Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2013, 79(4):1221. DOI: 10.1128/AEM.03056-12.**
Published Ahead of Print 7 December 2012.

AEM
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S-Layer Protein Mediates the Stimulatory Effect of *Lactobacillus helveticus* MIMLh5 on Innate Immunity

Valentina Taverniti,[a] Milda Stuknyte,[a] Mario Minuzzo,[b] Stefania Arioli,[a] Ivano De Noni,[a] Christian Scabiosi,[a] Zuzet Martinez Cordova,[c] Ilkka Junttila,[d,e] Sanna Hämäläinen,[c,d] Hannu Turpeinen,[c] Diego Mora,[a] Matti Karp,[f] Marko Pesu,[c,e] Simone Guglielmetti[a]

Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy[a]; Department of Biomolecular Sciences and Biotechnology, Università degli Studi di Milano, Milan, Italy[b]; Institute of Biomedical Technology, Biomeditech University of Tampere, Tampere, Finland[c]; School of Medicine, University of Tampere, Tampere, Finland[d]; Fimlab Laboratories, Tampere, Finland[e]; Institute of Environmental Engineering and Biotechnology, Tampere University of Technology, Tampere, Finland[f]

The ability to positively affect host health through the modulation of the immune response is a feature of increasing importance in measuring the probiotic potential of a bacterial strain. However, the identities of the bacterial cell components involved in cross talk with immune cells remain elusive. In this study, we characterized the dairy strain *Lactobacillus helveticus* MIMLh5 and its surface-layer protein (SlpA) using *in vitro* and *ex vivo* analyses. We found that MIMLh5 and SlpA exert anti-inflammatory effects by reducing the activation of NF-κB on the intestinal epithelial Caco-2 cell line. On the contrary, MIMLh5 and SlpA act as stimulators of the innate immune system by triggering the expression of proinflammatory factors tumor necrosis factor alpha and COX-2 in the human macrophage cell line U937 via recognition through Toll-like receptor 2. In the same experiments, SlpA protein did not affect the expression of the anti-inflammatory cytokine interleukin-10. A similar response was observed following stimulation of macrophages isolated from mouse bone marrow or the peritoneal cavity. These results suggest that SlpA plays a major role in mediating bacterial immune-stimulating activity, which could help to induce the host's defenses against and responses toward infections. This study supports the concept that the viability of bacterial cells is not always essential to exert immunomodulatory effects, thus permitting the development of safer therapies for the treatment of specific diseases according to a paraprobiotic intervention.

Lactobacilli are Gram-positive bacteria that include 10s of species living in a variety of habitats where high levels of soluble carbohydrates, protein-breakdown products, vitamins, and low oxygen tension occur (1). Certain lactobacilli are autochthonous inhabitants of the gastrointestinal tract of animals and humans, where they are considered a beneficial component of the microbiota. In fact, an increasing amount of scientific data demonstrates that intestinal lactobacilli can positively affect the host's health (2, 3). Due to their ability to interact with several gut physiological processes, certain human intestinal *Lactobacillus* species (particularly *Lactobacillus acidophilus*, *L. johnsonii*, *L. paracasei*, *L. reuteri*, and *L. rhamnosus*) are commonly employed as probiotics, i.e., live microorganisms that, when administered in adequate amounts, confer benefits to the host organism (4).

Other *Lactobacillus* species, such as *L. delbrueckii*, *L. helveticus*, and *L. plantarum*, are traditionally involved in the production of fermented dairy and vegetable foods. These *Lactobacillus* cells are ingested in high numbers during the consumption of fermented products and, consequently, may come into direct contact with the host's oropharyngeal and gastrointestinal mucosa. The potential influence of food-associated lactobacilli on a host's health compared to that of intestinal lactobacilli, however, has been poorly investigated. Nonetheless, a few studies demonstrate the efficacy of food-associated lactobacilli in the modulation of host physiology. For instance, *L. plantarum* CJLP133, a strain isolated from the traditional Korean fermented vegetable kimchi, exhibited therapeutic potential for atopic dermatitis in mice by increasing type 1 $CD4^+$ helper T cell and regulatory T cell activation (5). Furthermore, recent papers by our research group showed that *L. helveticus* MIMLh5 isolated from Grana Padano cheese natural whey starter can efficiently antagonize group A streptococci and modulate the immune response in epithelial cells, dendritic cells, and macrophages (6–8).

The cross talk between host and intestinal/probiotic bacteria principally relies on the capacity of host cells to recognize specific bacterial components or products, thus giving rise to responses that most frequently involve the mucosa-associated lymphoid tissue (MALT) and, therefore, the immune system (9, 10). Specifically, cell surface components of commensal or food/probiotic bacteria, known as microbe-associated molecular patterns (MAMPs), can be identified by pattern recognition receptors (PRRs) on cells constituting the innate immune system, resulting in the activation of immune responses. The molecular mechanisms of such immune modulations are largely unknown. Therefore, identifying and characterizing unique bacterial components that act as effectors of the immune system are crucial for the elucidation of host-microbial interplays and bacterial modes of action that result in immune modulation. In addition, a deeper understanding of the molecular mechanisms of cross talk between bacteria and the host organism's system is of great importance to better define the benefits and potential risks associated with the administration of probiotic therapies (11).

In this study, we investigated *L. helveticus*, a bacterium specialized for colonizing dairy environments and traditionally used in

Received 2 October 2012 **Accepted** 4 December 2012
Published ahead of print 7 December 2012
Address correspondence to Simone Guglielmetti, simone.guglielmetti@unimi.it.

doi:10.1128/AEM.03056-12

the manufacture of Swiss-type and long-ripened Italian cheeses. Phylogenetically, *L. helveticus* is closely related to *L. acidophilus* (the 16S rRNA gene sequences of these bacteria differ by just 1.6% [12]), an intestinal bacterium regularly used as a probiotic and thoroughly investigated for its ability to modulate immune responses (13–16). Studying the interaction of the dairy bacterium *L. helveticus* with the host immune system could provide important insights into the health-modulating potential of a bacterium that, in contrast to the intestinal/probiotic *L. acidophilus*, had its adaptive evolution in dairy environments and, therefore, outside a human/animal host. Specifically, we investigated the surface-layer (S-layer) protein (also known as SlpA) of *L. helveticus* because the corresponding protein in *L. acidophilus* was recently demonstrated to be involved in the modulation of human dendritic cell and T cell functions (13). We employed cells typically involved in the host innate immune system, including intestinal epithelial cells and three populations of macrophages: the human macrophage cell line U937, mouse bone marrow-derived macrophages (BMDMs), and macrophages isolated from the mouse peritoneal cavity. Our results showed that the S-layer protein SlpA plays a significant role in promoting the immunostimulatory activity of the strain *L. helveticus* MIMLh5.

MATERIALS AND METHODS

Bacterial strains, isolation, and growth conditions. *L. helveticus* MIMLh5 was grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI) at 42°C, whereas *L. acidophilus* NCFM was grown in the same medium at 37°C. *Lactobacillus* strains were inoculated from frozen glycerol stocks and subcultured twice in MRS broth using a 1:100 inoculum. For immunological experiments, bacterial cells from an overnight culture were collected, washed twice with sterile phosphate-buffered saline (PBS), and then resuspended in the same medium used to culture human or murine cells. Bacteria were tested at multiplicities of infection (MOIs) of 100 and 1,000.

Extraction, purification, and chemical characterization of the S-layer protein from *L. helveticus* MIMLh5. Extraction of the S-layer protein from *L. helveticus* MIMLh5 was performed with LiCl as described previously (17–20). Briefly, 500 ml of an overnight culture of MIMLh5 was harvested by centrifugation at 10,000 × *g* for 20 min at 4°C and washed with 1 volume of cold sterile Milli-Q water. The cell pellet was extracted with 0.1 volume of 1 M LiCl solution for 30 min at room temperature in the presence of protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) with slight agitation and centrifuged. The cell pellet was then extracted with 0.1 volume of 5 M LiCl solution for 1 h at room temperature in the presence of protease inhibitor cocktail and centrifuged. The supernatant was filtered through a 0.2-μm-pore-size filter and exhaustively dialyzed for 36 h at 4°C against distilled water using 12,000-kDa-cutoff membranes (Sigma-Aldrich), which were prepared for dialysis by boiling in 2% $NaHCO_3$ and 1 mM EDTA solution. At each water change, 0.001% protease inhibitor cocktail was added. The dialysate was collected and centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant was removed, and the pellet was resuspended in sterile Milli-Q water and freeze-dried. We calculated an approximately 4-fold underestimation in the quantification of the S-layer protein with the Bradford microassay method using bovine serum albumin (BSA) as a standard (probably due to the difference in the amino acid residue compositions between S-layer and BSA proteins). For this reason, for all the experiments in this study, the amount of S-layer protein was determined by measuring the weight of freeze-dried dialyzed protein on an electronic analytical balance. Protein purity was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and reverse-phase high-pressure liquid chromatography (RP-HPLC) analysis.

(i) SDS-PAGE. S-layer protein and total bacterial lysates were resuspended in SDS-PAGE (Laemmli) sample buffer, boiled for 5 min, and separated on a 10% polyacrylamide gel in Tris-glycine-SDS buffer on a Mini-Protean 3 system (Bio-Rad Italia, Milan, Italy). Gels were stained with Coomassie brilliant blue G-250 (Sigma-Aldrich) or silver.

(ii) RP-HPLC/ESI-MS analysis of the S-layer protein. RP-HPLC/electrospray ionization (ESI)-mass spectrometry (MS) analysis was performed on a Waters Alliance 2695 instrument (Waters, Vimodrone, Italy) connected to a quadrupole time of flight (Q-Tof) micro-mass spectrometer (Micromass; Waters) equipped with an orthogonal electrospray source (Z spray). S-layer protein was dissolved in 8 M urea and separated at 40°C on a polymeric reversed-phase PLRP-S column (2.1 mm [inner diameter]; particle size, 5 μm; 300 Å; Polymer Laboratories Ltd., Church Stretton, United Kingdom). The eluents used for the separation were solvent A (0.1% trifluoroacetic acid [TFA] in Milli-Q-treated water) and solvent B (0.1% TFA in acetonitrile). The linear elution gradient expressed as the solvent B proportion was as follows: 0 min, 25%; 0 to 5 min, 25%; 5 to 35 min, 25 to 55%; 35 to 36 min, 55 to 95%; 36 to 38 min, 95%; 38 to 39 min, 95 to 25% (run-to-run time, 40 min). Protein was eluted at a flow rate of 0.2 ml min^{-1} and monitored at 210 nm with a Waters 2996 photodiode array detector. Mass spectrum acquisition was performed in positive ion mode. The optimized mass spectrometry conditions were a capillary voltage of 3,200 V, a source temperature of 100°C, and a cone voltage of 45 V. The ESI gas was nitrogen. The time-of-flight analyzer collected data at between *m/z* 650 and *m/z* 2,000. The acquired MS spectrum was analyzed with MassLynx (version 4.1) software (Waters). MS data were processed using the MaxEnt 1 algorithm.

Experiments with Caco-2 cell layers. (i) *In vitro* cultivation. Caco-2 cells (human epithelial colorectal adenocarcinoma cell line; ATCC HTB-37) were routinely grown in Eagle's minimum essential medium (EMEM) supplemented with 10% (vol/vol) heat-inactivated (30 min at 56°C) fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids (NEAA), and 2 mM L-glutamine and were incubated at 37°C in a water-jacketed incubator in an atmosphere of 95% air and 5% carbon dioxide.

(ii) Study of NF-κB activation. A stable recombinant Caco-2 cell line was generated by transfecting cells with the plasmid pNiFty2-Luc (Invivogen, Labogen, Rho, Italy) as described by Guglielmetti et al. (21). This plasmid contains a promoter with five NF-κB-binding sites followed by the firefly luciferase reporter gene *luc*. Stimuli that activate NF-κB promote its binding to the vector promoter, resulting in the expression of the luciferase gene. After growth in the presence of 50 μg/ml zeocin, cell monolayers (approximately 3×10^5 cells/well) were carefully washed with 0.1 M Tris-HCl buffer (pH 8.0). Subsequently, 50 μl of a tester bacterial suspension containing 2.5×10^8 cells (or the purified S-layer protein) was added to 0.45 ml of fresh EMEM containing 100 mM HEPES (pH 7.4). The resulting 0.5 ml was finally pipetted into the microtiter plate well containing the Caco-2 cell layer, resulting in an MOI of approximately 1,000. Stimulation was conducted both in presence and absence of 2 ng/ml of interleukin-1β (IL-1β). After incubation at 37°C for 4 h, the samples were treated and the bioluminescence was measured as described by Stuknyte et al. (7). All conditions were analyzed in triplicate in at least two independent experiments.

Study of activation of U937 human macrophage cell line. (i) Cell culture, growth conditions, and stimulation protocol. The cell line U937 (ATCC CRL-1593.2) was derived from a human histiocytic lymphoma (22). These cells are maintained as replicative, nonadherent cells and have many of the biochemical and morphological characteristics of blood monocytes (23). When treated with phorbol myristate acetate (PMA), U937 cells differentiate to become adherent, nonreplicative cells with characteristics similar to those of tissue macrophages, including similar isoenzyme patterns (24) and other phenotypic markers (23). The normal growth medium for the U937 cells consisted of RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco-BRL, Life Technologies, Milan, Italy), 2 mM L-glu-

tamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). U937 cells were seeded at a density of 5×10^5 cells/well in 12-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 48 h. Afterwards, cells were washed once with sterile PBS buffer to remove all nonadherent cells. At 1 h before the bacteria were added to the cells, the culture medium was replaced with RPMI 1640 medium supplemented with 1% (vol/vol) FBS to allow the cells to adapt. Bacteria were used at MOIs of 100 and 1,000. Lipopolysaccharide (LPS; final concentration, 1 μg/ml) from *Escherichia coli* O127:B8 (Sigma-Aldrich) was used as a positive control for the proinflammatory stimulus in U937 cells. An untreated sample, i.e., a sample treated only with RPMI 1640 medium with 1% (vol/vol) FBS, was used as a control.

(ii) Inhibition assay with TLR-neutralizing Abs. Human anti-Toll-like receptor 4 (anti-TLR4) and anti-TLR2 antibodies (Abs; Invivogen) were added to U937 cells 1 h before the stimulation with bacterial cells. A human immunoglobulin A2 (IgA2) isotype (Invivogen) was used as a control to exclude nonspecific binding and the blocking activity of the antibody. Anti-TLR4, anti-TLR2, and the IgA2 isotype were all used at 5 μg/ml. This concentration was determined by examining the neutralizing efficacy with zymosan from *Saccharomyces cerevisiae* (Invivogen) as a ligand for anti-TLR2 and LPS from *E. coli* O127:B8 (Sigma-Aldrich); we observed that 5 μg/ml resulted in 5-fold and 2-fold reductions when, respectively, anti-TLR2 and anti-TLR4 were used to partially block the action of their corresponding ligand in triggering the level of expression of tumor necrosis factor alpha (TNF-α), a cytokine known to be induced in the downstream pathway activated by TLR2 and TLR4 (25, 26) (data not shown).

Isolation and differentiation of mouse BMDMs. Bone marrow cells from C57BL/6 mice were collected by flushing the femurs and tibias from 6- to 8-week-old mice with sterile PBS. Erythrocyte-depleted bone marrow cells were resuspended, counted, and seeded in six-well plates at a density of 5×10^6 cells/ml in RPMI 1640 medium (2 ml/well) supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), 50 μM 2-mercaptoethanol, 15 mM HEPES (Sigma-Aldrich), and 30 ng/ml murine macrophage colony-stimulating factor (M-CSF) (Peprotech, Rocky Hill, NJ). Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. After 24 h, nonadherent cells were transferred to new six-well plates with complete RPMI 1640 medium supplemented with 30 ng/ml murine M-CSF. On days 3 and 5, the cultures were fed by adding 1 ml/well of RPMI 1640 complete medium supplemented with 30 ng/ml murine M-CSF, and the cells were allowed to grow and differentiate for 7 to 8 days. The purity of BMDM populations (>90%) was acquired by flow cytometry (FACSCanto II; BD, San Jose, CA) and analyzed by FlowJo software. Allophycocyanin-labeled anti-CD11c (N418; eBioscience, San Diego, CA) and peridinin chlorophyll protein-Cy5.5-labeled anti-F4/80 (BM8; eBioscience) were used as antibody mixtures. Prior to stimulation, nonadherent cells were discarded by aspirating the medium and washing once with sterile PBS. Fresh RPMI 1640 medium supplemented with 1% FBS was added to adherent cells, and the mixture was kept at 37°C in a 5% CO_2 humidified atmosphere for 1 h. Subsequently, macrophages were stimulated for 4 h. After stimulation, the cells were scraped and collected for RNA extraction.

Isolation of mouse peritoneal cavity macrophages (PCMs). C57BL/6 mice were euthanized by CO_2 inhalation. Cells were elicited from the peritoneal cavity by injecting 5 ml of cold, sterile PBS supplemented with 3% FBS (Gibco) and 2 mM EDTA. A gentle massage of the peritoneum was performed to dislodge any attached cells into the PBS solution. The fluid was then aspirated through a syringe and immediately transferred to 50-ml polypropylene tubes kept on ice. This procedure was repeated twice. Peritoneal cells were then centrifuged and resuspended in RPMI 1640 medium supplemented with 10% (vol/vol) heat-inactivated FBS (Gibco), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), 50 μM 2-mercaptoethanol, and 10 mM HEPES (Sigma-Aldrich). The cells were counted, seeded on six-well plates at a density of 1×10^6 cells/ml, and cultured for 24 h at 37°C in a 5% CO_2 humidified atmosphere. After 24 h, cells were washed three times with PBS to remove nonadherent cells, and fresh RPMI 1640 medium supplemented with 1% FBS was added to adherent cells. After 1 h of incubation at 37°C in a 5% CO_2 humidified atmosphere, the macrophages were stimulated for 4 h. Subsequently, the cells were scraped and collected for RNA extraction.

Ethics statement. All experimental methods have been accepted by the National Animal Experiment Board (Finland).

Preparation of RNA and RT. For both *in vitro* and *ex vivo* experiments, after incubating macrophages at 37°C for 4 h, the supernatant was carefully removed from each well and the total cellular RNA was isolated from the adhered cells with an RNeasy minikit (Qiagen Inc., Valencia, CA). Afterwards, RNA concentration and purity were determined with a NanoDrop spectrophotometer (ND-1000; Thermo Fischer Scientific, Middletown, VA), and reverse transcription (RT) to cDNA was performed with an iScript Select cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

RT-qPCR. The mRNA expression levels of cytokines were analyzed with SYBR green technology in an RT-quantitative PCR (RT-qPCR) using SsoFast EvaGreen Supermix (Bio-Rad Italia, Segrate, Italy) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used are as follows (5′ → 3′): for human macrophages, 18S rRNA forward primer ATCCCTGAAAAGTTCCAGCA, 18S rRNA reverse primer CCC TCTTGGTGAGGTCAATG, IL-10 forward primer AGCAGAGTGAAGA CTTTCTTTC, IL-10 reverse primer CATCTCAGACAAGGCTTGG, TNF-α forward primer TCAGCTCCACGCCATT, TNF-α reverse primer CCCAGGCAGTCAGATCAT, COX2 forward primer CCCTTGGGTGT CAAAGGTAA, and COX2 reverse primer TGAAAAGGCGCAGTTT ACG; for murine macrophages, 18S rRNA forward primer GTGATCCC TGAGAAGTTCCAG, 18S rRNA reverse primer TCGATGTCTGCTTTC CTCAAC, IL-10 forward primer GCCCAGAAATCAAGGAGCAT, IL-10 reverse primer TGTAGACACCTTGGTCTTGGAG, TNF-α forward primer CTTCTGTCTACTGAACTTCGGG, TNF-α reverse primer CAG GCTTGTCACTCGAATTTTG, COX-2 forward primer TGTGCTGACA TCCAGATCAT, and COX-2 reverse primer GGCAAAGAATGCAAACA TCA. All primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), and the specificity of the primers was tested with melting curves during amplification and by 1% TAE (Tris-acetate-EDTA) agarose gel electrophoresis. Quantitative PCR was carried out according to the following cycle: initial hold at 96°C for 30 s and then 40 cycles at 96°C for 2 s and 60°C for 5 s. Gene expression was normalized to that of the housekeeping gene coding for the18S rRNA. The amount of template cDNA used for each sample was 12.5 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) in comparison to the induction of the control (namely, unstimulated macrophages), to which we attributed an FOI of 1.

Statistical analysis. The significance of the results was analyzed by unpaired heteroscedastic Student's *t* test with a two-tailed distribution. Differences with *P* values of <0.05 were considered significant.

Nucleotide sequence accession number. The *slpA* gene sequence data have been submitted to the EMBL database under accession number HE993893.

RESULTS

Extraction, purification, and analysis of MIMLh5 S-layer protein. The S-layer protein (SlpA) of *L. helveticus* MIMLh5 was extracted with LiCl and purified as described in the Materials and Methods section. We verified that the protocol based on LiCl washes efficiently removed most of the S-layer protein from the surface of *L. helveticus* cells. In fact, when we loaded the LiCl-treated and LiCl-untreated bacterial cells resuspended in SDS-

PAGE loading buffer on a polyacrylamide gel, the electrophoretic profiles displayed a nearly complete disappearance of the band corresponding to SlpA (Fig. 1A). SDS-PAGE and RP-HPLC analyses revealed that the protein was purified to apparent homogeneity (Fig. 1B to D). Furthermore, we did not consider the contamination of the S-layer preparation with lipoteichoic acids (LTA) to be possible because it was previously demonstrated that LiCl treatments do not remove LTA from *L. helveticus* cells (27).

Mass spectrometry indicated a molecular mass of 43,853 Da, which is consistent with the average molecular mass calculated from the amino acid sequence deduced from the *slpA* gene sequence (Fig. 1D).

***L. helveticus* MIMLh5 reduces NF-κB activation in transfected Caco-2 cells.** We performed a preliminary immunological characterization of *L. helveticus* MIMLh5 using a reporter cell line obtained by transfecting Caco-2 cells with a luciferase reporter vector induced by active NF-κB. In these experiments, *L. helveticus* MIMLh5 and *L. acidophilus* NCFM cells from an overnight culture were incubated with the transfected Caco-2 layer for 4 h at 37°C at an MOI of 1,000. *L. helveticus* MIMLh5 significantly reduced NF-κB activation both at the baseline and in the presence of IL-1β; therefore, bioluminescence was always significantly lower when bacterial cells were added to the Caco-2 layer than when they were added to the unstimulated control. Under both tested conditions, strain MIMLh5 was significantly more effective in immunomodulatory activity than *L. acidophilus* NCFM, which is an extensively studied commercial probiotic strain that was included as a reference in our experiments (Fig. 2).

S-layer protein from *L. helveticus* MIMLh5 reduces the activation of NF-κB in recombinant Caco-2 cells. In the following part of the study, we investigated the potential role of the S-layer protein in the immunomodulatory activity exerted by *L. helveticus*. We isolated the S-layer protein from strain MIMLh5 to employ it in experiments on recombinant Caco-2 cells. We found that similar to whole bacterial cells, purified S-layer protein was able to significantly reduce the activation of NF-κB at both concentrations tested (Fig. 2).

MIMLh5 strain and its S-layer protein elicit proinflammatory responses in human U937 macrophages. After a preliminary immunological investigation performed on epithelial cells, we employed cells belonging to the innate immune system to study the host's immune responses triggered by *L. helveticus* MIMLh5 and its S-layer protein. We quantified via RT-qPCR the gene expression of two proinflammatory factors, TNF-α and COX-2, and the anti-inflammatory/regulatory interleukin IL-10 in PMA-differentiated U937 human macrophages. After 4 h of stimulation of the U937 cells, MIMLh5 induced a pronounced proinflammatory profile at MOIs of 100 and 1,000, as evidenced by an enhanced induction of COX-2 and TNF-α compared to the induction of IL-10 (Fig. 3A). The cytokine expression profile of the purified S-layer protein was similar to that induced by the MIMLh5 strain itself. The involvement of the S-layer protein in the immunostimulating effects of MIMLh5 was also confirmed in experiments involving bacterial cells without the S layer (i.e., after LiCl extraction of the protein). In fact, we observed that at an MOI of 1,000, the removal of the S layer resulted in a decrease in COX-2 and TNF-α induction levels, whereas IL-10 levels did not change (Fig. 3A). A similar trend was observed at an MOI of 100, even if the data are not statistically significant (Fig. 3A). Furthermore, the addition of the purified S-layer protein to MIMLh5 cells without S-layer proteins resulted in a significant increase of COX-2 and TNF-α gene expression, while IL-10 remained unaffected (Fig. 3A). These results indicate that the S-layer protein from strain MIMLh5 could be primarily involved in inducing the expression of the proinflammatory factors COX-2 and TNF-α in human U937 macrophages.

BMDMs display a proinflammatory profile upon stimulation with MIMLh5 and its S-layer protein. After the *in vitro* evaluation of the immunological activity of *L. helveticus* MIMLh5 and the purified S-layer protein on human U937 macrophages, we used the same approach to study their effects in *ex vivo* experiments by isolating and differentiating macrophages from mouse bone marrow. Similar to our previous results with human macrophages, we found that *L. helveticus* MIMLh5 induced, at both MOIs tested, a clear proinflammatory profile, as evidenced by a strong induction of COX-2 and TNF-α but not IL-10 (Fig. 4). The results obtained by incubating BMDMs with S-layer protein confirmed the proinflammatory properties of the protein on murine macrophages (Fig. 4). Furthermore, when we exposed BMDMs to the bacterium after the elimination of the S layer, we observed a trend of reduction (although this result was not statistically significant) of TNF-α levels for MIMLh5 (particularly at the highest MOI tested), whereas the absence of the protein led to a slight increase of IL-10 levels (Fig. 4).

The S-layer protein from strain MIMLh5 induces proinflammatory activity in murine PCMs. Once we defined the immune profile induced by MIMLh5 and its S-layer protein *in vitro* on human U937 cells and *ex vivo* on BMDMs, we verified whether the same proinflammatory response could be confirmed in tissue-specialized macrophages. To this aim, we employed macrophages isolated from the mouse peritoneal cavity. Unlike the previous results, when we used the whole bacterium, we observed a balanced profile between pro- and anti-inflammatory cytokines in PCMs because the induction of IL-10 was not lower than the induction of TNF-α (Fig. 5). However, consistent with our findings in U937 cells and BMDMs, the S-layer protein triggered higher levels of COX-2 and TNF-α than IL-10 in peritoneal macrophages (Fig. 5). Furthermore, in contrast to U937 cells and BMDMs, the proinflammatory factor levels induced in PCMs by the S-layer protein were almost equal to the levels induced by LPS. This pronounced proinflammatory activity was shown to be dose dependent because when we tested a lower concentration of the protein, 0.1 μg/ml, the levels of TNF-α and COX-2 induced were lower than those induced by LPS, although the proinflammatory factor profile was qualitatively the same. The results obtained by employing PCMs, therefore, confirm the involvement of S-layer protein principally in promoting proinflammatory immune responses.

TLR2 is involved in the recognition of the S-layer protein from strain MIMLh5 in human U937 cells. To gain information about the possible signaling pathways underlying the immune response activated by the S-layer protein isolated from *L. helveticus* MIMLh5, we evaluated whether TLR2 and TLR4 could be involved in the recognition of this protein. To this aim, we used neutralizing Abs on U937 cells to partially block the ability of TLR2 and TLR4 to bind ligands prior to the addition of the S-layer protein. We found that TLR2 and, to a lesser extent, TLR4 are involved in mediating the proinflammatory response elicited by S-layer protein because the treatments with Abs significantly reduced the S-layer-induced activation of TNF-α and COX-2 but

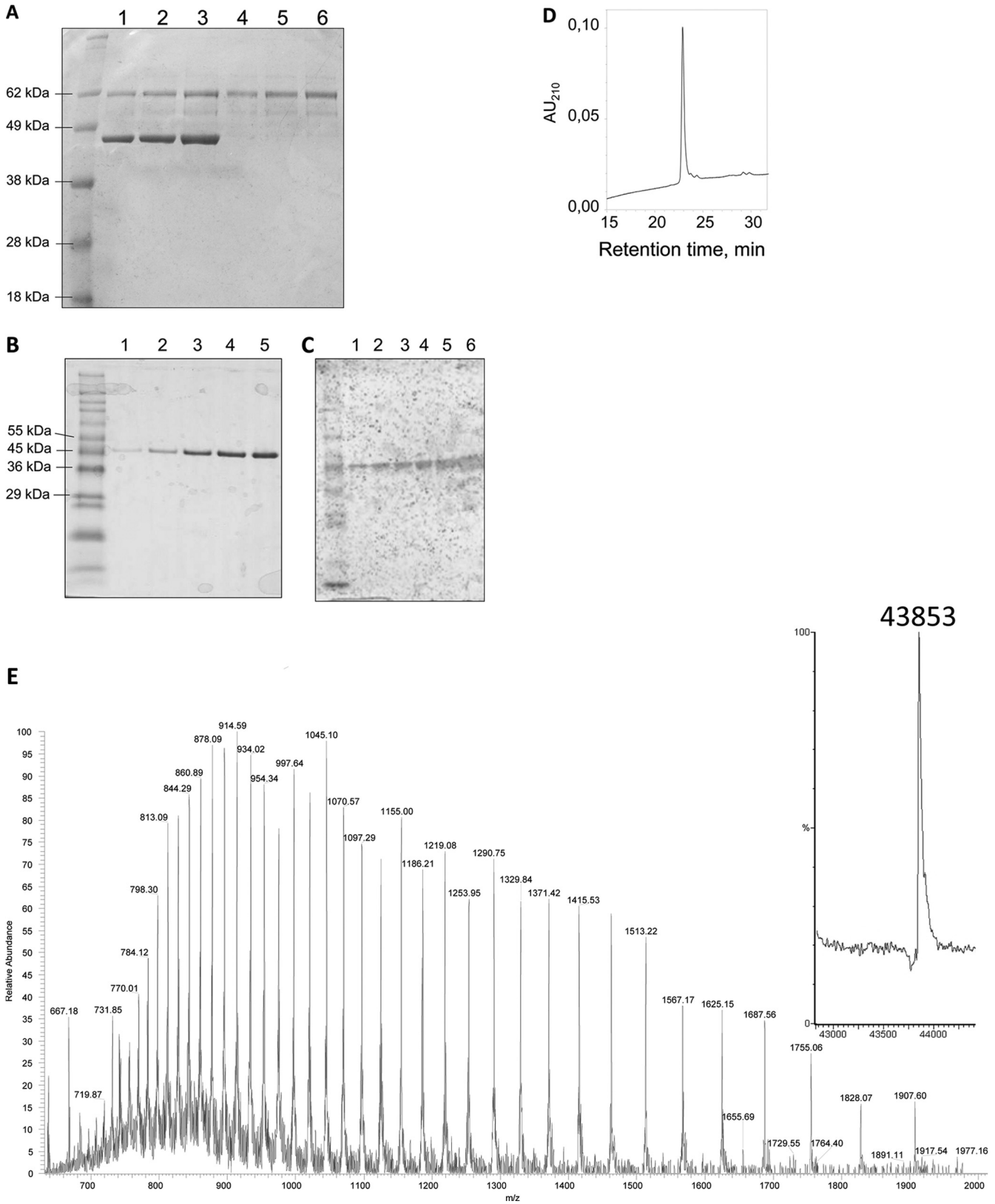

FIG 1 Biochemical analyses of *L. helveticus* MIMLh5 S-layer protein. (A) SDS-PAGE profile of MIMLh5 cells before (lanes 1 to 3) and after (lanes 3 to 6) treatment with LiCl solutions; (B and C) SDS-PAGE profile with Coomassie blue staining (B) and silver staining (C) of purified S-layer protein; 50 ng to 3 μg of protein was loaded per well on the gel; (D) HPLC profile of purified S-layer protein (AU_{210}, absorbance units at 210 nm); (E) ESI-MS spectrum of the S-layer protein and reconstructed mass spectrum, indicating an average mass value of 43,853 Da.

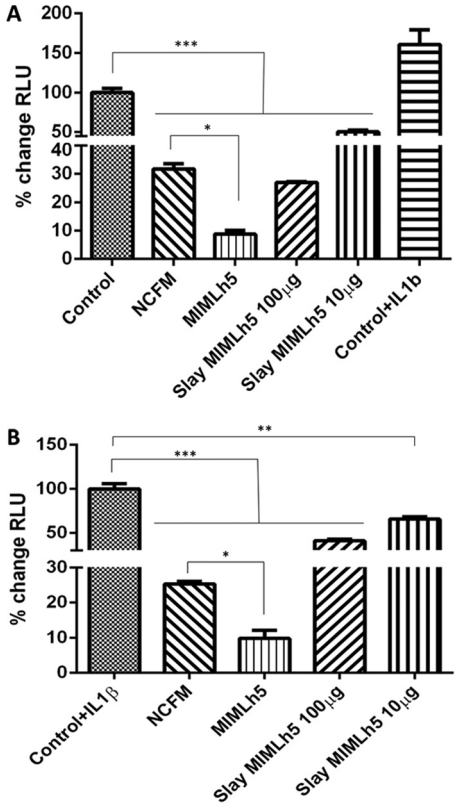

FIG 2 Effect of *Lactobacillus acidophilus* NCFM, *Lactobacillus helveticus* MIMLh5, and the purified S-layer protein (Slay) of MIMLh5 on human epithelial colorectal Caco-2 cells stably transfected with an NF-κB/luciferase reporter vector at baseline (A) or stimulated with 2 ng/ml of IL-1β (B). A recombinant Caco-2 cell layer was incubated with EMEM only (A) or with the addition of IL-1β (B). Bacterial strains were used at an MOI of 1,000 (bacterial cells per Caco-2 cell). S-layer protein was tested at two different concentrations (100 and 10 μg/ml). Data in the histograms are the means (+ standard deviations) from at least three independent experiments conducted in triplicate. Data are reported as percent variation of light emission (relative luminescence units [RLU]), assuming that the value for the corresponding control was 100%. Asterisks indicate statistically significant differences: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

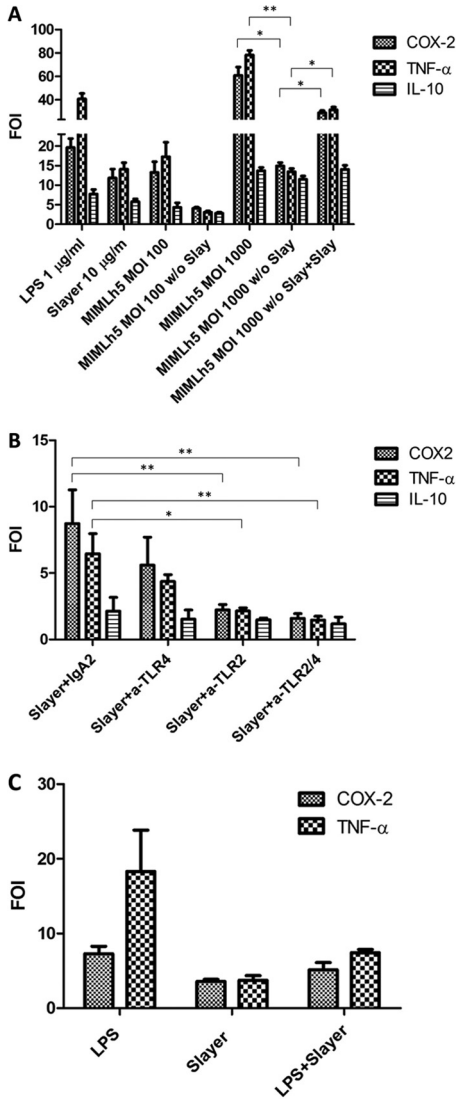

FIG 3 Quantitative analysis of cytokine gene expression in U937 human macrophages after 4 h of stimulation. Expression profiles of TNF-α, IL-10, and COX-2 are indicated as the FOI relative to the induction level of the control, which was set at a value of 1. Presented data are the means (+ standard deviations) for a result representative of three independent experiments. Asterisks indicate statistically significant differences: **, $P < 0.01$; *, $P < 0.05$. (A) U937 cells stimulated with *L. helveticus* MIMLh5 (MOIs, 1,000 and 100) and its S-layer protein (10 μg/ml); control, unstimulated U937 cells; MIMLh5 w/o Slay, MIMLh5 cells after LiCl extraction of the S-layer protein; MIMLh5 w/o Slay + Slay, S-layer-depleted MIMLh5 cells supplemented with 10 μg/ml of the purified S-layer protein; LPS was used as a positive control at a concentration of 1 μg/ml. (B) Cytokine expression profiles in the presence of neutralizing antibodies against TLR2 (a-TLR2) and TLR4 (a-TLR4); IgA2 was used as a control for nonspecific blocking activity; IgA2 and anti-TLRs were added at a concentration of 5 μg/ml on U937 cells 1 h before stimulation with the S-layer protein; anti-TLR2/4, anti-TLR2 and anti-TLR4 simultaneously added at a concentration of 2.5 μg/ml each; control, U937 cells incubated with IgA2. (C) Cytokine expression profiles in U937 cells stimulated with *L. helveticus* MIMLh5 S-layer protein and LPS; LPS and S-layer protein were added at concentrations of 1 μg/ml and 10 μg/ml, respectively, both when used alone and in association; control, unstimulated U937 cells.

not IL-10 (Fig. 3B). The differences observed when anti-TLR2 was used, either alone or in combination with anti-TLR4, were statistically significant compared to the results obtained with U937 cells stimulated with S-layer protein in the presence of IgA2, which was used as a control for nonspecific blocking activity. From these data, we can hypothesize that the S layer, which is the outermost part of the bacterial cell wall, might come into contact first with host cells, mediate bacterial recognition, and therefore play a key role in triggering the immune response.

S-layer protein modulates the proinflammatory response triggered by LPS in human U937 macrophages. In a final set of experiments, we tested the effects of the S-layer protein on U937 cells in the presence of LPS. Interestingly, we observed that the presence of the S-layer protein induced a clear reduction in the proinflammatory cytokine TNF-α compared with the levels induced by LPS alone (Fig. 3C). These results suggest that although the S-layer protein and LPS presented similar proinflammatory effects, the simultaneous presence of both stimuli did not result in an additive or synergistic effect on the proinflammatory response.

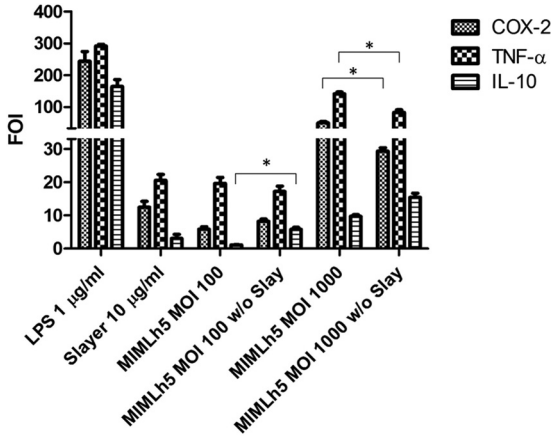

FIG 4 Quantitative analysis of cytokine gene expression in murine BMDMs after 4 h stimulation with *L. helveticus* MIMLh5 and its S-layer protein. Expression levels of TNF-α, IL-10, and COX-2 are indicated as the FOI relative to the induction level of the control (unstimulated BMDMs), which was set at a value of 1. LPS was used as a positive control at a concentration of 1 μg/ml. S-layer protein was tested at a concentration of 10 μg/ml. MIMLh5 was used at MOIs of 1,000 and 100. MIMLh5 w/o Slay, MIMLh5 cells after removal of the S-layer protein by LiCl extraction. Presented data are the means of measurements (+ standard deviations) for a result representative of three independent experiments. Asterisks indicate statistically significant differences compared to results for the corresponding control: *, $P < 0.05$.

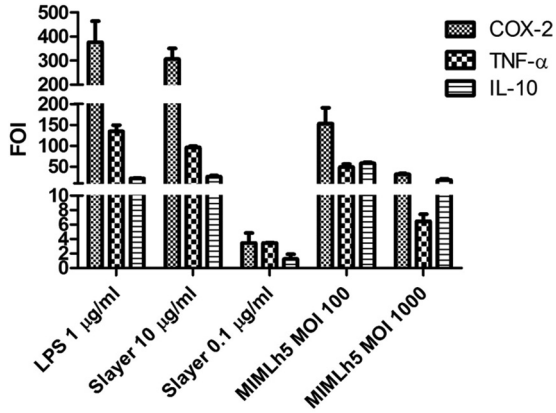

FIG 5 Quantitative analysis of cytokine gene expression in murine macrophages isolated from the peritoneal cavity after 4 h stimulation with *L. helveticus* MIMLh5 and its S-layer protein. Expression levels of TNF-α, IL-10, and COX-2 are shown as the FOI relative to the induction level of the control (unstimulated peritoneal macrophages), which was set at a value of 1. LPS was used as a positive control at a concentration of 1 μg/ml. S-layer protein was tested at concentrations of 10 and 0.1 μg/ml. MIMLh5 was used at MOIs of 1,000 and 100. Presented data are the means of measurements (+ standard deviations) for a result representative of three independent experiments.

Rather, the addition of the S-layer protein induced a reduction of the stimulating activity of LPS.

DISCUSSION

In this study, we focused our attention on *L. helveticus* MIMLh5, a dairy strain isolated from Italian Grana Padano cheese natural whey starter, which has already been described in our previous studies for its metabolic, biotechnological, and probiotic features (8, 28, 29). This strain was demonstrated to exert promising immunomodulatory effects (6–8). Due to the importance of understanding immunological aspects when defining the probiotic potential of a bacterial strain (30), we aimed to define which specific molecular component of the *L. helveticus* MIMLh5 bacterial surface could have a role in mediating the immunological activity exerted by the whole bacterium. First, in the Caco-2 epithelial cell line model, we analyzed the ability of strain MIMLh5 to reduce the activation of NF-κB, which is a transcriptional factor involved in the expression of several proinflammatory cytokines (31) and a therapeutic target in a wide range of human (auto)inflammatory diseases (32).

We compared MIMLh5 with the well-described commercial probiotic strain *L. acidophilus* NCFM, which we included as a reference. Similar to what we observed in our previous studies on the pharyngeal epithelial FaDu cell line (6), MIMLh5 efficiently reduced the activation of NF-κB in the Caco-2 cell model, both at baseline and in the presence of the proinflammatory stimulus IL-1β. Therefore, in light of the immunologic information available for strain MIMLh5 (6, 8), we proceeded with the characterization of this strain by studying the possible involvement of the S-layer protein (SlpA) in mediating bacterial effects on the host immune system. The SlpA protein is an abundant molecule present on the outer surface of all *L. helveticus* strains, constituting up to 15% of the total *Lactobacillus* protein content (33). For *L. helveticus*, SlpA forms approximately 45% of the cell wall dry weight (34). Even if many functions have been suggested/hypothesized (35–37), the actual role of the S-layer protein remains elusive.

When we tested the MIMLh5 S-layer protein in the *in vitro* Caco-2/NF-κB model, we observed that the immunomodulatory effects of the purified protein were similar to the effects of the whole bacterium. Studies concerning the effects of *Lactobacillus* S-layer proteins on intestinal epithelial cells have already been performed (19, 38). Specifically, the properties described to date mainly refer to the role of S-layer proteins in mediating the bacterium's ability to antagonize pathogens due to the capability of the protein to efficiently adhere to the intestinal epithelium. This effect has been demonstrated for *L. helveticus* (38, 39) and *L. acidophilus* (37, 40) strains. Nonetheless, the S-layer protein was also demonstrated to exert direct effects on the immune responses of intestinal epithelial cells. For instance, it was shown that the S-layer protein from *L. acidophilus* ATCC 4356^T reduced *Salmonella enterica* serovar Typhimurium FP1-induced apoptosis in Caco-2 cells, and this effect was dependent on the inhibition of caspase-3 activation (41). In another study, the oral immunization of mice with the purified SlpA from *L. helveticus* M92 induced an increase in total levels of serum IgG, IgM, and IgA (42).

We analyzed the immunological properties of the purified SlpA protein from *L. helveticus* MIMLh5 by using macrophages, which are professional phagocytes serving as sentinels to detect microbial host invaders (43). Macrophages undergo activation upon environmental signals, including microbial products and cytokines (44). We measured the gene expression induction of TNF-α (a cytokine involved in inflammatory responses [45]), IL-10 (a multifunctional cytokine whose principal routine function appears to be to limit and ultimately terminate inflammatory responses [46, 47]), and COX-2. COX-2 is a homodimeric enzyme involved in the synthesis of prostaglandins (PGs), which are hormones that participate in physiological processes such as inflammation, as well as in protecting the gastrointestinal mucosa (48, 49).

In experiments on human U937 macrophages and macrophages isolated from mouse bone marrow (BMDMs), MIMLh5 exerted proinflammatory effects by inducing high levels of TNF-α and COX-2 and lower levels of IL-10. Interestingly, the purified SlpA extracted from *L. helveticus* MIMLh5, according to the expression levels of TNF-α and COX2, seems to be responsible for the proinflammatory behavior of the whole bacterium, as also confirmed by the experiments performed with SlpA-depleted MIMLh5 cells (Fig. 3D).

The ability to induce proinflammatory cytokines has already been shown in lactic acid bacteria (LAB) (50–53). Nonetheless, such an ability of LAB should not be considered detrimental. In fact, even though cytokines belonging to the TNF-α superfamily are connected to the occurrence of inflammatory diseases (54), they have also been shown to participate in the rejection of tumors and infections (55–57). Furthermore, the induction of TNF-α could be important for the initiation of cross talk among immune cells without causing any inflammation or detrimental effects (30). In addition, a transient inflammatory state could aid host defense. As recently suggested, differences among pathogenic, probiotic, and commensal microorganisms lie in the magnitude of the immune response evoked, which can be defined as strong, intermediate, or homeostatic (58).

Similarly, the upregulation of COX-2 induced by LAB has been demonstrated both *in vitro* (59, 60) and *in vivo* (60, 61). The proinflammatory role of COX-2, however, has recently been questioned. In fact, it has been proposed that COX-2 can induce the resolution of inflammation (62) and an immunosuppressive phenotype in T cells (63) through the activity of PGs. In addition, it has also been shown that a rapid COX-2 upregulation in response to injury or inflammation helps to restore mucosal integrity (64). In this context, the effects of MIMLh5 and SlpA on COX-2 expression could potentially be beneficial for the host's mucosa.

The ability of specific cell wall components to drive the immune response elicited by LAB has rarely been described (65, 66). Concerning S-layer proteins, Konstantinov and colleagues (13) demonstrated that SlpA from *L. acidophilus* NCFM mediates the interaction of the bacterium with human monocyte-derived dendritic cells (DCs) by specifically binding to the ligand of the dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN). Even if the purified SlpA protein of NCFM did not affect DC maturation, it was responsible for the anti-inflammatory cytokine profile observed for *L. acidophilus* NCFM because the protein induced higher levels of IL-10 in the presence of LPS compared to the levels induced by SlpA or LPS alone. In comparison to the SlpA of *L. acidophilus* NCFM, our data suggest that the S-layer protein isolated from *L. helveticus* MIMLh5 exhibits more pronounced proinflammatory behavior. Although the S-layer proteins of *L. acidophilus* NCFM and *L. helveticus* MIMLh5 are highly similar (73% identity, 83% positivity; data not shown), it has been suggested that even small differences in surface proteins could alter the type of immune responses elicited by LAB (58, 67). Nonetheless, the most plausible explanation for the different immunological behaviors observed between MIMLh5 and NCFM SlpAs (13) could reside in the different cell models utilized.

When we tested strain *L. helveticus* MIMLh5 and its SlpA on macrophages, we observed an immunological effect (stimulation of the proinflammatory factors) that was substantially different from what was noted for epithelial cells (i.e., inhibition of NF-κB activation). These results are not surprising, considering that macrophages are immune cells that can express class and a number of receptors on their cell surface considerably different from those expressed by epithelial cells. This fact might result in recognition through diverse mechanisms and activation of alternative signaling pathways. Moreover, it is plausible (and desirable) that a potential probiotic candidate and its cell components do not trigger any inflammatory response at the host epithelium. This type of noninflammatory interaction can be assimilated to a mechanism of oral tolerance, which, *in vivo*, is due to the natural cohabitation of epithelial cells with commensal and/or food-associated bacteria. In contrast, when bacteria come in direct contact with immune cells—for instance, when they cross the epithelial barrier following loss of epithelial integrity—the immune system is typically alerted through proinflammatory signaling.

In our experiments on macrophages, the SlpA of MIMLh5 induced a cytokine profile qualitatively similar to that evoked by LPS; nonetheless, the cytokine induction levels on human U937 cells and murine BMDMs were lower for the S-layer protein than for LPS. In peritoneal macrophages, the S-layer protein triggered a cytokine induction similar to that triggered by LPS only when 10 times more S-layer protein (10 μg) than LPS (1 μg) was present. Furthermore, when we stimulated U937 cells with LPS and SlpA together, we observed a trend of reduced TNF-α induction compared to that achieved with LPS alone. These data indicate that the SlpA of MIMLh5 is not a proinflammatory stimulus as potent as LPS. We could hypothesize that the S layer alone, under basal conditions, may exert a mild stimulatory effect on the immune system. However, in the presence of an inflammatory stimulus, such as LPS, the MIMLh5 S layer could act as an immune modulator, representing a potential protective element against the septic shock caused by LPS, as already observed in murine models for certain probiotic bacteria (68, 69).

When we used murine macrophages isolated from the peritoneal cavity (PCMs), the MIMLh5 S-layer protein displayed a proinflammatory profile, whereas *L. helveticus* MIMLh5 cells induced a more balanced ratio between IL-10 and TNF-α. This finding suggests that MIMLh5 bacterial cells could be more prone to induce an anti-inflammatory response in this cellular system than U937 cells and BMDMs. Such different behavior can be explained by considering that different immune responses can be induced by the same stimulus depending on the cell type, origin, and polarization (70). Moreover, because BMDMs and U937 cells have been maturated *in vitro*, it is plausible that they are less physiological than PCMs and that they could not possess all the features of matured macrophages. It could also be speculated that macrophages from the peritoneal cavity can present phenotypes different from those presented by other macrophage populations due to the activity exerted on PCMs by the intestinal microbiota, which can promote a more tolerogenic activity on the basis of the differential expression of PRRs. Accordingly, the induction of an anti-inflammatory cytokine profile by lactobacilli has been observed in murine PCMs (71). Nonetheless, in comparison to whole bacterial cells, the proinflammatory effect of S-layer protein was particularly evident in PCMs. In fact, for MIMLh5 cells, the TNF-α/IL-10 ratios were 0.84 at an MOI of 100, 0.35 at an MOI of 1,000, and approximately 3.7 when purified SlpA protein was used as a stimulus.

Experiments with TLR-neutralizing Abs on U937 cells demonstrated an interaction of the S-layer protein with TLR2, a receptor

that was previously shown to be involved in mediating the immunological effects of LAB (13, 14, 72, 73). S-layer protein and LPS interact with different TLRs, and, consequently, the observed modulatory effect of the S-layer protein should not be attributed to a competition for the same host cells' receptors but, more likely, to an activation of different immune response pathways.

In conclusion, the immunological characterization of single bacterial components, according to the strategy undertaken during this study, represents a reductionist approach of key importance to elucidate the molecular mechanisms determining the final immune response. Furthermore, our study supports the concept that the viability of bacterial cells is not always essential to exert immunomodulatory effects (9). In fact, dead or inactivated bacterial cells or even their single molecular components might also be effective in exerting beneficial immunostimulating properties, thus permitting the development of safer therapies for the treatment of specific diseases. This approach has recently been defined as paraprobiotic intervention (10).

The possible medical and clinical uses of the S-layer protein are particularly intriguing, especially in light of the physicochemical properties of this molecule. The S-layer proteins are crystalline arrays of subunits forming a highly regular crystalline structure that has the capacity to reassemble spontaneously in suspension at the liquid-air interface, at solid surfaces, at floating lipid monolayers, on liposomes, and on nanocapsules (74, 75). These features could support the use of the S-layer protein as a bioactive coating material, as a matrix for the immobilization and delivery of different molecules, and as a template for the formation of regularly arranged bioactive nanoparticles (76). Future efforts will explore the feasibility of such applications.

ACKNOWLEDGMENTS

Part of this study was funded by the Fondazione Cariplo (grant 2010-0678). This study was also financially supported by the Academy of Finland (projects 128623 and 135980 to M.P.), a Marie Curie International Reintegration grant within the 7th European Community Framework Programme (to M.P.), the Emil Aaltonen Foundation (to M.P.), the Sigrid Jusélius Foundation (to M.P.), the Tampere Tuberculosis Foundation (to M.P.), and Competitive Research Funding of the Tampere University Hospital (grants 9M080 and 9N056 to M.P.).

We thank Annemari Uusimäki, Anna Oksanen, and William Remelli for important technical support and for fruitful scientific discussions.

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