Immuno-stimulation by *Lactobacillus kefiri* S-layer proteins with distinct glycosylation patterns requires different lectin partners

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S-layer (glyco)-proteins (SLPs) form a nanostructured envelope that covers the surface of different prokaryotes and show immunomodulatory activity. Previously, we have demonstrated that the S-layer glycoprotein from probiotic *Lactobacillus kefiri* CIDCA 8348 (SLP-8348) is recognized by Mincle (macrophage inducible C-type lectin receptor), and its adjuvanticity depends on the integrity of its glycans. However, the glycan’s structure has not been described so far. Herein, we analyze the glycosylation pattern of three SLPs, SLP-8348, SLP-8321, and SLP-5818, and explore how these patterns impact their recognition by C-type lectin receptors and the immunomodulatory effect of the *L. kefiri* SLPs on antigen-presenting cells. High-performance anion-exchange chromatography–pulse amperometric detector performed after β-elimination showed glucose as the major component in the O-glycans of the three SLPs; however, some differences in the length of hexose chains were observed. No N-glycosylation signals were detected in SLP-8348 and SLP-8321, but SLP-5818 was observed to have two sites carrying complex N-glycans based on a site-specific analysis and a glycomic workflow of the permethylated glycans. SLP-8348 was previously shown to enhance LPS-induced activation on both RAW264.7 macrophages and murine bone marrow–derived dendritic cells; we now show that SLP-8321 and SLP-5818 have a similar effect regardless of the differences in their glycosylation patterns. Studies performed with bone marrow–derived dendritic cells from C-type lectin receptor–deficient mice revealed that the immunostimulatory activity of SLP-8321 depends on its recognition by Mincle, whereas SLP-5818’s effects are dependent on SignR3 (murine ortholog of human DC-SIGN). These findings encourage further investigation of both the potential application of these SLPs as new adjuvants and the protein glycosylation mechanisms in these bacteria.

Glycosylation is considered the most popular post-translational modification targeting proteins. Although it was assumed that until the mid-1970s that the ability to glycosylate proteins was restricted to eukaryotic cells and archaeabacteria (1), nowadays it is known that this post-translational modification is not an exception in eubacteria, although it is early to predict the full extent of prokaryotic glycosylation (2). From the studies performed on eukaryotic cells, it is clear that the presence of glycans affect the expression, localization, and lifetime of numerous proteins, thus affecting its functions as well as several downstream biological events (3).

In eubacteria, protein glycosylation has been extensively studied in pathogens, emphasizing its relevance in virulence and pathogenicity. On the contrary, the nature and function of glycoproteins in nonpathogenic bacteria, including gut commensal species, remains largely unexplored (4). Because interactions between commensal bacteria, intestinal epithelial and immune cells play a crucial role in the maintenance of gut homeostasis, the study of protein glycosylation in gut commensal bacteria has become an expanding field of research because of the importance of the role of gut microbiota in health and disease (5, 6). This is particularly relevant in the case of surface proteins, which act as mediators of several interactions between microorganisms and their host.

The S-layer is a nanostructured proteinaceous envelope constituted by subunits that self-assemble to form a two-dimensional lattice that covers the surface of different species of *Bacteria* and *Archaea* (7). Given their ubiquitous presence, S-layers are considered as the result of evolutionary changes of the microorganisms to survive in harsh environments (8), although there are no reports on common functions for all of them (9). The presence of S-layer has been found in both Gram-negative and Gram-positive bacteria, including pathogenic and nonpathogenic species (10). Regarding the members of the genus *Lactobacillus*, microorganisms commonly retrieved in the gut of different mammalian hosts as well as in fermented foods, the S-layer has been detected in many but not all species (11). Because of their unique self-assembly ability, presenting repetitive identical physicochemical properties down to the subnanometer scale, the S-layer proteins have gained interest in distinct areas of biotechnology, biomimetics, and biomedicine. Indeed, different researchers have been conducted experiments focusing on the application of S-layer proteins

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for the development of new antigen/hapten carriers, adjuvants, or vaccination vesicles (11, 12).

After the presence of S-layer proteins in several strains of *Lactobacillus kefiri* formerly isolated from kefir grains and characterized as potentially health-promoting microorganisms was described (13), several studies have been performed in our laboratory to gain knowledge about structural and functional properties of these surface proteins. In particular, the S-layer proteins from *L. kefiri* strains show differences in their amino acid sequences, mainly in the C-terminal region of the polypeptide (14). Moreover, they are part of the small group of glycosylated S-layer proteins reported within the genus *Lactobacillus* (11), and until now, the detailed glycan structures have been described just for the strain *L. kefiri* CIDCA 83111 (15).

Noteworthy, although the S-layer proteins were the first glycoproteins described in prokaryotes, the studies of the role of the glycan residues in the functional properties of these proteins are scarce and are usually focused on some archaea (16) or pathogenic bacteria (17, 18). Regarding the genus *Lactobacillus*, there are some reports describing the involvement of the carbohydrate receptor DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN) in the functional activity of the S-layer proteins from *Lactobacillus acidophilus* NCFM (19), *Lactobacillus plantarum* (20), and *L. kefiri* JCM 5818 (21). In this sense, we have recently demonstrated that the S-layer glycoprotein from *L. kefiri* CIDCA 8348 is able to enhance the LPS-induced response in murine macrophages in a carbohydrate receptor–mediated process (22). Moreover, this SLP improves the OVA-specific cell immune response by triggering maturation of antigen-presenting cells through the recognition of glycan moieties by Mincle (23). However, the structure of its glycans has not been determined so far. Considering all this evidence, in the present work we aim to determine the glycosylation patterns of the S-layer proteins expressed on different strains of *L. kefiri* and to analyze the impact on the immunomodulatory activity on antigen-presenting cells.

**Results**

**Glycan structures of *L. kefiri* S-layer proteins**

We have previously reported the composition and structure of the glycans present in the S-layer protein from *L. kefiri* CIDCA 83111 (15). In this work, glycosylation of the S-layer protein from *L. kefiri* JCM 5818, CIDCA 8348, and CIDCA 8321 (SLP-5818, SLP-8348, and SLP-8321, respectively) were characterized. In this regard, the analysis of both O- and N-glycans was performed.

**O-Glycome analysis**

The SDS-PAGE bands corresponding to the purified SLPs of each strain were subjected to reductive β-elimination. Sugar composition was achieved by HPAEC-PAD. For this purpose, the released oligosaccharides were hydrolyzed, and monosaccharides were determined. In the three SLPs examined glucose was detected as the major component (Fig. 1, A–C). Also, in the three cases, when an alditol analysis was performed, sorbitol was detected. Taking into account that during the treatment the oligosaccharides linking the peptide are released and the reducing end of the oligosaccharides are reduced by NaBH₄, the result indicates that a glucose unit is the linkage between the O-linked oligosaccharides and the peptides (Fig. 1, D–F).
The O-glycoproteomic analysis of the SLPs was performed by nanoHPLC–ESI–Orbitrap–HCD. An in-gel trypsin digestion of the purified glycoprotein from each strain was performed followed by a glycopeptide enrichment step using cotton HILIC chromatography. In SLP-5818, the analysis of the extracted ion chromatogram showed a defined region of O-glycosylation detected by the extracted ion chromatograms of oxonium ions where up to four hexose units were detected (m/z = 163.06; 325.11, 487.16, and 649.21). It is known that glycosidic bonds are more labile under the HCD dissociation than peptides. However, notably in this case, a high coverage level of peptide fragmentation was achieved by detecting b and y ions from the glycosylated peptide. Moreover, peptide-glycan fragments (y ions) and the ion corresponding to the naked peptide were also detected (Fig. 2A). Deconvolution of the ions allowed the attribution of the signals eluting at retention time between 10 and 14 min to peptide 147SASASSASSASSAEQTTLTDALK170 carrying from 10 to 35 hexoses. Different glycoforms were resolved in the operating conditions of the reverse phase nanoHPLC employed. It is clearly evidenced that the chromatographic elution of O-glycopeptides is based on the hydrophilic nature of the attached glycans, therefore on their...
To determine the N-glycosylation sites in SLP-5818, we used PNGase F to release the N-glycans. It is known that the glycosylated asparagine residue is converted to aspartic acid during the enzymatic reaction (26). As a result, a mass increase of 0.984 Da is obtained in the modified peptide. Based on this strategy, a sample of enriched glycopeptides from SLP-5818 was N-deglycosylated using PNGase F and analyzed by nanoHPLC–ESI–Orbitrap. Because spontaneous deamidation might occur, the ratio asparagine/aspartic acid was considered to avoid misassignments (Table S2 shows peptide identification data). Thus, two N-glycosylation sites at NVAVN\(^{55}\)GTNA-LYTK and at DAALTSLN\(^{457}\)NTVK were determined. As shown in Fig. 4B, MS2 spectrum of peptide NVAVNGTNA-LYTK showed fragment ion y\(_9\) with a m/z = 982.82 (calc. m/z = 981.50, Δ 0.984 Da) confirming the N-glycosylation site at Asn\(^{55}\). In the same way, MS2 spectrum of peptide DAALTSLN\(^{457}\)NTVK showed y\(_7\)-fragment ion m/z = 576.30 (calc. m/z = 575.31, Δ 0.984 Da) evidencing an N-glycosidic linkage in Asn\(^{457}\) (Fig. 4C). Further on, to determine the N-glycan structures attached to SLP-5818, we performed an enzymatic hydrolysis of the N-oligosaccharides using PNGase F. Glycans were separated, permethylated, and analyzed by nanoHPLC–ESI–Orbitrap. The extracted ion chromatograms corresponding to m/z = 464.24 (HexNAc-Hex\(^1\)) and m/z = 189.11 (dHex\(^1\)) allowed detection of a family of complex permethylated N-glycans eluting between real times of 40 and 45 min (Fig. 5A). Main structures, m/z, and mass errors are detailed in Table 4. Fig. 6 shows two examples of MS2 spectra of selected ions assigned to two biantennary glycans differing in the composition of one antennae. The permethylated N-glycans were also examined using MALDI-TOF MS, and structures corresponding to biantennary species were also detected (Fig. 5B).

Stimulation of murine macrophages by L. kefiri SLPs

Because we have previously demonstrated that SLP-8348 can enhance LPS-induced activation of antigen-presenting cells (22, 23), we decided to test the ability of SLP-8321 and SLP-5818 to activate murine macrophage, using RAW 264.7 cells as first model. As was previously observed for SLP-8348 (22, 23), the SLP-8321 and SLP-5818 were not able to induce activation of RAW 264.7 cells by themselves. However, respective significant increments in secreted IL-6, as well as in surface expression of CD40 and CD86, were observed for macrophages simultaneously exposed to *Escherichia coli* LPS and *L. kefiri* S-layer glycoproteins compared with LPS-treated cells (Fig. 7, A and B). To note, no significant differences were observed among the stimulatory activity of strains despite the differences in their glycosylation patterns.

Interaction of SLPs with different C-type lectin receptors

Considering that it has been previously shown for SLP-8348 that the recognition of the glycan residues by a C-type lectin receptor (CLR) is a key event in the immunomodulatory activity of the protein (23), we decided to test the ability of SLP-8321 and SLP-5818 to interact with different CLRs. As shown previously for SLP-8348 (23), SLP-8321 and SLP-5818 bound to the...
CLRs DC-SIGN, SingR3, Langerin, and Mincle (Fig. 8A). As expected, a reduction of ∼70% was observed in the SLP–CLR interactions in the presence of the Ca²⁺-chelating agent EGTA (Fig. 8B).

**Activation of BMDC by SLP-8321 and SLP-5818**

Regarding the ability of SLP-8321 and SLP-5818 to interact with different CLRs and considering that both glycoproteins enhanced LPS-induced response in murine macrophages, we decided to use BMDCs from C57BL/6 mice as antigen-presenting cells to assess both internalization of SLPs and cell activation. As shown for SLP-8348 (23), we observed that both SLP-8321 and SLP-5818 were internalized by BMDCs in a dose-dependent way (Fig. 9A). However, a significant decrease in the uptake of SLP-8321 was observed when BMDCs from Mincle²⁻/² mice were used. On the contrary, the internalization of SLP-5818 was only reduced in BMDCs from SignR3²⁻/² mice and was not affected by the absence of Mincle (Fig. 9A).

Following the same methodology previously reported (23), we decided to test the immunomodulatory activity of SLP-8321 and SLP-5818 on BMDCs. Unlike in the case of RAW 264.7 cells, both SLPs were able to induce the maturation of BMDCs because a significant increment in the level of secreted IL-6 and TNFα, as well as in the surface expression of CD40 and CD80, was observed (Fig. 9, B and C). The stimulatory activity of SLP-8321 was lost when BMDCs from Mincle²⁻/² mice were used, whereas SLP-5818 was not able to activate BMDCs from SignR3²⁻/² mice (Fig. 9, B and C).

To further investigate the T-cell stimulatory function of the SLP-activated BMDCs, OVA-treated BMDCs from WT, Mincle²⁻/², SignR3²⁻/², and CARD9²⁻/² were incubated in the presence or absence of SLP-8321 or SLP-5818 with purified T cells from OT-II mice, as was previously performed with SLP-8348 (23). Both SLPs could enhance the OVA-specific T-cell response because a higher IFN-γ secretion as well as higher CD4⁺ T-cell proliferation were observed upon simultaneous stimulation of WT BMDCs with OVA and SLP-8321 or SLP-5818 compared with OVA-treated antigen-presenting cells (Fig. 10, A and B). The absence of Mincle abrogated the immunostimulatory activity of SLP-8321, whereas SLP-5818 was not able to enhance CD4⁺ T-cell response when BMDCs from SignR3²⁻/² mice were used (Fig. 10, A and B). As was expected, none of the SLPs assessed were able to potentiate CD4⁺ T-cell activation when assays were performed using BMDC from CARD9²⁻/² mice, a downstream signaling protein of Mincle and SignR3 (27) (Fig. 10, A and B).

**Discussion**

Considering the ubiquitous presence of S-layer–carrying microorganisms and the abundance of the S-layer proteins, it is evident that these structures reflect the evolutionary adaptation of the organisms to natural habitats and must have provided them with advantages in specific environmental and ecological conditions (8). Glycosylation is the post-translational modification most frequently found in SLPs, a feature shared with other surface-exposed proteins such as...
L. kefiri S-layer protein glycosylation on immune activation

Table 2
O-Linked glycosylated peptides of L. kefiri CIDCA 8348 S-layer protein

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Table 3
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flagellin and pilin. Within the genus Lactobacillus, the glycoprotein nature of the S-layer proteins has been reported for strains of Lactobacillus buchneri (28, 29), L. plantarum 41021/252 (28), L. acidophilus NCFM (19), L. acidophilus ATCC 4356 (30), and several strains of L. kefiri (14, 24). In this last species, we have previously described the composition and structure of the O- and N-glycans present in the SLP of the strain L. kefiri CIDCA 83111 (15). Herein, we show that SLP-5818 also presents O- and N-glycosylated chains. In this case, peptide 147–170 is O-glycosylated in average with eight hexose units, but at difference with the SLP from L. kefiri CIDCA 83111, is not decorated with galacturonic acid (15). Regarding N-glycans, both SLP-5818 and SLP-83111 present two N-glycosylation carrying bianntenary chains.

On the other hand, SLP-8321 and SLP-8348 show 100% of homology at primary sequence level (14), and although they present six potential N-glycosylation sites, no evidence of bearing N-glycans was found. On the contrary, O-glycosidic chains constituted by 4–12 glucose units were detected in both SLPs.

Interstrain differences in the glycosylation patterns of SLP were also seen some years ago in Thermoanaerobacter thermohydrodsulflcicus and Thermoanaerobacterium thermosaccharolyticum (31). Moreover, in Clostridium difficile the presence of a glycosylated SLP was only demonstrated in the strain Ox247, which encodes a putative glycosylation locus (18), whereas normally most C. difficile strains do not possess a glycosylated S-layer (32). On the other hand, Anzengruber et al. (29) reported that SLPB from L. buchneri CD034, SLPN from L. buchneri NRRL B-30929, and L. buchneri SLPB from 41021/251 showed identical O-glycans consisting of on average seven glucoses at four serine residues within the sequence 152SASSAS157 without any evidence of the presence of N-glycosidic chains in those SLP. In this sense, SLP-8321 and SLP-8348 show a higher similarity to the SLPs from L. buchneri strains than SLP-83111 and SLP-5818. Further studies are needed to elucidate the mechanisms through which the glycan chains are coupled to the peptide backbone in these surface proteins.

As surface-exposed structures, the SLPs could be a key mediator between bacteria and host immune system contributing to the shaping of the immunological response to pathogenic, commensal, and probiotic microorganisms. By using murine macrophages RAW 264.7 cells as a model, we have demonstrated that despite the differences between glycosylation patterns of SLP-8321 and SLP-5818, both proteins are able to enhance LPS-induced cell activation (Fig. 7), similarly to previously demonstrated for SLP-8348 (22). These results suggest that O-glycans present in these three mentioned L. kefiri SLPs could be responsible, at least in part, of their immunostimulatory ability.

On the other hand, as previously described with SLP-8348 (23), the assays using BMDCs from Mincle−/− and SignR3−/− mice allowed us to determine that the internalization of SLP-8321 and SLP-5818, as well as their ability to activate BMDCs, was mainly mediated by Mincle and SignR3 (the murine ortholog of human DC-SIGN), respectively (Fig. 9), which could be expected considering the differences in the glycosidic moieties present in those proteins. Moreover, the incubation of BMDCs with SLPs from different L. kefiri strains leading to the enhancement of activation of OVA-specific CD4+ T cells from OT-II mice, is also dependent on the presence of Mincle and SignR3 for SLP-8321 and SLP-5818, respectively (Fig. 10).

Some years ago, a pioneering work performed using a SlpA-knockout mutant L. acidophilus NFCM revealed that recognition of SlpA by DC-SIGN in presence of LPS induce an anti-inflammatory response in human monocyte-derived dendritic cells (19), suggesting the involvement of glycans in the immunomodulatory properties of SLPs. More recently, it was demonstrated that L. acidophilus NCK2187, a strain that solely expresses SlpA, and its purified SlpA binds to SignR3 to trigger regulatory signals that result in mitigation of experimental colitis, maintenance of healthy gastrointestinal microbiota, and mouse protection (33). In addition, the ability of DC-SIGN to recognize the SLP-5818 was recently suggested by Prado Acosta et al. (21), which agrees with our findings. However, the role of glycan residues, as well as the CLR involved in the internalization and immunostimulatory activity of SLP-5818, had not been reported so far. On the contrary, it is not surprising
that Mincle, the CLR responsible for the recognition and adjuvantivity of the SLP-8348 (23), mediates the immunostimulatory effect of SLP-8321 on BMDCs because both glycoproteins present not only the same amino acid sequence (14) but also the same \(O\)-glycans. It is known that Mincle recognizes diverse sugar-containing ligands including trehalose dimycolate glycolipid from mycobacteria; mannose-, glucose-, or fucose-containing glycoconjugates; and Lewis antigen from \textit{Helicobacter pylori} LPS (34). To note, it was recently reported that Mincle also recognize the \(O\)-linked oligosaccharides of the SLP from the Gram-negative oral pathogen \textit{Tannerella forsythia} and that interaction induces both pro- and anti-inflammatory cytokine secretion in macrophages (17).

To resume, despite the differences in glycosylation patterns, as well as the involvement of different CLRs, the glycosylated SLPs from distinct \textit{L. kefiri} strains can induce activation of antigen-presenting cells through the recognition of their glycans. Taken together, these findings encourage us to further investigate the potential application of these surface proteins derived from probiotic bacteria to the development of new adjuvants or carrier for vaccine antigens, as well as to get deeper insight into the protein glycosylation mechanisms in these bacteria.

**Experimental procedures**

**Bacterial strains and culture conditions**

\textit{L. kefiri} CIDCA 8321 and 8348 belonging to the collection of the CIDCA (35) and \textit{L. kefiri} JCM 5818 were used. Bacteria were cultured in deMan–Rogosa–Sharpe broth (DIFCO, Detroit) at 37 °C for 48 h, under aerobic conditions.

**S-layer protein extraction**

Extraction from bacterial cells at stationary phase was performed using 5 M LiCl as described previously (22). The samples were centrifuged, and the protein concentration in the
The supernatant was determined according to Bradford. The homogeneity of the protein extracts was tested by SDS-PAGE and stained with Coomassie Blue (36). S-layer proteins were filtrated through a membrane of 0.22-μm pore diameter for cellular stimulation assays.

**Composition and structure of glycan moieties**

Lectin-based N-glycoprotein purification

The enrichment of glycoproteins was performed using WGA lectin affinity chromatography. Briefly, 1 ml of WGA–Sepharose (Sigma) was loaded onto a 5-ml column. Prior to sample

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**Figure 5.** A, nanoHPLC–ESI–Orbitrap analysis of permethylated N-glycans. Panel a, total ion current chromatogram. Panels b and c, extracted ion chromatograms of permethylated oxonium ions: m/z 464.24 (panel b, Hex-HexNAc oxonium ion) and m/z 189.11 (panel c, dHex oxonium ion). Red triangles, dHex; green and yellow circles, Hex; blue squares, HexNAc. B, MALDI-TOF MS spectrum of permethylated N-glycans.
purification, the stationary phase was equilibrated with 20 mM Tris, pH 7.5, 0.5 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂. Then 50 μl of protein sample was loaded using the latter buffer solution. Nonglycosylated proteins were washed using 20 mM Tris, pH 7.5, containing 1 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂. Finally, the retained glycoproteins were eluted using the above buffer solution containing 0.5 M N-acetyl-D-glucosamine. The glycoprotein fraction was dialyzed against ultrapure water using Spectra/Por® molecular porous membrane tubing with a cutoff of 3.5 kDa. The lectin-based purification was monitored by SDS-PAGE and stained with Colloidal Coomassie Blue. 

Release of N-glycosidic chains by PNGase F treatment

The SDS-PAGE S-layer glycoprotein band was cut out from the gel, frozen for 3 h and successively washed with (a) acetonitrile, (b) 20 mM NaHCO₃, pH 7, and (c) acetonitrile. The gel pieces were dried, and the N-glycans were released by incubation with PNGase F (20 milliliters) (New England Biolabs, Inc., Beverly, MA) overnight at 37 °C in 20 mM NaHCO₃, pH 7. The gel pieces were thoroughly washed, and the supernatants were separated and filtered through an Ultrafree McFilter (M₅, 5000), dried, resuspended in 0.1% (v/v) formic acid (20 μl), and left at room temperature for 40 min. Finally, the glycans were dried and suspended in water.

Glycoprotein digestion

The protein band corresponding to the S-layer glycoproteins were cut out from the gel and washed with acetonitrile. The gel pieces were reduced with 10 mM DTT in 50 mM NH₄HCO₃ at 55 °C for 30 min. Then 50 μl of protein sample was loaded using the latter buffer solution. Nonglycosylated proteins were washed using 20 mM Tris, pH 7.5, containing 1 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂. Finally, the retained glycoproteins were eluted using the above buffer solution containing 0.5 M N-acetyl-D-glucosamine. The glycoprotein fraction was dialyzed against ultrapure water using Spectra/Por® molecular porous membrane tubing with a cutoff of 3.5 kDa. The lectin-based purification was monitored by SDS-PAGE and stained with Colloidal Coomassie Blue.

In gel-reductive β-elimination and sugar analysis

O-Linked glycans were released from each SDS-PAGE band of the S-layer glycoproteins using 0.05 M NaOH, 1 M NaBH₄ and incubated overnight at 50 °C. The solution was separated, and acetic acid was added until pH 7 followed by repeated evaporation with methanol. The sample was dissolved in water, desalted in a Dowex 50 W (H⁺) (Fluka) column, and dried in SpeedVac. The β-eliminated samples were further hydrolyzed in 2 N TFA for 4 h at 100 °C. The acid was eliminated by evaporation, and the hydrolysates were resuspended in water for HPAEC-PAD. Analysis was performed using a DX-500 Dionex BioLC system (Dionex Corp.). For neutral and amino sugars, a CarboPac PA20 with the corresponding guard column was used. Isocratic separations were achieved with 18 mM NaOH, and the flow rate was set to 0.4 ml/min. For aldolits, a CarboPac MA 1 column was used with a 0.4 M NaOH isocratic program and a flow rate of 0.4 ml/min.

Permethylation of released oligosaccharides

After PNGase digestion, the oligosaccharides were methylated with NaOH in DMSO followed by addition of CH₃I as described (37).

Glycopeptide enrichment

Sample purification and glycopeptide enrichment was achieved by solid-phase extraction cotton HILIC microtips as described (38). Briefly, HILIC microtips were equilibrated with three volumes of 85% ACN. Dried sample was resuspended in 85% acetonitrile. Prior to SPE clean-up step, samples were loaded onto the stationary phase by aspirating and dispensing the protein mixture. The peptides were washed off from the column with 85% ACN, 0.5% TFA, and the retained glycopeptides were eluted using water. The glycopeptide fraction was freeze-dried and stored at −20 °C until MS analysis.

MS analysis

The enriched glycopeptide mixtures were re-suspended in 50% acetonitrile, 1% formic acid/water (1:1). The digests were analyzed in a nanoLC 1000 coupled to an EASY-Spray Q Exactive mass spectrometer (Thermo Scientific) with a HCD and an Orbitrap analyzer. An EASY-Spray PepMap RSLC C18 column (50 μm × 150 mm; particle size, 2.0 μm; pore size, 100 Å) at 40 °C was used. Separation was achieved with a linear gradient from 5% to 35% solvent B developed in 75 min, at a flow of 300 nl/min (mobile phase A: water, 0.1% formic acid; mobile phase B: acetonitrile, 0.1% formic acid). Injection volume was 2 μl. Spray voltage was 3.5 kV (+) or 3.0 kV (−). A full-scan survey MS experiment was performed (m/z range, 400–2000; automatic gain control target, 3 × 10⁶; maximum IT, 200 ms; resolution, 400 m/z, 70,000). The data-dependent MS2 method was set to the centroid mode; resolution was 17,500; maximum IT was 50 ms; automatic gain control target was 105; the fragment included the top 15 peaks in each cycle; and normalized collision energy was 27. For the analysis of permethylated oligosaccharides, HPLC separation was achieved with a linear gradient of solvent B from 5% to 60% developed in 75 min (flow, 300 nl/min; solvent A was water, 0.1% formic acid; solvent B was ACN, 0.1% formic acid; injection volume, 5 μl).

MALDI-TOF-MS analysis

Measurements were performed using an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a solid-state laser (λ = 355 nm). The system
was operated by the Flexcontrol 3.3 package (Bruker Daltonics GmbsH, Bremen, Germany) using gentisic acid as matrix.

Data analysis
Proteome Discoverer 2.1 (Thermo Scientific) assisted protein identification. The mass tolerances for MS and MS/MS were 10 ppm and 0.02 Da, respectively. Missed cleavages allowed for tryptic digestion was 1. Carbamidomethylation of cysteine residues was set as static modification and oxidation of methionine as dynamic modification. For N-glycosite analysis, conversion of asparagine to aspartic acid was included as dynamic modification. Precursor mass tolerance was 10 ppm, and fragment mass tolerance was 20 ppm. Confident identification for peptides was set to a score of $>100$.

Glycopeptide search was performed with MaxQuant version 1.6.5, Byonic™ from ProteinMetrics, Proteome Discoverer 2.2.

Figure 6. Selected MS2 spectra of permethylated N-glycans detected by nanoHPLC–ESI–Orbitrap analysis. A, m/z 1009.5226. B, m/z 886.9614. Red triangles, dHex; green and yellow circles, Hex; blue squares, HexNAc.
and using a semiautomatic homemade software (A0A1C3S3I4 S-layer protein OS=Lactobacillus kefiri OX = 33962 GN=S-layer A0A1C3S3T6 S-layer protein OS=Lactobacillus kefiri OX = 33962 GN=S-layer). All glycopeptides identifications from both searches were manually curated. MS-Convert Tool (from ProteoWizard platform tools) converted raw data from enriched glycopeptides into text files for manual annotation. Deconvolution was assisted by Xtract on Thermo Xcalibur 3.0.63.

**C-type lectin receptor recognition of S-layer proteins**

The CLR reactivity on S-layer proteins from *L. kefiri* CIDCA 8321 (SLP-8321) and *L. kefiri* JCM 5818 (SLP-5818) was evaluated by an ELISA type assay as described previously (23, 39). A half-area microplate (Greiner Bio-One GmbH, Frickenhausen, Germany) was coated with 0.25 μg of SLP per well for 16 h at 4°C and blocked with 1% BSA (Thermo Fisher Scientific, Darmstadt, Germany) for 2 h at room temperature. Then 0.25 μg of each CLR–hFc fusion protein in lectin-binding buffer (50 mM HEPES, 5 mM MgCl2, and 5 mM CaCl2) was added and incubated for 1 h at room temperature. For inhibition assays, CLR–hFc fusion proteins were preincubated with 5 mM of EGTA (Sigma–Aldrich). After washes, horseradish peroxidase–conjugated anti-human IgG anti-body (Dianova) was added to each well for 1 h at room temperature. Finally, the plates were incubated with chromogenic substrate (o-phenylenediamine dihydrochloride substrate tablet (Thermo Fisher Scientific), 24 mM citrate buffer, 0.04% H2O2, 50 mM phosphate buffer in H2O). The reaction was stopped with 2.0 M sulfuric acid and the product was read at

**Figure 7. Modulation of LPS-induced maturation of macrophages by *L. kefiri* SLP-8321 and SLP-5818.** A, IL-6 concentration (pg/ml) by capture ELISA in the supernatant of the murine RAW 264.7 cultures after 24 h of stimulation. B, percentage of CD40+ and CD86+ RAW 264.7 cells after 24 h of stimulation. A representative figure of three independent experiments is shown (± S.D., indicated by error bars). *, *P < 0.05; n.s., not significant.

**Figure 8. C-type lectin receptor recognition of SLP-8321 and SLP-5818.** A, reactivity of CLR–hFc fusion proteins on SLP was evaluated by an ELISA type assay (using the same experimental model as in Ref. 23). B, inhibition assays were performed using EGTA, a Ca2+–chelating agent. These are representative figures of three independent experiments (± S.D., indicated by error bars).
495 nm using a Multiskan Go microplate spectrophotometer (Thermo Fisher Scientific).

Mice

The source of the CLR- and CARD9-deficient mice was described previously (40–42). Mincle<sup>−/−</sup>, SignR3<sup>−/−</sup> and CARD9<sup>−/−</sup> mice were backcrossed on C57BL/6 background over 10 generations in the animal facility of the Federal Institute for Risk Assessment (Berlin, Germany). All mouse lines (including WT and C57BL/6-Tg (TcraTcrb)425Cbn/J (OT-II) mice) were kept in the animal house of the University of Veterinary Medicine (Hannover, Germany) with water and food supplied ad libitum. Mice were sacrificed for the isolation of spleen cells (OT-II transgenic mice) or the preparation of bone marrow for BMDC generation (Mincle<sup>−/−</sup>, CARD9<sup>−/−</sup>, or SignR3<sup>−/−</sup> bone marrow precursors (2.5 × 10<sup>5</sup> cells/ml) that were plated in complete culture medium (Iscove’s modified Dulbecco’s medium supplemented with 2 mM glutamine, 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin) supplemented with a granulocyte/macrophage colony-stimulating factor containing supernatant from P3-X63 cells. Medium was exchanged every 48 h, and BMDCs were used after 8–10 days of differentiation to ascertain that ≥80% of the cell population expressed the marker CD11c (43).

Cell cultures

The monocyte/macrophage murine cell line RAW 264.7 was cultured in Dulbecco’s modified Eagle’s medium supplemented with: 10% (v/v) heat-inactivated (30 min/60 °C) fetal bovine serum (FBS), 1% (v/v) nonessential amino acids and 1% (v/v) penicillin-streptomycin solution (100 units/ml penicillin G, 100 g/ml streptomycin). All cell culture reagents were from Gibco BRL Life Technologies (Rockville, MD).

BMDCs were generated from C57BL/6 WT, Mincle<sup>−/−</sup>, CARD9<sup>−/−</sup>, or SignR3<sup>−/−</sup> bone marrow precursors (2.5 × 10<sup>5</sup> cells/ml) that were plated in complete culture medium (Iscove’s modified Dulbecco’s medium supplemented with 2 mM glutamine, 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin) supplemented with a granulocyte/macrophage colony-stimulating factor containing supernatant from P3-X63 cells. Medium was exchanged every 48 h, and BMDCs were used after 8–10 days of differentiation to ascertain that ≥80% of the cell population expressed the marker CD11c (43).

Cell stimulation assays

These experiments were performed as previously described (22, 23). RAW 264.7 cells (2.5 × 10<sup>5</sup>) were distributed onto 24-well microplates (JET BIOFIL<sup>®</sup>, China), and the medium volume was adjusted to 0.5 ml. The plates were incubated for 48 h
at 37 °C in a 5% CO2 95% air atmosphere to allow cell adherence prior to experimentation. After that, the cells were treated with SLP-8321 or SLP-5818 (10 μg/ml), in the presence or absence of LPS 0.1 μg/ml (LPS from *E. coli* O111:B4, Sigma), in Dulbecco’s modified Eagle’s medium for 24 h at 37 °C in a 5% CO2 95% air atmosphere. The cells were incubated with anti-CD40 (3/23), and anti-CD86 (P03.1) and then analyzed by flow cytometry.

BMDCs from WT or CLR-deficient mice (1 × 10^5 cells/well) were distributed onto 96-well microplates (Sarstedt, Germany) and stimulated with SLP-8321 or SLP-5818 (10 μg/ml), LPS (0.25 μg/ml), or the combination of both for 16 h at 37 °C in a 5% CO2 95% air atmosphere. Culture supernatants were collected and analyzed by ELISA for IL-6 and tumor necrosis factor secretion. The cells were incubated with anti-CD16/32 (93) to block cell-surface FcgRII/RIII receptors; stained with anti-CD11c (N418), CD40 (3/23), and CD80 (16-10A1); and then analyzed by flow cytometry.

**BMDCs/T cell co-culture assay**

Splenocytes were isolated from OT-II transgenic mice by flushing the spleen with complete Iscove’s modified Dulbecco’s medium. After erythrocyte lysis, the cells were resuspended in magnetic-activated cell separation buffer (0.5% BSA, 2 mM EDTA in PBS). T cells were obtained by magnetic-activated cell separation using Pan T cell isolation kit II, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (Sigma–Aldrich) and seeded on a 96-well round bottom culture plate (7 × 10^4 cells/well). After 30 min, BMDCs treated with OVA (Endo Grade® Ovalbumin, LIONEX GmbH, Germany) or OVA/SLPs were added and incubated for 72 h. Culture supernatants were collected for IFN-γ secretion. Proliferation index of CD4+ T cells within each experimental group was calculated as the ratio between the percentage of CFSE<sup>low</sup> CD4<sup>+</sup> cells and CFSE<sup>low</sup> CD4<sup>+</sup> cells from OVA-treated BMDCs.

**Cytokine quantification in culture supernatants**

Production of IL-6 by macrophages was analyzed by ELISA from BD-Pharmingen (San Diego). Secretion of IL-6 and TNFα by BMDCs and IFN-γ from purified T cells were analyzed by ELISA from PeproTech. The assays were performed according to the manufacturer’s instructions. After determining optical densities, cytokine levels in cell culture supernatants were calculated using the GraphPad Prism 7.0 program.

**Immunocytostaining and flow cytometry**

After stimulation experiments, the cells were washed twice with PBS containing 2% (v/v) FBS and then labeled with specific monoclonal antibodies for 30 min at 4 °C. The cells were washed twice and then fixed with 1% (v/v) formaldehyde. The cells were analyzed using a FACSCalibur Analyzer (BD Biosciences) or Attune NxT flow cytometer (Thermo Fisher Scientific). Data analysis was performed using the FlowJo Software (FlowJo, Ashland, OR).

**Statistical analysis**

The values from at least three independent experiments were analyzed by using a one-way or two-way analysis of
variance with Tukey’s post hoc test ($p < 0.05$ was considered statistically significant). Statistical analysis was performed with the GraphPad Prism program (GraphPad Software, San Diego, CA).

Data availability

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (44) partner repository with the data set identifier PXD020358.


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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: SLP, S-layer glycoprotein; CLR, C-type lectin receptor; HPAEC, high-performance anion-exchange chromatography; HCD, high collision dissociation; WGA, wheat germ agglutinin; ESI, electrospray ionization; BMDC, bone marrow-derived dendritic cell; ANPCyT, Agencia Nacional de Promoción Científica y Tecnológica; CIDCA, Centro de Investigación y Desarrollo en Criotecnología de Alimentos; PNGase F, peptide:N-glycosidase F; FBS, fetal bovine serum; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; PAD, pulse amperometric detector; OVA, ovalbumin; DC-SIGN, dendritic cell–specific intercellular adhesion molecule–3 grabbing non-integrin; ACN, acetonitrile; HILIC, hydrophilic interaction liquid chromatography.

References

L. kefiri S-layer protein glycosylation on immune activation


