MANα1-2MAN decorated liposomes enhance the immunogenicity induced by a DNA vaccine against BoHV-1

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Abstract
New technologies in the field of vaccinology arise as necessity for treatment and control of many diseases. Currently modified live virus and inactivated vaccines used for Bovine Herpesvirus-1 (BoHV-1) have several disadvantages. Previous works for preventive treatment of BoHV-1 with DNA based vaccines have demonstrated the capability to induce humoral and cellular immune response. Nevertheless, it is well known that "naked" DNA induces low immunogenic response. Thus, loading of antigen encoding DNA sequences in liposomal formulations targeting dendritic cell receptors could be a promising strategy to better activate these antigen presenting cells (APC). In this work, DNA based vaccine encoding the truncated version of gD glycoprotein (pCIgD) of BoHV-1 was investigated alone and upon encapsulation on liposomal formulation coated with MANα1-2MAN-PEG-DOPE and LPS from *Brucella ovis* (pCIgD-Man-L) in mice and cattle assay. Results showed that the use of pCIgD-Man-L was capable to enhance the immune response in both animal models. Significant levels of humoral immunity were achieved when total antibody titers and isotypes were detected in sera and mucosa. For cellular immunity, specific viral lymphoproliferation was detected in the animals inoculated with pCIgD-Man-L. In addition, positively modulation of CD40 molecules on the surface of bovine dendritic cells (DCs) was observed when cells were stimulated and activated with vaccine formulations. When challenge assay was performed, bovines inoculated with pCIgD and liposome decorated with MANα1-2MAN-PEG-DOPE elicited better protection and diminished viral excretion.

The results demonstrate the targeting of the MANα1-2MAN coated liposomes toward dendritic cells and their ability to boost the immunogenicity according to an adjuvant effect that results in long-lasting immunity.

Liposome decorated with MANα1-2MAN-PEG-DOPE were for the first time tested as DNA based vaccine in cattle as preventive treatment of BoHV-1. These results open up new perspectives for the design of vaccine for the control of bovine rhinotracheitis.

Keywords: adjuvant, BoHV-1, DNA, DC targeting vaccine, liposome

1. Introduction

Bovine herpesvirus 1 (BoHV-1), an enveloped virus belonging to the alphaherpesvirus subfamily, infects cattle of all ages and breeds worldwide (Babiuk et al., 1987; Tikoo et al., 1995). This virus is pathogen whose infection can severely impact cattle production. It causes a variety of symptoms in cattle including infectious bovine rhinotracheitis (IBR), conjunctivitis, abortions and shipping fever, which is a complicated infection of the upper respiratory tract (Jones, 2003). The pathogenesis is responsible for considerable economic losses due to decreased milk production, weight loss and abortion and has been recognized as an important component of the bovine respiratory complex.

BoHV-1 initiates the disorder through immunosuppression that could render the animals more susceptible to secondary bacterial infections, leading to pneumonia and occasionally to death. BoHV-1 establishes latency. Latently infected animals should always be considered a potential source of infection (Bitsch,

1973), although vaccination can considerably reduce the amount of virus excreted following reactivation (Mars et al., 2001).

In general, there are control programs with the use of conventional modified and killed vaccines inactivate but there are no specific international programs to eradicate BoHV-1. In Europe, only a small number of countries has achieved the goal of IBR-eradication (Blickenstorfer et al., 2010), by the destruction of a great number of healthy, seropositive animals because they are persistently infected with BoHV-1. In others European countries, control strategy against this virus has been vaccination with live and killed gE-deleted marker (van Drunen Littel-van den Hurk, 2006; Romera et al., 2014). In endemic countries like Argentina, Brazil and Spain, voluntary intensive vaccination programs are implemented to reduce the prevalence of infected animals.

Vaccination remains one of the most cost-effective strategies to prevent and control the clinical signs and transmission of these viruses. Nevertheless, these vaccines have a series of disadvantages for BoHV-1. In the case of conventional vaccines, they may protect individual animals against clinical disease, but they cannot prevent either the efficient transmission of the virus or the establishment of latency. Inactivated vaccines do not provide complete protection because they are generally poor inducers of cellular immunity, while attenuated vaccines despite giving good levels of protection are not completely safe (Deshpande et al., 2002; Blome et al., 2013; Quattrocchi et al., 2017).

BoHV-1 uses a variety of mechanisms to elude the host's immune system. By spreading intracellularly, it can exist in the presence of anti-viral specific antibodies (Fuller and Lee, 1992; Miethke et al., 1995). For this reason, cytotoxic T-lymphocytes (CTL) are critical for the elimination of the virus (Langellotti et al., 2011).

DNA vaccines emerge as an alternative to conventional vaccines. These vaccines have the potential to induce both cellular and humoral immune responses against antigen encoded by recombinant DNA (Kanthesh et al., 2018) and are highly specific; the expressed immunizing antigen is subject to the same modifications as natural infection (Lee et al., 2015) without the risk of trace pathogenicity due to incompletely inactivated virus or due to reverted attenuated virus (Rajčáni et al., 2005). It is well known that the use of naked DNA vaccines in small animal models function as an adjuvant or immunomodulator itself, (Yamamoto et al., 1992; Krieg et al., 1995) but in some cases and especially in large animals, the protection conferred against disease is inefficient. For this reason, there are many trials in the use of plasmids with adjuvants and delivery systems to improve and enhance the immune response (Quattrocchi et al., 2017).

The aim of this study was to evaluate the immune response elicited in mice model and in cattle by a plasmid encoding BoHV-1 secreted gD (pCIgD) with the use of a liposome engineered through their coating with a ligand that specifically target dendritic cells (Man-L) and the inclusion of an adjuvant that helps stimulate the immune response. The liposomes were decorated with a patented synthetic molecule (Pappalardo et al., 2013), whose specific α1,2Mannobiose ligand has an affinity for DC-SIGN (Feinberg et al., 2007; Yamakawa et al., 2008). The MANα1-2MAN-PEG-DOPE derivative was selected because it can target dendritic cells (DCs) of different species (Pappalardo et al., 2013) and thus provide selective delivery of different antigens and nucleic acids that can then be processed for presentation in the contexts of MHC-I and II. *Brucella ovis* HS antigen (LPS), which contains LPS and some OMP proteins, is associated to this nanovaccine as adjuvant. The formulation of DNA loaded nanovaccine confers advantages such as increased antigen uptake by antigen presenting cells (APCs), cytokine secretion stimulation by APC or lymphocytes, increased antigen stability and decreased antigen degradation (Zaman et al., 2013). Previous studies have demonstrated the ability to better deliver an antigen to DC by anchoring this di-mannose molecule on liposome surface in nanovaccine formulation. It has been proven effective *in vitro* on murine and human DCs. There are also *in vivo* results of the effectiveness of this mannose ligand used in nanovaccines against *Brucella ovis* in sheep (Pappalardo et al., 2016).

To our knowledge, BoHV-1 DNA based vaccine was for the first time formulated with liposome targeted to DC with MANα1-2MAN-PEG-DOPE and tested in cattle. These results could be useful to design a vaccine for the control of bovine rhinotracheitis.

2. Materials and Methods

2.1 Plasmid construction

Construction of the pCIgD plasmid, which expresses the secreted form of BoHV1 glycoprotein D (gD), has been previously described (Langellotti et al., 2011). pCIgD and pCIneo empty plasmid (Promega negative control) were amplified in transformed *Escherichia coli* DH5α and purified using anion exchange columns (Qiagen Plasmid Purification GIGA Kit). They were analyzed based on 260/280 absorbance ratios and

restriction digests. Integrity and concentration of the plasmids were evaluated before and after each vaccine formulation.

2.2 Liposome formulation

A lipid film was prepared by rotary evaporation from a mixture of phosphatidylcholine (PC), cholesterol (Chol), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), *Brucella ovis* HS extract (LPS), and the α1,2-Mannobiose-PEG$_{2kDa}$-DOPE derivative (Manα) (Pappalardo et al., 2013) in chloroform. The *B. ovis* HS extract was kindly provided by Mgtr. Carlos Robles (Robles, 2009). The lipid film was rehydrated for 30 minutes in sterile normal saline (0.9% NaCl), pH 7.4, at a PC concentration of 1.2 mg/mL (mice vaccines) or 4.8 mg/mL (bovine vaccine) in the final suspension and then vortexed for 5 minutes. The liposomes were prepared by extrusion of the suspension through a 200 nm pore Whatman® Nucleopore® Polycarbonate membrane using an Avanti Polar Lipids® Mini Extruder™.
For mice vaccines: formulation of Man-L had PC:Chol:DOTAP:LPS:Manα with a 60:30:10:4.3:2 molar ratio [2.13 mg/mL total lipids]. These were incubated ON with plasmids resulting in a final dose of 15 μg DNA/300 μL liposomes.
For bovine vaccines: formulation of Man-L had PC:Chol:DOTAP:LPS:Manα with a 60:30:30:4.3:2 molar ratio [9.96 mg/mL total lipids]. These were incubated ON with plasmids, resulting in a final dose of 600 μg DNA/1500 μL liposomes. "Bovine vaccine" was formulated with a higher ratio of DOTAP with respect to "mice vaccine" to increase liposome stability due to the higher concentration of lipids in medium.

2.3 Vaccine formulations and animal treatment

Adjuvanted PC:Chol:DOTAP:LPS:Manα liposome (Man-L) was included as follows.
In mice: vaccines were formulated using 15 μg dose of a secreted gD coding plasmid (pCIgD) according to previous dose–response results (Langellotti et al., 2011) or plasmid without gD insert (pCIneo) as negative control. Groups of BALB/c (n=5) were intradermally (i.d.) inoculated at 0 and 20 days with 15 μg /0.3 mL of pCIgD or pCIneo alone; 15 μg /0.3 ml of pCIgD-Man-L or pCIneo-Man-L.
In cattle: a dose of 600 μg pCIgD was chosen based on a dose–response curve (data not shown). Seronegative bovines to BoHV-1 (1–2 years old) were vaccinated i.d. in the back of the ear with 600 μg/1,5 mL (distributed in five sites) of pCIgD (n = 5), pCIgD-Man-L (n = 5) and pCIneo as negative control (n = 4). Bovines were vaccinated at 0, 30 and 60 days.

2.4 Enzyme-linked immunosorbent assay for detection of anti-BoHV-1 antibodies

Before each animal vaccination, sera were tested by anti BoHV-1 enzyme-linked immunosorbent assay (ELISA). Briefly, Immulon 1B (Dynatech, Laboratories) microtiter plates were coated with inactivated BoHV-1 (iBoHV) LA Strain (1/300) in 0.1 M carbonate-bicarbonate buffer, pH 9.6 and incubated overnight (ON) at 4 °C. Plates were blocked with PBS/0.05% Tween 20 (PBST) containing 1% ovalbumin (PBST-OVA). Serial dilutions of mice or cattle sera were prepared in PBST-OVA and dispensed in 50 μL/well. Plates were washed three times with PBST-OVA and incubated with anti-mouse or anti-bovine IgG peroxidase conjugate (KPL) respectively for 1 h at 37 °C. After extensive washing with PBST, ortho-phenylene-diamine (1,2-benzenediamine) dihydrochloride (OPD, SIGMA) and H$_2$SO$_4$ were added as substrate and absorbance was measured at 492 nm using MR 5000 microplate reader (Labsystems, MN, USA). Cut-off was established as the mean A492 of the negative sera +2 standard deviation (SD). Titers were expressed as the log$_{10}$ of the reciprocal of the highest serum dilution giving an OD higher than the cut-off.

2.5 Immunoglobulin isotyping ELISA

Isotype profile by indirect ELISA was evaluated to determine the IgG1, IgG2a, IgG2b and IgG3 subtypes in immunized mice at 40 days post vaccination (dpv) and the titer isotype of IgG1, IgG2 from sera of immunized cattle at 90 dpv and to determine IgA from nasal swabs at 6 days post challenge (dpc).
Greiner Microlon microtiter plates were coated with iBoHV-1 and dilutions were conducted as described previously. Bound antibodies in mice sera were detected with biotinylated goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Caltag Laboratories, San Francisco, CA). After incubation for 60 minutes, plates were washed with PBST and a dilution of streptavidin/alkaline phosphatase (SIGMA) was added to each plate. The results for IgG1, 2a, 2b, and IgG3 obtained in 1/50 sera dilution were expressed as OD at A492 nm.

For isotype detection in cattle, the same ELISA described in chapter 2.4 was used with modifications: anti-bovine IgG1, IgG2, or IgA mouse monoclonal antibodies (provided by Dr. S. Srikumaran, University of Nebraska, USA) were used, followed by incubation with horse radish peroxidase (HRP) conjugated anti-mouse Ig. Finally, the reaction was visualized, and the cut-off was established as described before for IgG1 and IgG2. The results for IgA 1/2 dilution in nasal swabs were expressed as OD at A492 nm.

2.6 ALDCs Stimulation

Afferent lymph dendritic cells (ALDCs) from bovines were obtained by cannulation of pseudo afferent lymph vessels and characterized as previously described (Hope et al., 2006). Dendritic cells were incubated with culture medium RPMI 10% FBS (mock) or with 1 μg/mL of vaccines pCIgD, pCIgD-Man-L or plasmid free α1,2-Mannobiose-PEG$_{2kDa}$-DOPE coated liposome (Man-L) with (+) or without (-) LPS from *Brucella ovis*. After 16 h incubation, a direct surface staining of cells was performed using monoclonal antibody DEC205-FITC (SEROTEC, UK), and an indirect surface staining was performed using monoclonal antibody anti CD40 (SEROTEC, UK), and anti-mouse IgG PE conjugated (Jackson laboratories, USA). Cells were fixed with 0.2% paraformaldehyde and acquired using FACScalibur cytometer and CellQuest software (BD).

2.7 Splenocytes in mice and Peripheral blood mononuclear cells (PBMCs) in cattle isolation

Spleen cells from immunized mice were removed at 120 dpv, stimulated *in vitro* with iBoHV-1, and evaluated for antigen-specific proliferation. Splenocytes were obtained by gentle homogenization of spleens in supplemented RPMI 1640 medium/10% fetal calf serum (FCS, GIBCO)/1% ampicillin-streptomycin and gentamycin. Cells were counted using Turk.
Cattle blood samples were collected by venipuncture in syringes containing sodium citrate as an anticoagulant. PBMCs were isolated by centrifugation on Ficoll-Paque™ PLUS (density 1.077 g/mL; GE Healthcare Bio-Sciences AB) as described elsewhere (Romera et al., 2000). Cells were counted using Trypan blue.

2.8 Lymphoproliferation Assay

Splenocytes and PBMCs suspensions obtained from each animal were labeled with 3 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 30 min at 37 °C. Cells were washed and resuspended in RPMI 1640 complete medium (RPMI 1640 10% FBS, 10 mM HEPES, 100 U/ml penicillin, 100 mg/mL streptomycin, and 50 mM 2-mercaptoethanol). CFSE-labeled PBMCs or splenocytes were added to a 96-well plate (U-bottom) at a concentration of $5x10^5$ cells per well, in 100 μL of complete medium containing moi 1 of inactivated BoHV-1, concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO, USA), or medium as a positive or negative proliferation control, respectively. Cells were maintained at 37 °C in 5% CO$_2$ atmosphere. After 4 days incubation of cells from mice or 5 days for cells from cattle, cells were fixed with 0.2% paraformaldehyde. Cell proliferation was analyzed by flow cytometry, using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) and CellQuest software (Becton Dickinson).

2.9 Viral challenge assay in cattle

At 90 dpv, cattle were challenged by aerosol exposition as described previously (Romera et al., 2014; Quattrocchi et al., 2017) with a total of 5 mL in each nostril of $1x10^{6.81}$ TCID$_{50}$/mL with BoHV-1 LA strain. At 0, 3, 5, 6, 7, 8, and 12 dpc, calves were clinically examined, and rectal temperature was recorded. Clinical score after viral challenge was established according to: Grade 0 = normal; grade 1 = slight rhinitis with serous mucus with or without mild serous conjunctivitis; grade 2 = moderate/heavy rhinitis with fibrinous serous mucus with or without moderate serous conjunctivitis; grade 3 = fibrinopurulent mucus with moderate or severe conjunctivitis; grade 4 = rhinotracheitis with or without conjunctivitis (Romera et al., 2014).
To measure viral excretion, nasal swabs were obtained at 0, 3, 5, 6, 7, 8, and 12 dpc by inserting tampons into each nostril and dipping them in MEM containing 5,000 IU/mL penicillin, 2,500 μg/mL streptomycin, and 10 μg/mL amphotericin B. For virus titration in nasal swabs, samples were serially diluted and inoculated onto MDBK cell monolayers, which were inspected for cytopathic effect (cpe). Virus titration was performed by the end point dilution method (Reed and Muench, 1938).

2.10 Statistical analysis

The InfoStat program was used. ANOVA test and Bonferroni post-hoc test were performed to evaluate if the difference is significant. Differences between two means were analyzed using Student's t-test. A value of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1 pCIgD-Man-L increased specific BoHV-1 antibodies in mouse

In order to study the humoral immune response induced by pCIgD-Man-L vaccine, an ELISA test to detect anti-BoHV-1 antibodies was used. Vaccines pCIgD and pCIgD-Man-L were capable to induce specific sera antibody at 20 and 40 days after administration, but the formulation with liposome improved notably the IgG titer with respect to pCIgD ($p<0.01$, $p<0.05$ respectively). In addition, it was possible to detect titers at 120 days; indeed, in case of pCIgD-Man-L, antibody titer levels remained higher ($p<0.001$) with respect to those observed on the pCIgD treated group, where the titer declined (**Fig.1a**).

When the profile of antibody isotype was analyzed, a marked difference between the group treated with pCIgD-Man-L and the group treated with pCIgD alone was observed. Group treated with pCIgD-Man-L had elevated IgG1 ($p<0.001$), IgG2a ($p<0.05$) and IgG2b ($p<0.01$) levels with respect to the group treated with pCIgD (**Fig.1b**).

3.2 pCIgD-Man-L induces viral specific proliferation of lymphocytes in mice

A lymphoproliferative assay was performed to study the cellular immune response against the viral antigen. After splenocytes extraction from mice, they were stimulated *in vitro* with iBoHV-1. As a result, the percentages of specific lymphoproliferation was higher in pCIgD-Man-L group ($p<0.05$) than in pCIgD group (**Fig.2**). Taking together, these results indicate that pCIgD vaccine formulated with liposome Man-L could induce a higher humoral and cellular response against BoHV-1 in mice. Therefore, this formulation was further tested in a trial in bovines, natural host species.

3.3 pCIgD-Man-L increased specific BoHV-1 antibodies titers in cattle

Cattle received vaccination at 0, 30 and 60 days. When antibody kinetics was evaluated, pCIgD and pCIgD-Man-L were able to induce specific anti-BoHV-1 antibody levels (**Fig.3a**). However, significant differences in pCIgD-Man-L at 60 dpv ($p<0.05$) were found compared with pCIgD group. After challenge assays (90 dpv) all animals seroconverted (data not shown).

In addition, ELISA isotype titer at 90 dpv against BoHV-1 was analyzed (**Fig. 3b**). Interestingly, there were significant differences in IgG1 ($p<0.001$) for the pCIgD and pCIgD-Man-L group versus pCIneo, but in IgG2 difference was observed only for pCIgD-Man-L ($p<0.05$) with respect to the pCIneo group.

No significant differences among groups were found when neutralizing antibodies were measured; however, a trend of increasing neutralizing antibodies was observed in pCIgD-Man-L over pCIgD treated groups (data not shown).

Antibodies in nasal swabs were also evaluated. It is reported that the presence of antibody in nasal mucosa confer protection against respiratory viral penetration. Here, IgA isotype anti-BoHV-1 level in nasal swabs at 6 days post challenge (dpc) was evaluated (**Fig. 3c**). The results showed that the IgA titer in swabs of the pCIgD-Man-L treated group was significantly different compared with pCIneo treated group ($p<0.05$).

3.4 pCIgD and Man-L induce Dendritic Cells Activation *in vitro*

Dendritic cells are the major antigen presenting cell (APC) and are key initiators of antiviral responses (Ludewig et al., 2000). They endocitose, process and present antigens to naive T cells and act as messengers between the adaptive and innate immune responses. Afferent lymph dendritic cells (ALDCs) from bovine were used to elucidate if DNA alone or with loaded targeted liposomes were able to positively modulate dendritic cells. ALDCs were incubated *in vitro* with vaccines pCIgD, pCIgD-Man-L (with or without LPS from *Brucella ovis*) or Man-L alone (with or without LPS from *Brucella ovis*). CD40 surface molecules were significantly upregulated with respect to mock for pCIgD ($p<0.01$), pCIgD-Man-L with ($p<0.05$) and without LPS ($p<0.01$). Also, CD40 was upregulated positively when treated with Man-L with LPS ($p<0.001$) but not with Man-L without LPS. These results indicate that pCIgD alone and liposomes including LPS can activate dendritic cells (**Fig.4**).

3.5 pCIgD-Man-L induces BoHV-1 specific proliferation of lymphocytes

Lymphoproliferation was evaluated at 12 dpc in PBMCs after *in vitro* stimulation with inactivated BoHV-1. The results showed that pCIgD-Man-L treated group underwent the highest degree of proliferation with respect pCIneo ($p<0.01$) and pCIgD group ($p<0.05$) (**Fig. 5**). This result indicates that liposomes possess a remarkable "adjuvant" effect and are capable of enhancing cellular immune response elicited by pCIgD in bovines.

3.6 Viral excretion is decreased in pCIgD-Man-L group

Bovines were challenged at 90 dpv via aerosol with BoHV-1 strain Los Angeles. In order to measure the viral excretion after virus challenge trial, nasal secretions were obtained by inserting tampons into each nostril and collected the secretion.
In this assay, pCIgD and pCIgD-Man-L treatment were capable to reduce viral excretion compared to pCIneo treated group but the bovines that received pCIgD-Man-L formulation presented a markedly reduction of excretion at 5 dpc compared with pCIneo group ($p<0.01$). This phenomenon was not observed in the pCIgD group (**Fig 6**). In addition, these results were analyzed as area under curve (AUC) of the viral titer. In this way, AUC of the viral titer in animal treated with pCIgD-Man-L was the lowest with respect to the other groups.

3.7 pCIgD and pCIgD-Man-L presented similar clinical score

Clinical score was obtained by measuring the symptoms acquired by the cattle after the viral challenge.
At different days post challenge, calves were clinically examined, and rectal temperature was recorded. Clinical score after viral challenge was established according to: grade 0 = normal; 1 = slight rhinitis with serous mucus with or without mild serous conjunctivitis; 2 = moderate/heavy rhinitis with fibrinous serous mucus with or without moderate serous conjunctivitis; 3 = fibrinopurulent mucus with moderate.
Therefore, animal vaccinated with pCIgD and pCIgD-Man-L had a lower clinical score and there were significant differences compared with pCIneo group ($p<0.05$) at 6 dpc for pCIgD and at 7 dpc for pCIgD-Man-L ($p<0.05$) but there were no significant differences between them (**Fig. 7**). Clinical score AUC was analyzed and again pCIgD-Man-L treated animals had the lowest level respect the other groups.

4. Discussion

DNA vaccines have taken great interest in the field of vaccinology because of their simplicity, safety, stability, and versatility for development (Kanthesh et al., 2018). Previous studies with these vaccines have shown to induce good levels of humoral and cellular immunity in different species model depending on the type of encoded antigen, route of administration and promoter efficiency (Huang et al., 2005). Glycoprotein D is involved in virus penetration and has been considered the major target in vaccine development against bovine and human herpesvirus. A major limit of DNA vaccine is the low immunogenicity due to difficulties in delivering DNA plasmid into the host cell for further synthesis of the encoded antigenic protein (Suschak et al., 2017). Here, the use of suitable delivery systems and specific molecular activators can be combined to generate adjuvants that help to improve the efficiency of DNA based vaccines. In the search to set up a vaccine that generates both types of immunity against BoHV-1, we investigated DNA based vaccines using a DNA sequence encoding for the truncated version of gD glycoprotein (pCIgD). Our previous studies using the same DNA plasmid combined with chemical adjuvants in mice provided good protection evidences (Zamorano et al., 2002; Langellotti et al., 2011; Di Giacomo et al., 2015) while studies in cattle with a subsequent viral challenge showed the protection was only partial (Quattrocchi et al., 2017) which prompted us to further search for new formulation strategies.
In this context, we have designed here a complex DNA based nanovaccines based on liposomes decorated with mannose molecules for DC targeting and including LPS as molecular adjuvant which is expected to enhance the transfection efficiency in DCs and thus promote immunity. This will result in virtue of the ability of the liposomes to selectively target the DC-SIGN receptor in DCs. Liposomes were selected because they represent a self-assembling carrier that allow to combine different functional components on their surface resulting in a cooperative adjuvant effect. In addition, liposomes protect DNA from deoxyribonuclease attack (Gregoriadis et al., 2002).
The intradermal inoculation is one of the best performing administration strategy to deliver a DNA based vaccine (Zonouzi et al., 2016). The rational for this is that the skin possess associated lymphoid tissue, including Langerhans cells, dendritic cells, keratinocytes and other immune cells. Here, we inoculated

animals by intradermal route, and we observed remarkable humoral and cellular immune response. In agreement with evidences that truncated glycoprotein D can induce good levels of humoral immune response (Van Drunen Littel-van den Hurk et al., 1998; Lewis et al., 1999; Huang et al., 2005) we obtained and increasing antibody titers upon administration of pCIgD which was significantly increased when the plasmid DNA encoding for truncated glycoprotein D was formulated with DC targeted liposome including LPS adjuvant (pCIgD-Man-L). It is interesting to note that although no new inoculation was made after 40 dpv in mice, there were high levels of antibody even at 120 dpv with pCIgD-Man-L, but this phenomenon was not observed with pCIgD alone. This can be attributed to the local depot effect, and the selective delivery and activation capacities of the liposomes combined with LPS from *B. ovis* (Fredriksen and Grip, 2012) thus enhancing and prolonging the exposure of the immune cells to the antigen which results in specific immune response and long-lived immunity. When isotype antibody was analyzed, the specific effect of the liposome-based vaccine resulted in the increase of the levels of IgG1 and the profile of IgG2a, IgG2b when compared with the pCIgD treated group where the levels of antibodies were significantly lower. Notably IgG2a is an isotype that is crucial for the defense against viruses. Previous works have shown that IgG2a antibodies are produced in mice upon viral infections (Coutelier et al., 1991) and there are also triggered when DNA based vaccines are administered for immunizations (Raz et al., 1996) as a result of T helper 1 cells involvement. This isotype is efficient at complement fixation and acts as opsonizing agent. It is reported that IFNγ can stimulate the production of IgG2a both by *in vitro and in vivo* activated B lymphocytes. This increase of IgG2a in the animals treated with our free DNA plasmid suggest that the gD plasmid could be internalized by DCs and that interaction between DCs-B cells occurred according to CD40:CD40L dependent manner (Bao et al., 2014). The B cell switching was confirmed by the presence of the IgG2a isotype in sera of mice and by the positive modulation of the T CD4+ cells response to a Th1/Th2 profile.

The immune system quick response to a viral infection confirm a cellular memory to the viral antigens. This would prove that the viral antigens were previously processed by the host and CD4+/CD8+ memory lymphocytes were generated. In this work, we observed that splenocytes from mice immunized with gD plasmids were able to lymphoproliferate against inactivated BoHV-1 in cell culture. Indeed, it is important to highlight that these animals were last immunized 100 days before their splenocytes were exposed to inactivated BoHV-1. This ability to lymphoproliferate in the pCIgD-Man-L group was certainly provided by the adjuvant effect of the targeted liposome because this was not observed with the DNA plasmid administered alone.

We then tested the novel liposome based nanovaccine in a field trial in cattle. When we assessed BoHV-1 specific antibodies in blood the difference was significant between the pCIgD-Man-L immunized group compared to the control group. This increase was also observed in the isotype antibody titres. While in the murine model, the transcription factors in T helper lymphocytes such as GATA-3 associated with enhanced type 2 cytokine gene transcription (IL-4) or T-bet associated to type 1 cytokine (IFNγ) have been widely shown, these factors are not evident in bovine (Estes and Brown, 2002). Bovine IL-4 could induce IgG1 while IFNγ may enhance IgG2 over IgG1 (Estes et al., 1995). The results obtained in our study for bovine isotypes elicited by the vaccines, showed a relationship IgG1 and IgG2 increases as it was observed in previous tests with other DNA based vaccines or adjuvants (Arulkanthan et al., 1999; Brown et al., 1999; Quattrocchi et al., 2017). However, it could be observed that there is a greater IgG2 titer in the groups of animals that received pCIgD-Man-L than the DNA vaccine alone.

IgA was assessed from nasal swabs and evaluated 6 days post challenge resulting in higher titre in the pCIgD-Man-L treated group with respect to the control pCIgD treated group. Reasonably, the persistence and enhanced exposure to glycoprotein D post challenge can be responsible for the expansion of DC-primed cell and IgGs and IgA secreting B cell populations as hypothized by Manoj (Manoj et al., 2004).

CD40 is a co-stimulatory cytokine for various types of cells including B cells, macrophages, non-hematopoietic cells and DCs, for which it has an crucial role in immunity development and in activation of the latter cell types (Elgueta et al., 2009). DCs are the major antigen presenting cells (APC) and they are crucial mediators between immune innate and adaptive response (Vázquez et al., 2012). When DCs are inactivated, CD40 is expressed constitutively at relatively low levels on their surface (Banchereau et al., 1994). Upon DC cells encounter pathogens, antigens (Kawai and Akira, 2007) or apoptotic cells (Ip and Lau, 2004), CD40 expression is up-regulated on their surface and is activated. In this work, ALDCs were stimulated and activated after incubation with free DNA plasmid and DNA loaded targeted liposomes embedding LPS from *Brucella ovis*. It is well known that DNA possess CpG domain which can be recognize by APC and trigger their response (Kuwajima et al., 2006). Here, we observed that ALDCs incubated with free DNA plasmid showed positive up regulation of CD40 molecule. Also, targeted liposome alone or associated with DNA and functionalized with LPS from *Brucella ovis* are capable to enhance the expression of CD40 on the surface of DCs. This result proves that the nanovaccines represent

a promising platform that comply the idea that a performing vaccine must addressed APC. Notably, the liposome obtained without functionalization with LPS were not able to activate DCs. In this way, the MANα1-2MAN targeting provides a crucial function to selectively target the dendritic cells while addressing the LPS as adjuvant of the cell response.

Since it is well known that cell-mediated immunity is very important for virus clearance upon BoHV-1 and other viruses infection, a better protection induced after challenge in animals immunized with targeted DNA loaded liposome was observed. Animals from pCIgD-Man-L treated group had less viral excretion with significant decrease of viral titer at 5 dpc respect to control group. Furthermore, induction of cellular immune response was observed at 12 dpc in pCIgD-Man-L treated group with positive response to viral-specific PBMCs proliferation. This could be correlated with the enhancement of IgG1 isotype as an indicator of activation of cellular immune response (Sin et al., 1999). The increased of the lymphoproliferative effect in pCIgD-Man-L treated group shows a better armed immune response, in which memory cells became established and were able to recognize the viral antigen more quickly than in pCIgD treated and control groups.

Although, the clinical score was similar for the animals vaccinated with free DNA plasmid and those immunized with the targeted DNA loaded liposomes, these animals presented less clinical symptoms than the control animals during viremia.

The development of performing vaccines for BoHV-1 based on DNA selective delivery to DC cells using new carriers including liposomes may allow better control of virus circulation in livestock. Although this trial as a proof of concept demonstrates that animals vaccinated with the engineered liposomal formulation, we set up improved humoral and cellular response levels, the impact of formulation parameters including vaccine dose and booster/lipid ratio still need to be systematically elucidated. Nevertheless, this study opens up perspectives for the generation of a new generation vaccine for robust protection of the animals against viral exposure.

To our knowledge, this is the first time that DNA based vaccine for BoHV-1 immunization is generated with liposomes decorated with MANα1-2MAN-PEG-DOPE as immune targeting agent and tested in cattle.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

Handling and housing of animals were made in accordance with the institutional guide for the care and use of experimental animals (Council resolution number 14/07). The Institutional Committee for the Care and Use of Experimental Animals, CICUAE-INTA Castelar, and CICUAE INTA-CeRBAS, Balcarce, Argentina, has given its approval for work with mice (44/2018) and cattle (123/2017) respectively.

Conflict of Interest Statement

The authors do not have any kind of association that might pose a conflict of interest.

Data Availability Statement

The data supporting the findings of this study are available on request from the corresponding author.

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