resting BM-DM (ENDO/LLO), LLO loaded endosomes from IFN-γ activated BM-DM (ENDO-IFN/LLO), LM infected resting BM-DM (1 x 10^6 cells) or LM infected and IFN-γ activated BM-DM (1 x 10^6 cells) for 7 days (n = 5/vaccination type). Next, mice were inoculated with LM (5 x 10^3 bc/mice) for 3 days. Splenocytes were obtained from homogenized spleens after the vaccination protocol. Samples were performed in triplicates and results expressed the mean ± SD ($p < 0.01$). [b]Splenocytes from vaccinated mice were stimulated for 5 hours with LLO_{91-99} or $LLO_{189-201}$ peptides in the presence of brefeldin A for intracellular cytokine staining. Stimulated cells were surface stained for CD4 FITC-labelled or CD8 FITC-labelled. Percentages of $CD4^+$ or $CD8^+$ T cells are expressed for each vaccination protocol. Samples were done in triplicates and results expressed the mean ± SD ($p < 0.05$). [c]LLO peptide stimulated splenocytes surface stained for CD4 or CD8 were fixed and permeabilized using a cytofix/cytoperm kit (BD. Biosciences). Cells were stained for IFN-γ using an anti-IFN-γ antibody PE-labelled. Samples were acquired using a FACSCanto flow cytometer. Data were gated to include exclusively $CD4^+$ or $CD8^+$ events, and the percentage of these cells expressing IFN-γ was determined. Samples were done in triplicates and results expressed the mean ± SD ($p < 0.05$).

examined after incubation of spleen cells with the specific LLO_{91-99} or $LLO_{189-201}$ peptides. The $CD4^+/LLO_{189-201}$ IFN- producers and $CD8^+/LLO_{91-99}$ IFN- producers reflect those sub-sets of $CD4^+$ and $CD8^+$ responding to each specific LLO peptide (lower flow histograms in **Figure 3**). In this regard, we observed higher percentages of splenic $CD8^+$ T cells recognizing LLO_{91-99} peptide after vaccination with ENDO-IFN/LLO than with ENDO/LLO endosomes (**Table 2**, 0.71 ± 0.004 *vs* 0.47 ± 0.002 values, respectively. $p<0.05$), values that were only slightly lower than the percentages observed after vaccination with LM infected BM-DM from resting or IFN- activated MØ (**Table 2**, 0.99 ± 0.007 *vs.* 1.4 ± 0.02 values, respectively. $p<0.01$). We observed comparative results when we analyzed the percentages of splenic $CD4^+$ T cells recognizing $LLO_{189-201}$ peptide. Vaccination with ENDO-IFN/LLO endosomes presented higher levels of $LLO_{189-201}$ specific $CD4^+$ T cells than vaccination with ENDO/LLO endosomes (**Table 2**, 0.56 ± 0.04 and 0.40 ± 0.02 values, respectively. $p<0.01$). Similarly, vaccination with LM infected BM-DM rendered slightly higher values, 0.68 ± 0.06 values for BM-DM/LM and 0.78 ± 0.05 values for BM-DM. These results suggested that LLO specific immune response is elicited in all vaccinated mice. However, LLO loaded endosomes from IFN- activated BM-DM and LM infected BM-DM presented higher numbers of both LLO_{91-99}-$CD8^+$ and $LLO_{189-201}$-$CD4^+$ T cells. These results confirmed that ENDO-IFN/LLO endosomes were efficient LLO processing compartments able to generate both LLO_{91-99} and $LLO_{189-201}$ epitopes to bind to MHC class I and class II molecules. These results are highly specific and significant since percentages of $CD8^+$ producers of IFN- after LM infection with 3 bursts are 8% containing all putative LM antigens and epitopes [15]; while $CD8^+/LLO_{91-99}$ and IFN- producers show values of 1.44 % ± 0.02 with only a single vaccine challenge or burst. Therefore, they support that ENDO-IFN/LLO endosomes appeared as high efficient vaccine carriers.

Mechanisms of different vaccines to prime the immune system

Vaccine efficiency is highly related with the recruitment of DC to the vaccination sites, a process known as priming of the immune system [6, 9, 10]. DC might capture antigens from other cells such as MØ and migrate to target organs to elicit a strong immune response. In this regard, mature DC shows a $CD11c^+IA^{k+}$ phenotype, immature DC show a $CD11c^+Ly6C^+$ phenotype and MØ corresponds to $CD11b^+IA^{k+}$ or $CD11b^+IA^{k-}$ phenotypes [9]. Therefore, we analyzed the phenotypes of the peritoneal exude cells (PEC) recovered after vaccination with the different vesicles or cell based systems using

Table 3. Mechanisms of priming the immune system by the different vaccines

Vaccine type[a]	Percentages of cells in PEC after vaccination[b]				
	NK	PMNs	CD11b⁺ (MØ)	CD11c⁺IAk⁺ (DCm)	CD11c⁺Ly6C⁺ (DCi)
NV	10 ± 0.01	13 ± 0.01	75 ± 0.06	3 ± 0.04	7 ± 0.05
BM-DM/LM	5 ± 0.02	9 ± 0.02	5 ± 0.01	55 ± 0.05	45 ± 0.03
BM-DM-IFN/LM	5 ± 0.02	7 ± 0.03	5 ± 0.02	70 ± 0.03	25 ± 0.02
ENDO/LLO	2 ± 0.01	5 ± 0.01	30 ± 0.03	7 ± 0.05	20 ± 0.03
ENDO-IFN/LLO	3 ± 0.02	5 ± 0.02	45 ± 0.05	27 ± 0.02	25 ± 0.05

[a]CBA/J mice were vaccinated *i.p* or not (NV) with 30 µg of LLO loaded endosomes from resting BM-DM (ENDO/LLO), LLO loaded endosomes from IFN-γ activated BM-DM (ENDO-IFN/LLO), LM infected resting BM-DM (1 x 10^6 cells) or LM infected and IFN-γ activated BM-DM (1 x 10^6 cells) for 7 days (n = 5/vaccination type). Next, mice were inoculated with LM (5 x 10^3 bc/mice) for 3 days. [b]PEC were obtained after 2-fold ice-cold washings with Hank´s balanced buffer and surface stained with the following FITC or PE-labelled antibodies: CD11b (MØ), CD11c (DC). Next, PE-CD11c⁺ cells were analyzed for the percentages of APC-labelled Ly6C or 40F anti-IA^k antibodies to distinguish for mature DC (DCm) (CD11c⁺IA^{k+}) or immature DC (CD11c⁺Ly6C⁺). Samples were acquired using FACSCanto flow cytometer and percentages of positive cells for each antibody are shown. Results are expressed as the mean ± SD of triplicates (p<0.05).

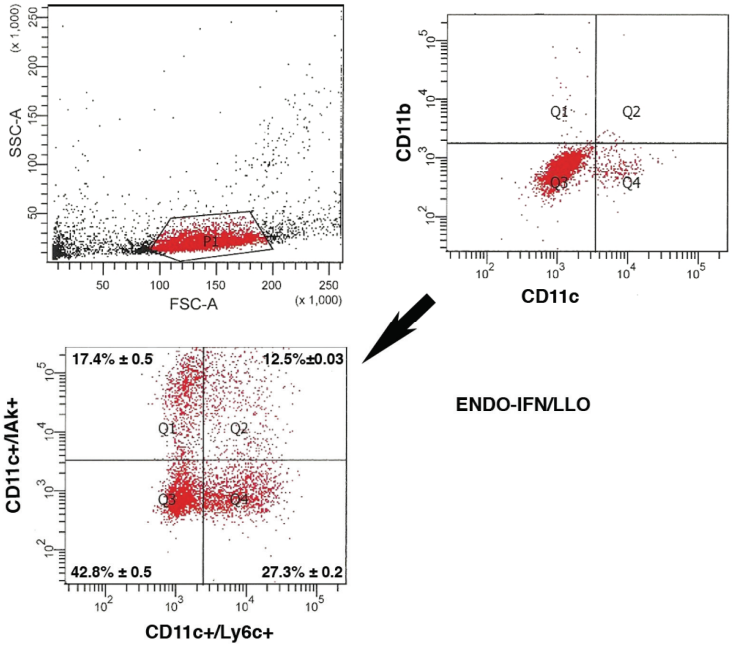

Figure 4. Analysis of cells recruited to the PEC of animals vaccinated with ENDO-IFN/LLO vaccine. CBA/J mice were vaccinated *i.p* with 30 µg of LLO loaded endosomes from IFN-γ activated BM-DM (ENDO-IFN/LLO) for 7 days (n = 5/vaccination type). Next, mice were inoculated with LM (5 x 10^3 bc/mice) for 3 days. PECs were obtained after 2-fold ice-cold washings with Hank´s balanced buffer and surface stained with the following FITC or PE-labelled antibodies: CD11b (MØ), CD11c (DC) (upper right flow histogram) and CD11c⁺ cells were gated (Q3 + Q4 regions in plots). Next, PE-CD11c⁺ cells were analyzed for the percentages of APC-labelled Ly6C or 40F anti-IA^k antibodies to distinguish for mature DC (DCm) (CD11c⁺IA^{k+}) or immature DC (CD11c⁺Ly6C⁺) (lower flow histogram). Samples were performed in triplicates and results of the double positive DCm or DCi cells are expressed as the mean ± SD of triplicates (p<0.01).

analysis of PEC populations by flow cytometry. We observed that LLO loaded endosomes from resting BM-DM recruited very low amounts of mature or immature DC, percentages of 7 ± 0.3 and 20% ± 0.5, respectively (**Table 3**, ENDO/LLO rows). While MØ were recruited at high 30% ± 0.7 rates. On the contrary, LLO loaded endosomes from IFN- activated BM-DM showed significant amounts of mature and immature DC with percentages of 27% ± 0.5 and 25% ± 0.2, respectively and also high 45% ± 0.7 rates of MØ (**Table 3**, ENDO-IFN/LLO rows). The percentages of mature and immature DC recruited to the vaccination sites by resting BM-DM infected with LM were high, with values of 55% ± 0.7 and 45% ± 0.5, respectively (**Table 3**, BM-DM/LM rows). Similarly, IFN- activated BM-DM infected with LM recruited also high 70% ± 0.7 rates of mature DC and 25% ± 0.3 rates of immature DC (**Table 3**, BM-DM-IFN/LM rows). Interestingly, the percentages of recruited MØ by both cell based vaccine systems were very low, with values of 5% ± 0.05 (**Table 3**). In **Figure 4**, we detail the protocol followed to stain for double positive CD11c⁺IA^{k+} (DCm) or CD11c⁺Ly6C⁺ (DCi) cells of the vaccine type selected as safer

and high efficiency, ENDO-IFN/LLO. In summary, these results suggested that vaccine efficiency correlates only with the recruitment of mature or immature DC to the vaccination sites showing the phenotypes $CD11c^+IAk^+$ and $CD11c^+Ly6C^+$, respectively. However, MØ ($CD11b^+$ cells) recruitment to the vaccination sites plays no role in the protection abilities of vaccine vectors.

Discussion

This study shows that LLO loaded endosomes derived from BM-DM activated with IFN- induce protective immunity against murine listeriosis and shows no cytotoxicity at all, therefore, they postulate as safer vaccines. Immune protection is associated with their high competences as MIIC, abilities to Ctsd-mediated LLO processing and the presence of a Ctsd degraded LLO_{1-491} form with binding abilities to MHC class II and an hypothetic structural accessibility to MHC class I molecules. Protection is achieved by recruitment of mature and immature DC to the vaccination sites that effectively may activate specific T cells with listericidal potential, both $CD4^+$-$LLO_{189-201}$ and $CD8^+$-LLO_{1-99} sub-sets. While their efficiency as vaccine vectors is moderate compared to BM-DM infected with LM. We aimed to develop an immunization system that preserved the immune-stimulatory properties of live BM-DM, contained no pathogenic agent and showed a non-cytotoxic LLO form, LLO_{1-491}. Using LLO loaded endosomes derived from BM-DM activated with IFN- we avoid the need to inject live cells, which might induce tumours in the recipients. Therefore, we envision that this vaccine vector is safe, confers significant protection against listeriosis and constitutes a novel and promising prophylactic immunotherapy.

What are the features that make these cell-free vesicles as suitable to confer protection against listeriosis? Endosomes from IFN- activated MØ are vesicles loaded with high levels of soluble lysosomal proteases such as Ctsd as well as other lysosomal membrane proteins involved in antigen presentation such as LAMP-2 and LIMP-2 (13, 14). Therefore, they contain all putative components that might degrade efficiently the LLO_{1-491} form. The high levels of MHC class II molecules observed in these endosomes, allows the formation of the immuno-dominant $LLO_{189-201}$ epitope but also other LLO epitopes. In this context, a wide range of MHC class II molecules loaded with different LLO epitopes will create an environment able to elicit a strong and polyclonal T cell response by itself. But also, the putative transference of $LLO_{189-201}$ epitopes from MHC class II molecules in these endosomes to recruited DC will enhance this immune response. Moreover, LLO_{91-99} loaded MHC class I molecules of these endosomes might transfer this epitope to DC and elicit a strong $CD8^+$ T cell expansion. In summary, LLO loaded endosomes from IFN- activated MØ showed the two requirements to induce protective immunity, enhanced antigen presentation and stimulation of effector and memory T cells (4, 7, 8, 10, 20-22). In fact, vaccination of mice with LLO loaded endosomes derived from BM-DM activated with IFN-, elicited significant numbers of IFN- producers $CD4^+$-$LLO_{189-201}$ specific and $CD8^+$-LLO_{91-99} specific T cells. These LLO specific T cells were reported as effector listericidal cells conferring protection against a LM infection (7, 8, 10, 20). These vaccine vectors are also able to stimulate the innate immunity by recruiting mature and immature DC to the vaccination sites as well as MØ. Moreover, their cellular-based composition might mediate signalling through different DC receptors that would expand the innate immune response.

We envision that LLO endosomes might rise after potent pro-inflammatory responses exerted by neutrophils liberating damaged bacteria to the inflammatory sites and bacterial antigens (21). Bacterial antigens might be captured by MØ that substitute neutrophils at the inflammatory sites. In this regard, a potent pro-inflammatory response leaded by IFN-, might induce MØ pyroptosis and the release of LLO loaded endosomes (23). Therefore LLO loaded endosomes, similar to exosomes (24-26), might function as natural occurring vaccines at the inflammation sites and develop T cell memory. When, a second challenge with the pathogen occurs, mice were naturally vaccinated with LLO loaded endosomes and protection is easily achieved.

LLO loaded endosomes from BM-DM activated with IFN- are promising vaccine vectors able to elicit a good protection pattern with only one vaccine challenge. However, studies should improve their potency up to the levels obtained with whole BM-DM infected with LM. Nevertheless, their safety for vaccine protocols in humans not using a pathogenic bacteria and showing no cytotoxicity, justifies these imple-

mentation measures.

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Abbreviations

APC, antigen presenting cells; BM-DM, bone-marrow derived macrophages; Ctsd, cathepsin-D; DC, dendritic cells; IFN-, interferon gamma; i.p., intraperitoneal; LLO, listeriolysin O; MHC, major histocompatibility complex; MIIC, MHC class II compartments or antigen presenting compartments; MØ, macrophages; SRBC, sheep red blood cells; Th1, T helper type 1 response.

Disclosure statement:

None of the authors have any conflicts of interest.

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