Supporting Information for:

Characterization of a Nanovaccine Platform Based on an α1,2-Mannobiose Derivative Shows Species-non-specific Targeting to Human, Bovine, Mouse, and Teleost Fish Dendritic Cells

Juan S. Pappalardo[1,2,3,]; Stefano Salmaso[4]; Tatyana S. Levchenko[3]; Francesca Mastrotto[4]; Sara Bersani[4]; Cecilia A. Langellotti[2,5]; Monica Vermeulen[5,6]; Federica Ghersa[1,7]; Valeria Quattrocchi[2]; Patricia I. Zamorano[2,5]; William C. Hartner[3]; Micaela Toniutti[3]; Tiziana Musacchio[3]; and Vladimir P. Torchilin[3,].*

[1]Veterinary Nanomedicine Group, Instituto de Investigaciones Forestales y Agropecuarias Bariloche (IFAB, INTA-CONICET), EEA Bariloche, Instituto Nacional de Tecnología Agropecuaria, Bote Modesta Victoria 4450, San Carlos de Bariloche, Río Negro, R8403DVZ, Argentina.

[2]Immunology and Immunomodulators Group, Instituto de Virología e Innovaciones Tecnológicas (IVIT, INTA-CONICET), IV, Instituto Nacional de Tecnología Agropecuaria, Nicolás Repetto 2799, William Morris, Buenos Aires, B1681FUU, Argentina.

[3]Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University. 360 Huntington Avenue. Boston, MA 02115, USA.

[4]Department of Pharmaceutical and Pharmacological Sciences, School of Medicine, University of Padova. Via F. Marzolo, 5. 35121 Padova PD, Italy.

[5]National Council of Scientific and Technical Research (CONICET). Av. Rivadavia 1917. Ciudad de Buenos Aires C1033AAJ, Argentina.

[6]Institute of Experimental Medicine (IMEX, ANM-CONICET), Academia Nacional de Medicina, Pacheco de Melo 3081. Ciudad de Buenos Aires C1425AUM, Argentina.

[7]Parasitology Laboratory, Instituto de Investigaciones en Biodiversidad y Medioambiente (INIBIOMA, UNCo-CONICET) Universidad Nacional del Comahue, Quintral 1250, San Carlos de Bariloche, R8400FRF, Río Negro, Argentina.

*Corresponding author:

- JS Pappalardo: The Veterinary Nanomedicine Group, EEA Bariloche, Instituto Nacional de Tecnología Agropecuaria (INTA). Bote Modesta Victoria 4450. San Carlos de Bariloche R8403DVZ, Rio Negro, Argentina.

E-mails: pappalardo.juans@inta.gob.ar / jspappalardo@gmail.com

This Supporting Information section contains the following materials:

Figure S1, Expression of specific markers CD11c and DC-SIGN for mouse bone marrow-derived dendritic cells by flow cytometry.

Figure S2, Expression of Gr1 marker for mouse bone marrow-derived granulocytes by flow cytometry.

Figure S3, Expression of specific marker DEC205 for bovine afferent lymph dendritic cells (ALDC) by flow cytometry.

Figure S4, Expression of specific marker CD1a for human MoDC by flow cytometry.

Figure S5, Size and ζ-potential profiles of micelles and liposomes.

Figure S6, Comparison of dendritic cell binding of control liposome versus the liposomal nanoplatform.

Figure S7, Expression of CD11c marker in mouse splenocytes.

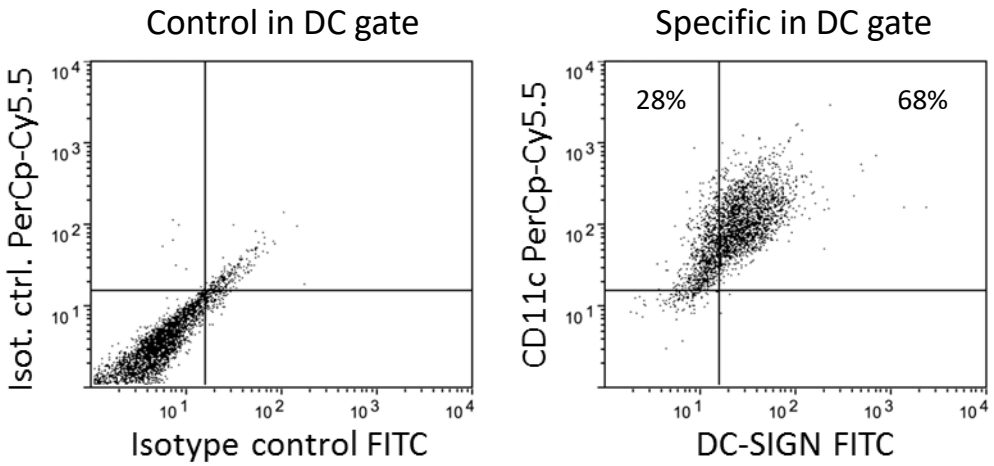
Figure S8, Comparison of liposome uptake by fresh splenocytes treated with plain-L, Man-L and Manα-L.

Figure S9, Comparison cell specific binding in human DC.

Figure S10, Scheme of the oxidation of α1'-2mannobiose.

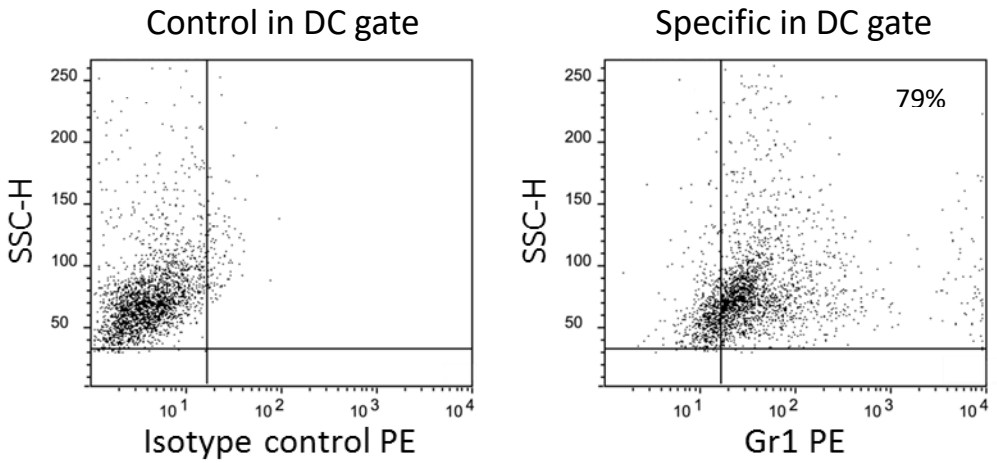
Figure S11: Comparison of uptake percentage by flow cytometry per species

FIGURE S1:



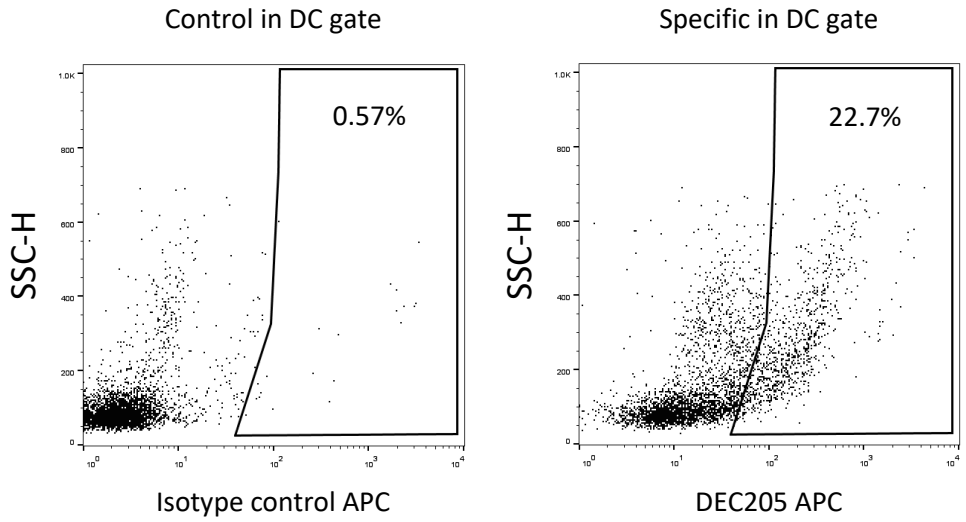
Expression of specific markers CD11c and DC-SIGN for mouse bone marrow-derived dendritic cells at the DC gate. Mouse bone marrow cultures were treated with antibodies against CD11c and DC-SIGN markers to evaluate specific expression which was 96% for CD11c, 68% of which also expressed DC-SIGN, in comparison with the isotype controls. Analysis of markers expression in mouse BMDC was performed by flow cytometry at day 4, after culturing the bone marrows with mouse recombinant GM-CSF and IL-4.

FIGURE S2:



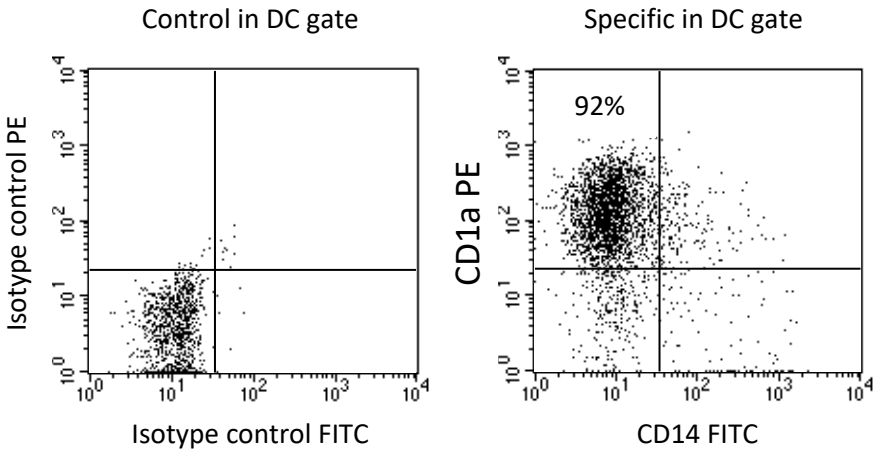
Expression of Gr1 marker for mouse bone marrow-derived granulocytes at the granulocyte gate. The culture was treated with antibodies against Gr1 marker to evaluate specific expression at its corresponding gate, which was 79%, in comparison with the isotype control. Analysis of marker expression in mouse bone marrow primary cultures was performed by flow cytometry at day 4, after culturing the bone marrows with mouse recombinant GM-CSF and IL-4.

FIGURE S3:



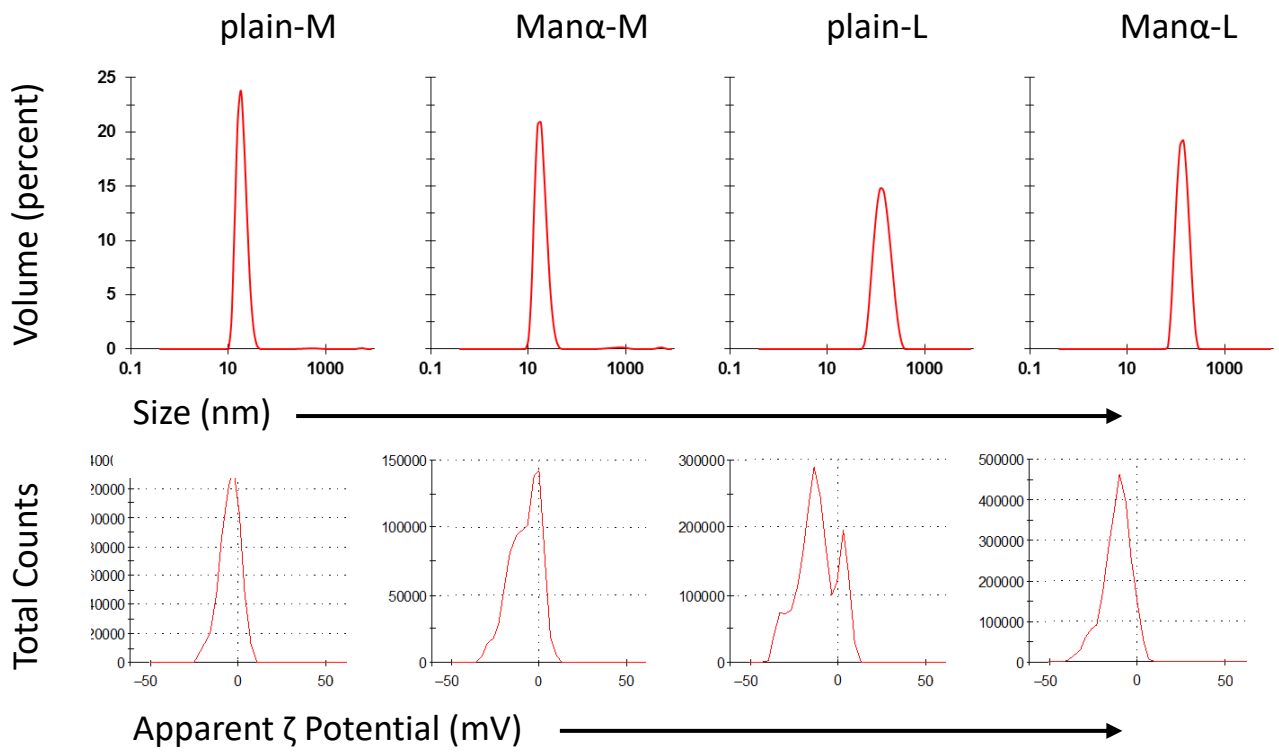
Expression of specific marker DEC205 for bovine afferent lymph dendritic cells (ALDC). After performing the cannulation as described, the lymph was collected and characterized by flow cytometry as $DEC205^{+}/FSC^{high}$.

FIGURE S4:



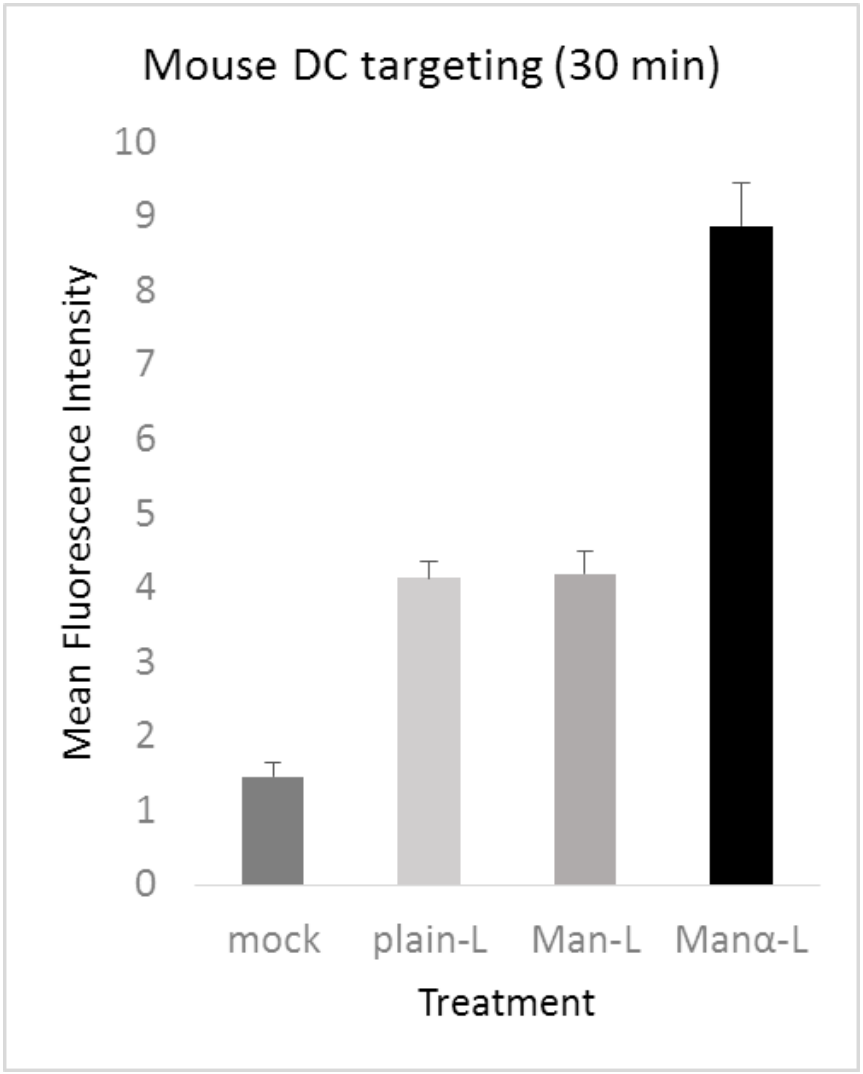
Expression of specific marker CD1a for human MoDC by flow cytometry. Human monocyte-derived cultures were treated with antibodies against CD1a and CD14 markers to evaluate specific expression (CD1a+/CD14-). Analysis of CD1a and CD14 expression in human MoDC was performed at day 5, after culturing with human recombinant GM-CSF and IL-4. Human MoDC express CD1a and have lost the CD14 expression from original monocytes.

FIGURE S5:



Size and ζ-potential profiles of plain and α1',2-mannobiose micelles (plain-M and Manα-M, respectively) and liposomes (plain-L and Manα-L, respectively).

FIGURE S6:



Comparison of dendritic cell binding of control liposome versus the liposomal nanoplatform: Control liposomes decorated with a single mannose derivative "Man-$PEG_{3.4kDa}$-DOPE" (Man-L) were compared to the nanoplatform based in 2α-mannobiose "Manα1'-2ManOx-PEG_{2kDa}-DSPE" (Manα-L). Flow cytometry analysis (at DC gate, expressed in MFI) of mouse DC cultures treated with rhodamine liposomes for 30 min. Treatments were mock, plain-L, Man-L and Manα-L. Man-L were formulated using a control derivative synthesized with mannosamine (1 mannose with amino group at the anomeric carbon, from Sigma). The unspecific binding of Man-L is the same as plain-L.

In order to perform this experiment, Man-PEG-DOPE was synthesized:

Synthesis of pNP-PEG-DOPE

pNP-PEG-PE was synthesized according to a previously published procedure [1]. Approximately 30 μmol of DOPE was dissolved in chloroform to obtain a 50 mg/mL solution and supplemented with 80 μL (around 2-fold molar excess over $PEG_{3.4kDa}$-$(pNP)_2$) of triethylamine. Then 300 μmol of $PEG_{3.4kDa}$-$(pNP)_2$ dissolved in 5 mL of chloroform was added to the mixture and the sample was incubated overnight at RT with stirring under argon. The organic solvents were removed using a rotary evaporator. The pNP-$PEG_{3.4kDa}$-DOPE micelles were formed in 0.01 M HCl, 0.15 M NaCl using water bath sonication. The micelles were separated from the unbound PEG and the released pNP on a CL-4B column using 0.01 M HCl, 0.15 M NaCl as an eluent. Pooled fractions containing pNP-$PEG_{3.4kDa}$-DOPE were freeze-dried, and the pNP-$PEG_{3.4kDa}$-DOPE was extracted with chloroform and stored as a chloroform solution at −80°C.

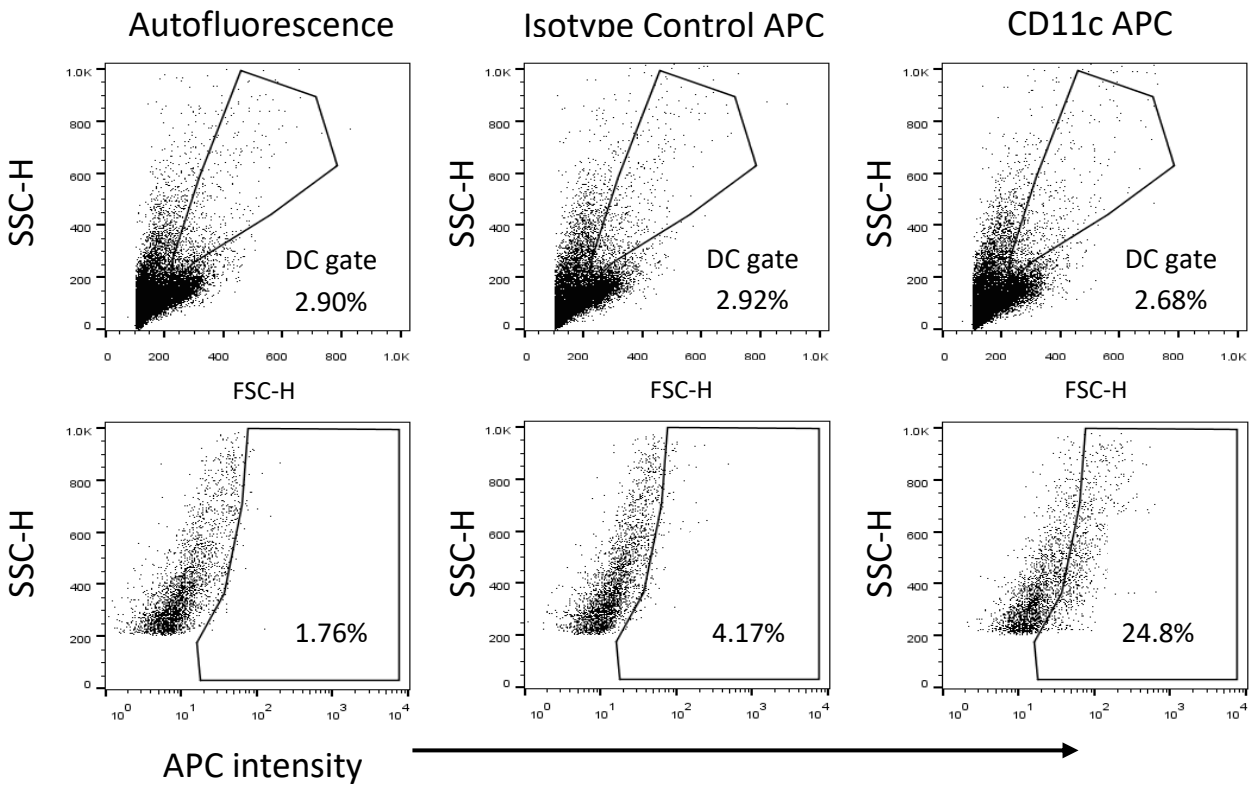
Synthesis of Man-PEG-DOPE

The attachment of D-mannosamine hydrochloride (Man-Nh_2, from Sigma-Aldrich, St. Louis, MO, USA) to pNP-$PEG_{3.4kDa}$-DOPE was carried out according to a previously published procedure. [1] Briefly, chloroform was removed from the pNP-$PEG_{3.4kDa}$-DOPE solution by rotary evaporation. The pNP-$PEG_{3.4kDa}$-DOPE micelles were formed in an Na-citrate buffer, pH 5.1, and mixed with Man-Nh2 in a borate buffer, pH 8.5. The Man-$PEG_{3.4kDa}$-DOPE sample was incubated overnight at RT, and dialyzed against water to eliminate the unbound Man-NH_2 and free pNP, freeze-dried and stored at −80°C.

Formulation of Man-liposomes

Mannose liposomes (Man-L) were prepared by a thin film hydration technique. Briefly, a lipid film was prepared by rotary evaporation of chloroform in a warming bath below 40 °C from a mixture of PC, Chol, rhodamine-DOPE and Man-$PEG_{3.4kDa}$-DOPE at a 60:30:0.25:2 molar ratio. The film was dried under vacuum overnight at -48 °C to remove traces of chloroform. The films were rehydrated for 30 min in sterile HBS, pH 7.4, at a concentration of 1.78 mg lipid/ mL buffer. The suspension was vortexed for 5 min, and then extruded through a polycarbonate filter (pore size, 200nm) by using a Mini Extruder (Avanti Polar Lipids).

FIGURE S7:



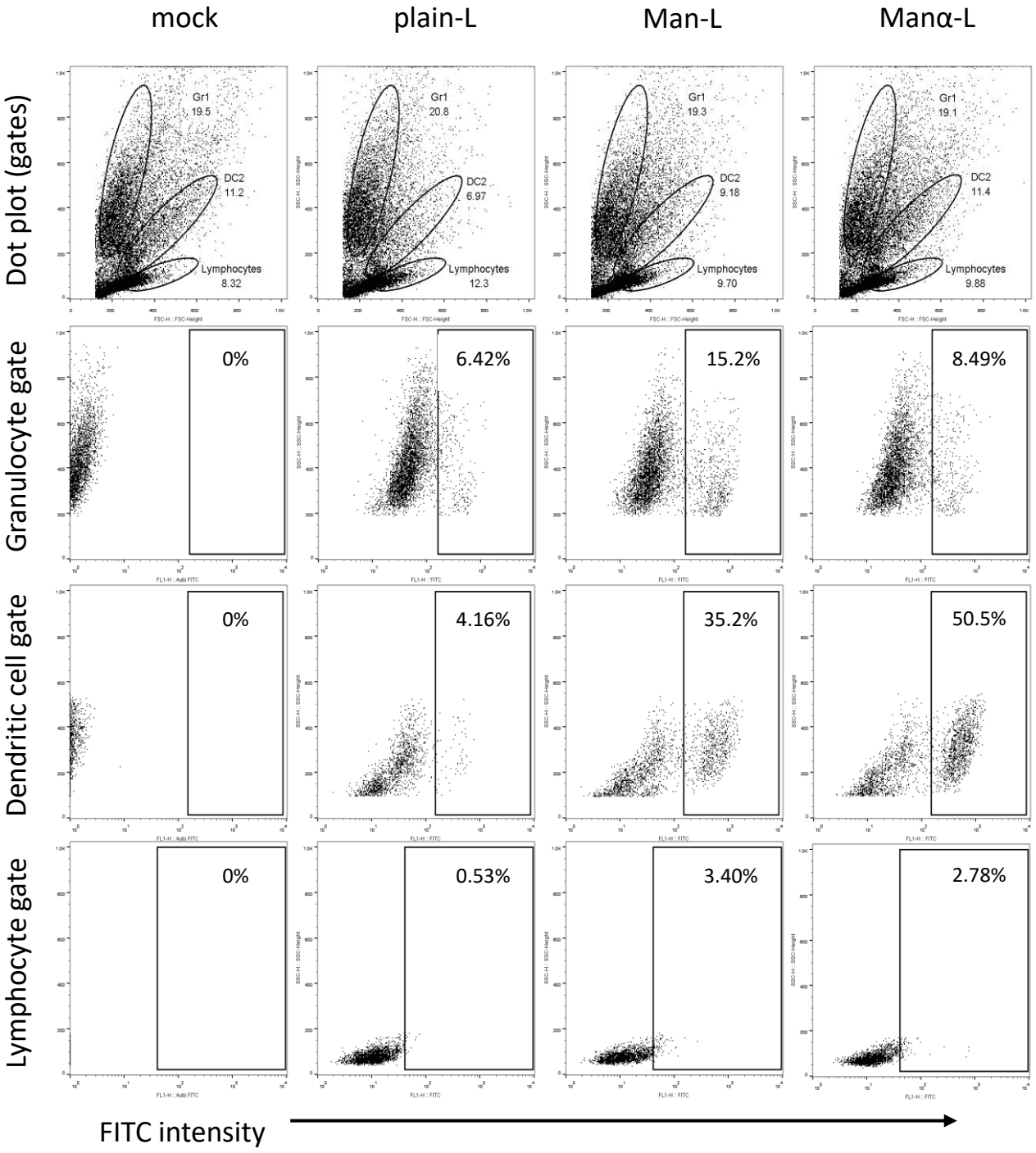
Expression of CD11c marker in mouse splenocytes. Fresh splenocytes were labelled with antibodies against CD11c marker to evaluate specific expression at its corresponding gate, which was around 20% after subtracting the isotype control, typical percentage of DC in spleen (in DC gate) as seen in previous research. [2] Analysis of marker expression was performed by flow cytometry immediately after obtaining the splenocytes from normal mice.

In order to perform this experiment, mouse splenocytes were collected:

Mouse splenocytes isolation:

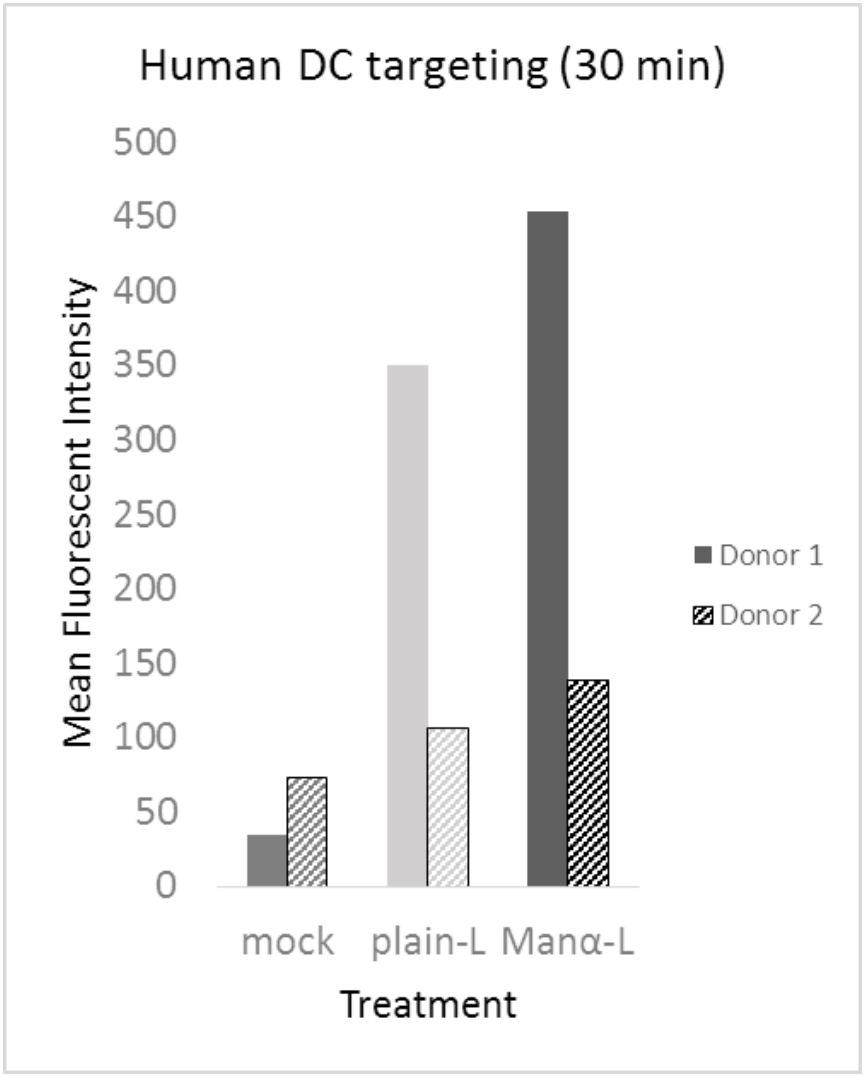
Splenocytes from normal male mice (6–8-week-old) were collected, incubated *in vitro* with various formulations and the uptake was characterized by flow cytometry. Briefly, after antesthesia and cervical dislocation, spleens were obtained in sterile conditions. Then splenocytes were obtained by gentle homogenization of spleens in compRPMI (10% FBS) and counted using Turk method. [3]

FIGURE S8:



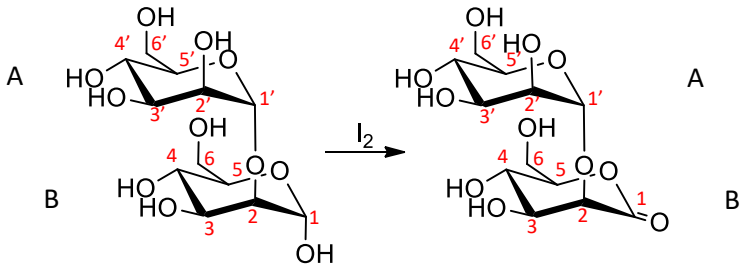
Comparison of liposome uptake by fresh splenocytes treated with plain-L, Man-L and Manα-L. Mock treated group was used as negative control. Cell culture dishes (24 well plates) were coated with the splenocytes and after 24-hour adaptation to culture conditions, the cells were treated for 30 min as previously described. Analysis was performed considering the 3 main populations of interest: granulocytes, dendritic cells and lymphocytes.

FIGURE S9:



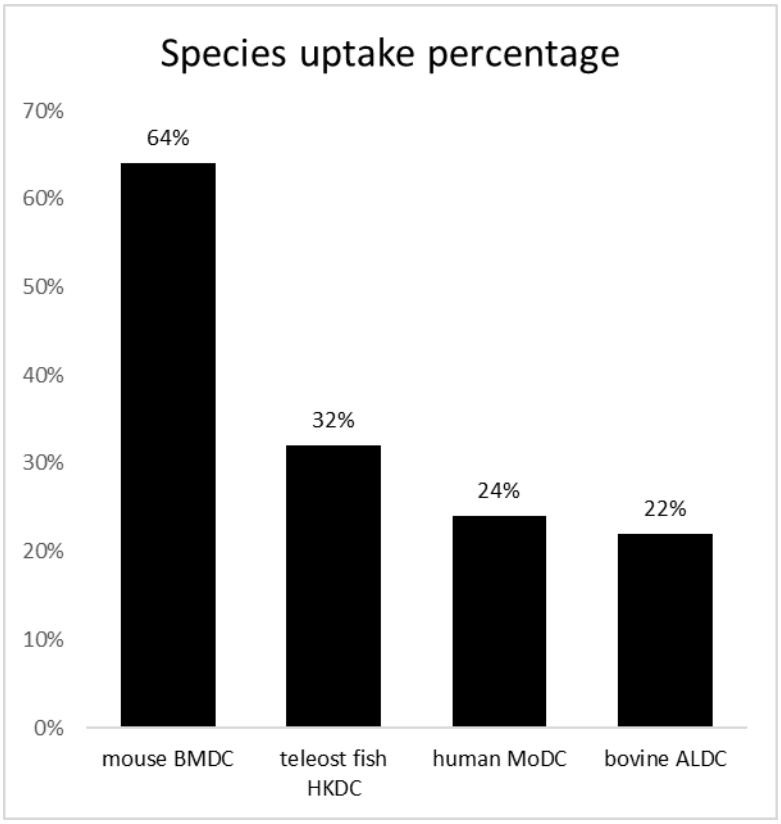
Comparison cell specific binding in human DC: Flow cytometry analysis of human DC culture from 2 donors. Donor 1 has been shown in the manuscript. Donor 2 has shown similar results with a lower MFI.

FIGURE S10:



Scheme of the oxidation of α1'-2mannobiose with carbons numbered for identification. Carbon 1 of reducing "Mannose B" is the only carbon loosing chirality upon treatment with Iodine.

FIGURE S11:



Comparison of uptake percentage by flow cytometry per species (mouse BMDC, teleost fish HKDC, human MoDC, bovine ALDC).

References:

1. Torchilin, V. P.; Levchenko, T. S.; Lukyanov, A. N.; Khaw, B. A.; Klibanov, A. L.; Rammohan, R.; Samokhin, G. P.; Whiteman, K. R. p-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via p-nitrophenylcarbonyl groups. *Biochim Biophys Acta* **2001,** *1511*, (2), 397-411.
2. Langellotti, C.; Quattrocchi, V.; Alvarez, C.; Ostrowski, M.; Gnazzo, V.; Zamorano, P.; Vermeulen, M. Foot-and-mouth disease virus causes a decrease in spleen dendritic cells and the early release of IFN-alpha in the plasma of mice. Differences between infectious and inactivated virus. *Antiviral Res* **2012,** *94*, (1), 62-71.
3. Kornuta, C. A.; Bidart, J. E.; Soria, I.; Gammella, M.; Quattrocchi, V.; Pappalardo, J. S.; Salmaso, S.; Torchilin, V. P.; Cheuquepan Valenzuela, F.; Hecker, Y. P.; Moore, D. P.; Zamorano, P. I.; Langellotti, C. A. MANalpha1-2MAN decorated liposomes enhance the immunogenicity induced by a DNA vaccine against BoHV-1. *Transbound Emerg Dis* **2020**.