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S-layer glycoprotein from *Lactobacillus kefiri* CIDCA 8348 enhances macrophages response to LPS in a Ca^{+2}-dependent manner

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ABSTRACT

The S-layer is a (glyco)-proteinaceous envelope constituted by self-assembled subunits that form a two-dimensional lattice covering the surface of different species of *Bacteria* and *Archaea*. It could be considered as one of the most abundant biopolymers in our planet. Because of their unique self-assembly features, exhibiting repetitive identical physicochemical properties down to the subnanometer scale, as well as their involvement in specific interactions with host cells, the S-layer proteins (SLPs) show a high potential application in different areas of biotechnology, including the development of antigen carriers or new adjuvants. The presence of a glycosylated SLP on potentially probiotic *Lactobacillus kefiri* strains was previously described by our research group. In this study, we aim to investigate the role of carbohydrates present in the SLP from *L. kefiri* CIDCA 8348 (SLP-8348) in their internalization by murine macrophages, as well as to analyze their immunomodulatory capacity and their effect on LPS-stimulated macrophages. RAW 264.7 cells internalized the SLP-8348 in a process that was mediated by carbohydrate-receptor interactions since it was inhibited by glucose, mannose or EGTA, a Ca^{+2} chelating agent. These results correlated with the recognition of SLP-8348 by ConA lectin. We further show that while SLP-8348 was not able to induce the activation of macrophages by itself, it favored the LPS-induced response, since there was a significant increase in the expression of surface cell markers MHC-II, CD86 and CD40, as well as in IL-6 and IL-10 expression at both transcript and protein levels, in comparison with LPS-stimulated cells. The presence of EGTA completely abrogated this synergistic effect. Taken together, these results strongly suggest the involvement of both glycosidic residues and Ca^{+2} ions in the recognition of SLP-8348 by cellular receptors on murine macrophages. Moreover, these results suggest the potentiality of the SLP-8348 for the development of new adjuvants capable of stimulating antigen presenting cells by interaction with glycan receptors.

1. Introduction

The S-layer, a monomolecular array of protein or glycoprotein subunits that self-assemble to form a two-dimensional lattice that completely covers the organism during all stages of growth. It is found on both Gram-positive and Gram-negative bacteria and is highly prevalent in archaea [1]. It is considered as the most ancient biological membrane that has remained through the microbial evolution as well as one of the most abundant biopolymers in our planet [2]. S-layer proteins (SLPs) are in direct contact with bacterial environment and thus they may be involved in many of their surface properties, including adherence to different substrates, self-aggregation, co-aggregation with other microorganisms, and bacterial recognition [3,4]. Because of their unique self-assembly features, exhibiting repetitive identical physicochemical properties down to the subnanometer scale, SLPs have attracted considerable interest in the biotechnology field. In fact, they have high potential to be applied in different areas of (nano)-biotechnology,

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biomimetics, biomedicine, and synthetic biology. As surface components that frequently mediate specific interactions with host cells, several experiments focusing on the use of SLPs as antigen/hapten carriers, as adjuvants, or as part of vaccination vesicles have been conducted by different research groups [5].

Lactobacillus kefiri is one of the most predominant lactobacilli present in kefir fermented milk [6] and several *in vitro* and *in vivo* studies support its potential as a probiotic microorganism [7,8]. The presence of a S-layer in kefir-isolated *L. kefiri* strains was previously demonstrated by our group [9]. Even though the function of the SLPs in *L. kefiri* surface remains unknown, their role in bacterial interaction with yeasts [10] as well as in the resistance to environmental conditions [11] has been reported. Moreover, we have demonstrated that isolated *L. kefiri* SLPs interact with *Clostridium difficile* toxins and antagonize their effect on eukaryotic cells [12], inhibit *Salmonella enterica* invasion to Caco-2 cells [13] and are able to enhance the adhesion of *L. kefiri* to gastrointestinal mucus [14].

Recognition of microbial components by host immune cells is mediated by different pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) [15] and C-type lectins receptors (CLRs) [16]. Myeloid CLRs are specialized in the recognition of glycosylated molecules. They share a common fold that harbours a Ca^{+2} for contact to the sugar, and are expressed by antigen-presenting cells (APCs) such as dendritic cells (DC) and macrophages. These APCs sense and internalize pathogens and present microbe-derived antigens on MHC molecules to T cells, thereby initiating adaptive immune responses [17].

Glycosylation is the post-translational modification most frequently found in SLPs and, indeed, they were the first glycoproteins detected in prokaryotes [18]. Interestingly, differences in sugar moieties have been described even between strains belonging to the same species [19]. Some studies have shown that carbohydrates present in the SLPs of different *Lactobacillus* species play a critical role in their adhesion and immunomodulatory properties. In this sense, it was shown that the CLR DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) mediates the modulation of dendritic cells by SlpA of *L. acidophilus* NCFM [20] and S-layer like proteins from *L. plantarum* [21], as well as cellular adhesion of SLP from *L. kefiri* JCM 5818 [22].

Previous studies performed in our laboratory revealed the presence of glycosidic residues in the SLPs from different *L. kefiri* strains [23,24]. In this study, we aim to investigate the role of the carbohydrates present in the SLP from *Lactobacillus kefiri* CIDCA 8348 (SLP-8348) in their internalization by the macrophage cell line RAW264.7. Furthermore, we analyzed the immunomodulatory capacity of SLPs and their effect on LPS-stimulated macrophages.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. kefiri CIDCA 8348 isolated from kefir grains was used [25]. The strain was cultured in de Man-Rogosa-Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) at 37 °C for 48 h in aerobic conditions. Frozen stock cultures were stored at −80 °C in skim milk until use.

2.2. S-layer proteins extraction

S-layer protein extraction from bacterial cells at stationary phase was performed using 5 M LiCl as previously described [12]. The sample was centrifuged and the protein concentration in the supernatant was determined according to Bradford [26]. SLPs extracts were tested by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS–PAGE) in 12% separating and 4% stacking gels using the discontinuous buffer system according to Laemmli [27]. Gels were migrated on a BioRad Mini-Protean II (BioRad Laboratories, Richmond, CA, USA) and revealed using Colloidal Blue Staining. SLPs were filtrated through a membrane of 0.45 μm pore diameter.

2.3. Lectin recognition of S-layer protein

The lectin-reactivity on SLP-8348 was evaluated by an ELISA-type assay [28]. Nunc microtiter plates (SIGMA, USA) were coated with 1 μg/well of SLP and blocked with 1% gelatin in Na_2CO_3/$NaHCO_3$ 100 mM buffer for 1 h at 37 °C. Then, different concentrations of biotin coupled lectins from *Canavalia ensiformis* (ConA: αMan>αGlc), were added and incubated for 1 h at 37 °C. For inhibition assays, ConA was pre-incubated for 30 min at 37 °C with 50 mM of glucose (Glc), mannose (Man) or galactose (Gal) (SIGMA, USA), or 5 mM EGTA (AMRESCO, USA). After three washes, streptavidin conjugated to horseradish peroxidase (HRP, BioLegend, USA) was added to each well for 30 min at 37 °C. Plates were then washed and incubated with chromogenic substrate (o-phenylenediamine dihydrochloride, SIGMA, USA). The reaction was stopped with sulfuric acid and the colored product was read at 492 nm.

2.4. Cell cultures

The monocyte/macrophage murine cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with: 10% (v/v) heat-inactivated (30 min/60 °C) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids and 1% (v/v) penicillin–streptomycin solution (100 U/mL penicillin G,100 g/mL streptomycin). All cell culture reagents were from GIBCO BRL Life Technologies (Rockville,MD, USA).

2.5. Binding and internalization of S-layer protein from L. kefiri CIDCA 8348 by macrophages

The *in vitro* binding and internalization of SLP-8348 was analyzed by flow cytometry. Labeling of SLP-8348 with Atto 647 N was performed according to the manufacturer's instructions (SIGMA, USA). RAW 264.7 cells (5×10^5/well) were incubated with Atto 647N-labeled SLP-8348 for 1 h at 37 °C in complete medium (to assess uptake), or at 4 °C in complete medium (to assess binding) [28]. Cells were then washed and analyzed by flow cytometry. For inhibition assays, Atto 647N-labeled SLP-8348 was previously incubated with 5 mM EGTA or 50 mM of Glc, Man or Gal. Internalization was calculated as the difference between the mean fluorescence intensity (MFI) at 37 °C and MFI at 4 °C. The percentage of internalization was normalized to the condition obtained for Atto 647N-labeled SLP-8348. For confocal microscopy, cells were washed with cold PBS and fixed with 0.1% paraformaldehyde solution for 15 min at 4 °C and incubated with mouse anti-CD11c IgG antibody FITC-conjugated for 30 min at 4 °C. Nuclei were stained with propidium iodide at 1 μg/mL for 15 min. Chambers slides after mounting were analyzed in a TCS SP5 Confocal Microscope combined with ImageJ software.

2.6. RAW 264.7 cells stimulation assays

RAW 264.7 cells (2.5×10^5) were distributed onto 24-well microplates (JET BIOFIL®, China), and the medium volume was adjusted to 0.5 mL. The plates were incubated for 48 h at 37 °C in a 5% CO_2 95% air atmosphere to allow cellular adherence prior to experimentation. After that, cells were treated with LPS 0.1 μg/mL (LPS from *Escherichia coli* O111:B4, SIGMA, USA), SLP-8348 (10 μg/

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mL) or combinations of both in DMEM during 4 h (for cytokine's mRNA quantification) or 24 h (for secreted cytokine's quantification) at 37 °C in a 5% CO_2 95% air atmosphere. Cells incubated with DMEM were used as negative control.

2.7. RNA extraction and quantitative real time PCR analysis

Stimulated cells were harvested after 4 h of stimulation and homogenized in RA1 lysis buffer (GE Healthcare, UK) to perform the extraction of total RNA using the illustraRNAspin Mini RNA Isolation Kit (GE Healthcare, UK). Reverse transcription was performed with 100 ng of RNA using random primers and MMLV-Reverse transcriptase (Invitrogen, USA). Resulting cDNA was amplified in triplicate using the SYBR-Green PCR assay, and products were detected on an iCycler thermal cycler (BioRad, USA). The primers used for the gene expression are in Table 1. The geometric mean of housekeeping gene HPRT was used as an internal control to normalize the variability in expression levels. All results were expressed as fold increase of each treatment vs. the mean of medium treatment ($2^{-\Delta\Delta Ct}$ method). Melting curves were used to determine the specificity of PCR products.

2.8. Cytokine quantification in culture supernatants

Production of IL-6 and IL-10 cytokines by macrophages was analyzed by sandwich ELISA using commercially available capture and detection antibodies from BD-Pharmingen (San Diego, USA). The assay was performed according to the manufacturer's instructions. After determining optical densities, cytokine levels in cell culture supernatants were calculated using the GraphPad Prism 6.0 program.

2.9. Immunocytostaining and flow cytometry

After stimulation experiments, RAW 264.7 cells were washed twice with PBS containing 2% FBS and then labeled with different monoclonal antibodies: anti-CD40 (clone 3/23) PE-conjugated from BD-Biosciences (CA, USA), anti-MHCII (clone M5/114.15.2) and anti-CD86 (clone PO3.1) PE-conjugated from Thermo-Fisher (USA) for 30 min at 4 °C. Cells were washed twice with PBS containing 2% FBS and 0.1% sodium azide, and then fixed with 1% formaldehyde. Cells were analyzed using a FACSCalibur Analyzer (BD Biosciences).

2.10. Statistical analysis

The statistical analysis was performed with the GraphPad Prism program. Values from at least three independent experiments were analyzed by using *t* test with a $P < 0.05$ (*) or $P < 0.001$ (**).

3. Results and discussion

Glycosylation is the post-translational modification most frequently found in S-layer proteins [18]. Considering that at least one O-glycosylation site located in the N-terminal region was recently described in the SLPs from different *L. kefiri* strains [23], and also that we have recently reported the presence of both Glc and Man in glycan structures of the SLP from the strain *L. kefiri* CIDCA 83111 [29], we first carried out a lectin-reactivity assay using *Canavalia ensiformis* (ConA: αMan>αGlc). As it is shown in Fig. 1A, ConA strongly recognized SLP-8348. Considering that ConA interacts with both mannosylated and glucosylated residues, we further investigated whether these residues mediated recognition of SLP-8348. Pre-incubation with Glc, Man or EGTA (a Ca^{+2} chelating agent) produced a statistically significant inhibition of SLP-8348 recognition (Fig. 1B) indicating that ConA interacts with either of these residues present on SLP-8348 in a Ca^{+2}-dependent manner.

Regarding both the unique structural features and the self-assembly ability of these bacterial SLPs, they can be considered as very interesting candidates for the development of vaccine adjuvants or antigen carriers. Thus, their interaction with APCs is a crucial issue to be addressed. To determine if the glycan chains participate in the binding or/and the internalization by macrophages, a series of experiments were performed. As it is shown, SLP-8348 both interacted with RAW 264.7 cells and was actively internalized by them (Fig. 2A and B). We found that SLP-8348 internalization was inhibited (around 50%) by Man or Glc, indicating that the uptake depends on the recognition of the glycan chains present in the protein. Moreover, the presence of EGTA induced a significant decrease in the SLP-8348 uptake, suggesting the participation of a CLR in this process. On the other hand, none of the competitors tested were able to affect the SLP-8348 binding to RAW 264.7 cells, suggesting that other receptors might be also involved in this process. This SLP-8348-macrophage interaction was also visualized by confocal microscopy (Fig. 2C). It is important to note that, to our knowledge, the internalization of a lactobacilli's SLP by macrophages has not been reported hereto.

Several studies have shown that after interaction with lactobacilli, APCs like macrophages and dendritic cells undergo maturation, as measured by expression of surface cells markers and production of cytokines [20,30–34]. These immune responses are reported to be stimulated not only by intact bacterial cells, but also by some of their components, including cell wall (CW) fractions and SLPs [35]. Here, we analyzed the capacity of glycosylated SLP-8348 to induce the synthesis of cytokines by RAW 264.7 cells. Although at the tested conditions the treatment with SLP-8348 did not induce macrophage activation, it could enhance the cellular response to stimulation with *E. coli* LPS. In this sense, macrophages exposed to a simultaneous incubation with SLP-8348 and LPS showed higher expression and secretion of IL-6 and IL-10 (Fig. 3A and B), as well as a higher expression of co-stimulatory molecules as CD40, CD86 and for MHCII compared to LPS-stimulated cells (Fig. 3C). These results suggest that although SLP-8348 and LPS present different immune stimulation profiles, the combination of both results in a synergistic effect on the inflammatory response to LPS. These findings partially agree with those reported by Konstantinov et al. who have demonstrated that the binding of SlpA from *L. acidophilus* NCFM on dendritic cells is not sufficient to induce a strong maturation, but the combination with LPS could induce a higher secretion of IL-10 compared with LPS alone [20], without affecting the secretion of IL-6 or TNF-α, even assessing the same SlpA/LPS ratio than we have tested in our experiments. However, the enhancement of cellular response to LPS is not a general rule for the lactobacilli SLPs. In fact, Taverniti et al. have reported that SlpA from *L. helveticus* MIMLh5 inhibits the proinflammatory response of human macrophages to LPS [36].

Interestingly, when EGTA was added to the combination of SLP-

Table 1
Primers used for Real-Time PCR analysis.

Target	Primers	Sequence 5′- 3′
TNF-α	Forward	CATCTTCTCAAAATTCGAGTGACAA
	Reverse	CCTCCACTTGGTGGTTTGCT
IL-6	Forward	GTTCTCTGGGAAATCGTGGAAA
	Reverse	AAGTGCATCATCGTTGTTCATACA
IL-10	Forward	CATTTGAATTCCCTGGGTGAGA
	Reverse	TGCTCCACTGCCTTGCTCTT
HPRT	Forward	CAATGCAAACTTTGCTTTCC
	Reverse	CAAATCCAACAAAGTCTGGC

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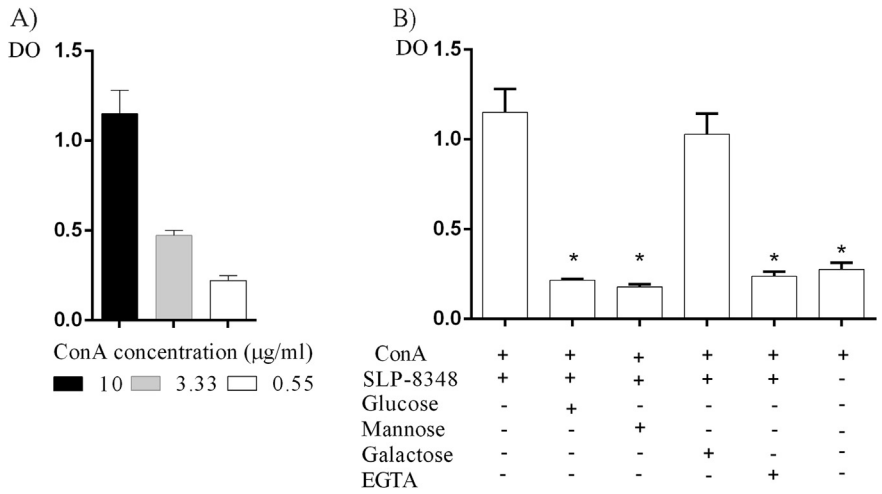

Fig. 1. Lectin from *Canavalia ensiformis* (ConA) recognizes the S-layer protein from *L. kefiri* CIDCA 8348 (SLP-8348). ConA reactivity to SLP-8348 at different lectin concentrations (A). Carbohydrate specificity was demonstrated by performing inhibition assays with specific carbohydrates (glucose and mannose) or a non-specific sugar (galactose) at 50 mM and 5 mM EGTA, using ConA at 10 µg/mL (B). *Significant difference relative to ConA + SLP-8348 ($P < 0.05$).

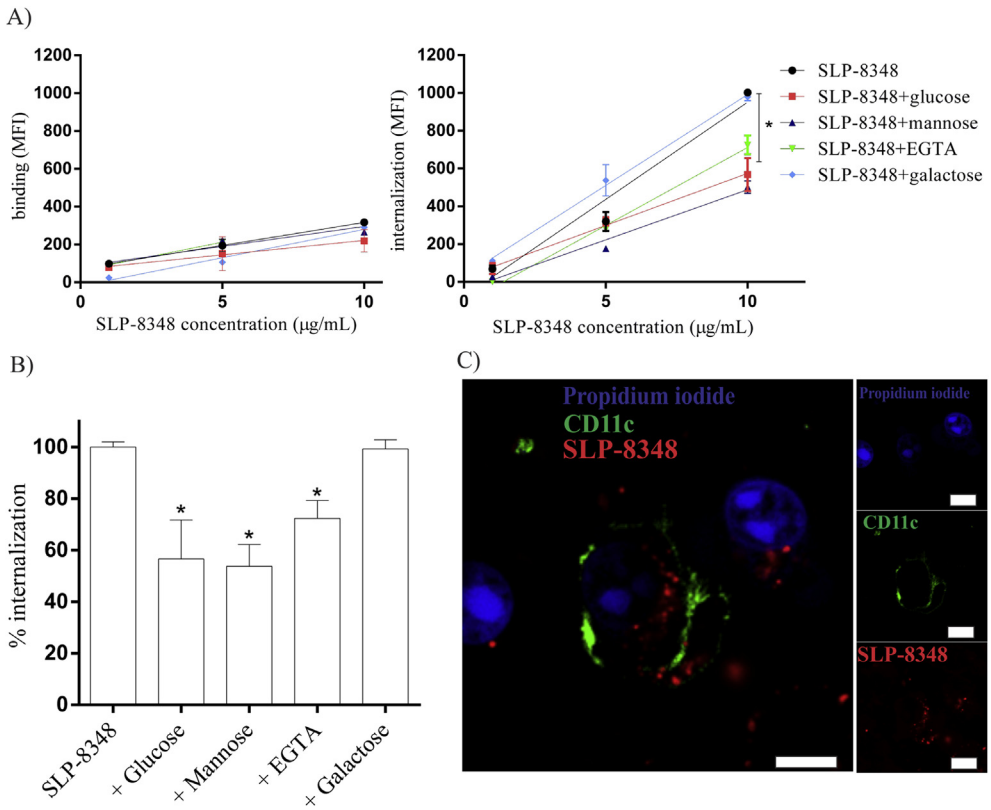

Fig. 2. SLP-8348 interacts with RAW 264.7 cells. Binding and internalization of Atto 647N-labeled-SLP-8348 at different concentrations in presence of 50 mM glucose, mannose or galactose, or 5 mM EGTA. Internalization was calculated as the difference between the MFI at 37 °C and MFI at 4 °C (A). Percentage of internalization in inhibition assays with Atto 647N-labeled-SLP-8348 at 10 µg/mL (B). Confocal microscopy of RAW 264.7 cells incubated with Atto 647N-labeled-SLP-8348 at 10 µg/mL (red), anti-CD11c-FITC (green) and propidium iodide (blue). Images were obtained with an optical magnification of 630X. White scale bar = 100 µm (C). *Significant difference relative to SLP-8348 ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

8348 and LPS in the stimulation assay, the level of secreted IL-6 and IL-10 decreased to those corresponding to the stimulation with LPS (Fig. 4), indicating that the interaction between SLP-8348 and LPS-activated macrophages is mediated by a CLR. To note, in the tested conditions, the addition of EGTA did not affect the pro-inflammatory response of RAW264.7 cells to LPS (Fig. 4). Even though SLP-DC-SIGN engagement was demonstrated for SlpA of *L. acidophilus* NCFM [20] and also has been recently suggested for the SLP from *L. kefiri* JCM 5818 [22], recent reports from Chinthamani et al. revealed that other CLRs such as macrophage inducible C-type lectin receptor (Mincle) are involved in the recognition of the SLP from the oral pathogen *Tannerella forsythia* [37]. Since

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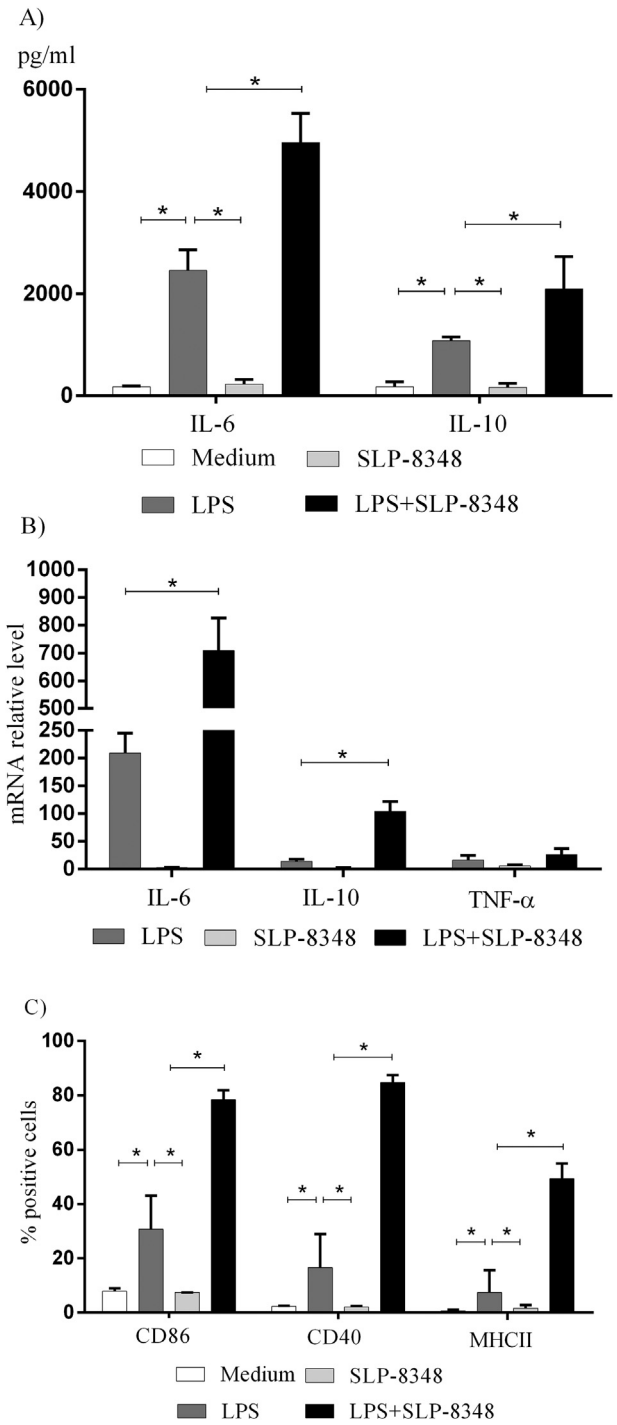

Fig. 3. SLP-8348 enhances the LPS-induced activation of macrophages. Cytokine's concentration (pg/mL) by capture ELISA in the supernatant of the murine RAW 264.7 cultures after 24 h of stimulation (A). Quantitative analysis of cytokine gene expression in murine RAW 264.7 cells after 4 h of stimulation. Expression levels of IL-6, IL-10, and TNF-α are indicated as the relative expression to the induction level of the control (medium stimulated RAW cells), which was set at a value of 1 (B). Percentage of $MHCII^{+}$, $CD40^{+}$ and $CD86^{+}$ RAW 264.7 cells after 24 h of stimulation (C). *P < 0.05.

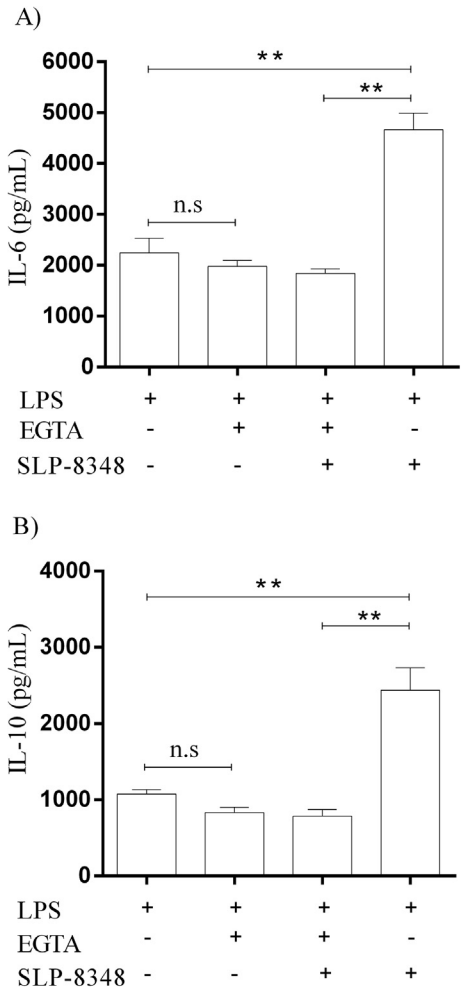

Fig. 4. SLP-8348-induced activation of LPS-stimulated macrophages is Ca^{+2}-dependent. IL-6 (A) and IL-10 (B) concentration (pg/mL) by capture ELISA in the supernatant of the murine RAW 264.7 cultures after 24 h of stimulation in presence or absence of 5 mM EGTA. **P < 0.001. n.s: not significant difference.

differences in SLP glycan structures have been described, even among strains of the same species, it is reasonable to think that different kind of carbohydrate receptors could be involved in bacterial SLP recognition.

At this point, all the results obtained in this work, strongly suggest the engagement of CLRs in the recognition of SLP-8348. Although that interaction could not be enough to induce a high response on macrophages itself, when SLP-8348 is combined with LPS (TLR4 agonist) the activation of RAW264.7 cells is higher than LPS stimulation alone. In this sense, several studies have demonstrated that cross-talk between CLRs and TLRs can occur [38]. Moreover, it has been shown that the crosstalk between TLR4 and DC-SIGN depends on the prior activation of NF-κB by TLR signaling and is therefore not limited to TLR4, but also includes triggering of other NF-κB inducing receptors, such as TLR3 and TLR5 [39]. Since CLR engagement could not only mediate endocytosis but also might influence intracellular signaling pathways, CLR targeting may allow the modulation of cellular functions [40]. The simultaneous triggering of several pattern recognition receptors can induce different innate immune responses, which provides the diversity that is required to shape an effective adaptive immune response [17]. Considering that CLRs such as DC-SIGN or MR usually display low affinities for their carbohydrate ligands, the engagement through multivalent ligands is a means to overcome these low affinities [40]. In this sense, the ability of SLP-8348 monomers to self-assemble could determine the presentation of glycan ligands in a multivalent form, resulting in an elevated binding affinity [41].

Further studies are needed to demonstrate which the receptor

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involved in the recognition of SLP-8348 on the surface of immune cells is. However, the immunomodulatory capacity of SLP-8348, together with its ability to self-assembly, do make this SLP a unique structure with high biotechnological potential in the search for adjuvants to development of new vaccines.

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Transparency document

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