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## Surface Layer of *Lactobacillus helveticus* MIMLh5 Promotes Endocytosis by Dendritic Cells

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1 **S-layer of *Lactobacillus helveticus* MIMLh5 promotes endocytosis by**  
2 **dendritic cells**

3 Running title: MIMLh5's S-layer drives endocytosis in DCs

4

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18 **ABSTRACT**

19 S-layers are proteinaceous arrays covering the cell wall of numerous bacteria. Their suggested  
20 properties, like the interaction with host immune system, have been only poorly described. Here, we  
21 aimed at elucidating the role of S-layer from the probiotic bacterial strain *Lactobacillus helveticus*  
22 MIMLh5 in the stimulation of murine bone marrow-derived dendritic cells (DCs). MIMLh5  
23 induced a higher production of IFN- $\beta$ , IL-12 and IL-10 compared to S-layer-depleted MIMLh5 (n-  
24 MIMLh5), whereas the isolated S-layer was a poor immunostimulator. No difference was found in  
25 the production of TNF- $\alpha$  and IL-1 $\beta$ . Inhibition of the MAP kinases JNK1/2, p38 and ERK1/2  
26 modified IL-12 production similarly in MIMLh5 and n-MIMLh5, suggesting the induction of the  
27 same signaling pathways by the two bacterial preparations. Treatment of DCs with cytochalasin D  
28 to inhibit endocytosis before addition of fluorescence-labeled MIMLh5 cells led to a dramatic  
29 reduction in the proportion of fluorescence-positive DCs, and to decreased IL-12 production.  
30 Endocytosis and IL-12 production were only marginally affected by cytochalasin D pre-treatment  
31 when using fluorescent n-MIMLh5. Treating DCs with S-layer-coated fluorescence-labeled  
32 polystyrene beads (SI-beads) resulted in a much higher uptake of beads compared to non-coated  
33 beads. Pre-stimulation of DCs with cytochalasin D reduced the uptake of SI-beads more than plain  
34 beads. These findings indicate that S-layer plays a role in the endocytosis of MIMLh5 by DCs. In  
35 conclusion, this study provides evidence that the S-layer of *L. helveticus* MIMLh5 is involved in  
36 endocytosis of the bacterium, which is of importance for a strong Th1 inducing cytokine  
37 production.

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39 **IMPORTANCE**

40 Beneficial microbes may positively impact on host's physiology at various levels, *e.g.* by  
41 participating in immune system maturation and modulation, boosting defenses and dampening  
42 reactions, therefore affecting the whole homeostasis. As a consequence, the use of probiotics is  
43 increasingly regarded as suitable for a more extended application for health maintenance, not only

44 restricted to microbiotas balancing. Evidently, this implies a deep knowledge of the mechanisms  
45 and molecules involved in host-microbes interaction, to the final purpose to fine-tune the choice of  
46 a probiotic strain for a specific outcome. To this aim, studies targeted to the description of strain-  
47 related immunomodulatory effects and individuation of bacterial molecules responsible for specific  
48 responses are indispensable. In this perspective, this study provides a new insight in the  
49 characterization of the food-origin probiotic bacterium *L. helveticus* MIMLh5 and its S-layer  
50 protein as driver for the cross-talk with dendritic cells.

51  
52 **Keywords:** probiotic, nanoparticles, MAPKs, cytokines, cytochalasin D

## 54 INTRODUCTION

55 Surface (S)-layers are bi-dimensional crystalline arrays of proteins, which form an outer self-  
56 assembled envelope on the bacterial cell wall. S-layer proteins are ubiquitously present in Archea,  
57 Gram-positive and Gram-negative bacteria (Fagan & Fairweather, 2014). They are composed of  
58 numerous identical subunits forming a symmetrical, porous, lattice-like layer that completely covers  
59 the cell surface. Considering the metabolic efforts that S-layer biogenesis, translocation and  
60 assembly imply for bacterial cell, these proteins are expected to play important functions for the  
61 organism. Several studies have evidenced different functions connected to the presence of S-layer  
62 proteins on bacterial surface, such as virulence, adhesion, protection, degradative activities (*e.g.*,  
63 amidase), and molecular sieving (Zhu et al., 2017). Within beneficial bacteria, several *Lactobacillus*  
64 species are equipped with S-layer proteins, including *L. helveticus*. In comparison with other  
65 bacteria, *Lactobacillus* S-layer proteins are characterized by their small size and high *pI* (Hynönen  
66 & Palva, 2013). Mostly, S-layers of lactobacilli have been shown to hold adhesive (Sun et al., 2012;  
67 de Leeuw et al., 2006) and immunomodulatory properties (Lightfoot et al., 2015; Taverniti et al.,  
68 2013; Li et al., 2011; Konstantinov et al., 2008). However, our understanding of S-layer's role in  
69 immune modulation is still limited.

70 We have previously described *L. helveticus* MIMLh5 as a probiotic strain (Taverniti et al.,  
71 2017; Taverniti et al., 2012; Guglielmetti et al., 2010a; Guglielmetti et al., 2010b). We also reported  
72 that the isolated S-layer protein induced the expression of TNF- $\alpha$  and COX-2 in the human  
73 monocyte-derived cell line U937, and in murine bone marrow-derived and peritoneal cavity-  
74 isolated macrophages (Taverniti et al., 2013). In those studies, we observed that depletion of the S-  
75 layer from the surface of *L. helveticus* MIMLh5 decreased the ability of the bacterium to induce  
76 TNF- $\alpha$  and COX-2, leaving the expression of IL-10 unaltered. In contrast, Konstantinov and  
77 collaborators demonstrated a role of the S-layer (SlpA) from *L. acidophilus* NCFM in eliciting the  
78 production of the anti-inflammatory cytokine IL-10 in human dendritic cells (DC) *via* interaction  
79 with the C-type lectin DC-SIGN receptor, whereas a more pro-inflammatory profile emerged in  
80 presence of a *L. acidophilus* NCFM knockout mutant lacking the SlpA (Konstantinov et al., 2008).

81 Dendritic cells (DCs) use two different strategies dependent on actin polymerization to  
82 endocytose bacteria and other particles larger than 800 nm: (i) phagocytosis, an endocytic process  
83 that requires the interaction between multiple microbial ligands and DCs receptors (Savina &  
84 Amigorena, 2007); and (ii) macropinocytosis, a non-specific uptake of components present in the  
85 surrounding fluid (Liu & Roche, 2015). Reportedly, endocytosis of *Lactobacillus acidophilus*  
86 NCFM by bone marrow-derived DCs induced IFN- $\beta$  production, that in turn activated the  
87 expression of numerous genes, including IL-12 (Weiss et al., 2010a,b, 2012). In addition, evidence  
88 was provided that both phagocytosis and constitutive macropinocytosis contribute to the uptake of  
89 strain NCFM (Boye et al., 2016). Lack of stimulation of plasma membrane Toll-Like receptors  
90 (TLRs) prior to endocytosis was also shown to be a prerequisite for a strong INF- $\beta$ /IL-12 induction  
91 (Boye et al, 2016) by *L. acidophilus* NCFM, whose S-layer protein shares high similarity with that  
92 of *L. helveticus* MIMLh5 (73% identity, 83% positivity; Stuknytė et al., 2014).

93 Here we investigated the role of MIMLh5 S-layer in the induction of IL-12 production by DCs  
94 and its possible role in endocytosis of the bacterium, by comparing the effects of DC stimulation  
95 with untreated MIMLh5 and S-layer-depleted MIMLh5 (naked (n)-MIMLh5). We also tested the

96 purified MIMLh5 S-layer protein, and S-layer-coated polystyrene beads (SI-beads, ~ 800 nm  
97 diameter) to mimic the interaction of the protein with immune cells when the protein is anchored on  
98 the surface of particles having size of a bacterium.

99

## 100 **MATERIALS AND METHODS**

101 ***L. helveticus* MIMLh5 preparation and growth conditions.** *L. helveticus* MIMLh5 was  
102 grown in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI, USA)  
103 inoculated from frozen glycerol stocks and sub-cultured twice in MRS using 1:100 inocula. To  
104 prepare cultures to be used in immunological experiments, bacteria from an overnight culture were  
105 collected, washed twice with sterile PBS, counted with Neubauer counting chamber, resuspended at  
106 a concentration of  $5 \times 10^9$  cells ml<sup>-1</sup> in PBS, and stored in aliquots at -80 °C. For preparation of n-  
107 MIMLh5, the cell pellet obtained after LiCl treatment (described below) was collected, washed 3  
108 times with PBS to remove residual LiCl, resuspended in PBS, counted and brought to the same cell  
109 concentration as MIMLh5, and stored in aliquots at -80°C.

110 **Extraction, purification and chemical characterization of the S-layer protein from *L.***  
111 ***helveticus* MIMLh5.** Extraction of the S-layer protein from *L. helveticus* MIMLh5 was performed  
112 with high-molarity LiCl as described previously (Taverniti et al., 2013; Smit et al., 2001). Briefly,  
113 cells from 500 ml of an overnight culture of MIMLh5 were harvested by centrifugation at 10,000 g  
114 for 20 min at 4 °C and washed with 1 volume of cold sterile MilliQ water. The cell pellet was  
115 extracted with 0.1 volume (referred to the starting broth culture volume) of 1 M LiCl for 30 min at  
116 room temperature in the presence of a Protease Inhibitor Cocktail (Sigma-Aldrich, Darmstadt,  
117 Germany) with slight agitation. After centrifugation, the pellet was extracted with 0.1 volumes of 5  
118 M LiCl for 1 h at room temperature in the presence of Protease Inhibitor Cocktail, and centrifuged.  
119 The residual pellet was used to prepare n-MIMLh5 cells (described above), whereas the supernatant  
120 was filtered through a 0.2 µm filter and exhaustively dialyzed for 36 h at 4 °C against distilled water  
121 containing 0.001% of Protease Inhibitor Cocktail. Dialysis was carried out with 12000 kDa cut-off

122 membranes (Sigma-Aldrich) that were previously boiled in 2% NaHCO<sub>3</sub> and 1 mM EDTA. The  
123 dialysate was collected and centrifuged at 20,000 g for 20 min at 4 °C. The supernatant was  
124 removed, and the pellet was resuspended in sterile MilliQ water and freeze dried. The lyophilized  
125 pellet was afterwards resuspended at a concentration of 1 mg ml<sup>-1</sup> in PBS and stored as aliquots at -  
126 80°C. Protein purity was determined by sodium dodecyl sulphate-polyacrylamide gel  
127 electrophoresis (SDS-PAGE) and reverse phase (RP)-HPLC/ESI-MS analysis as previously  
128 described (Taverniti et al., 2013). *SDS-PAGE*. S-layer protein and total bacterial lysates were  
129 resuspended in SDS-PAGE (Laemmli) sample buffer, boiled for 5 min, and separated on 10%  
130 polyacrylamide gel in TRIS-glycine-SDS buffer on Mini-PROTEAN 3 system (Bio-Rad). Gels  
131 were stained with Coomassie Brilliant Blue G-250 (Sigma-Aldrich, St Louis, MO).

132 **Generation of bone marrow-derived dendritic cells.** Bone marrow-derived DCs were  
133 prepared as described previously (Christensen et al., 2002). Briefly, bone marrow from C57BL/6  
134 mice (Taconic, Lille Skensved, Denmark) was flushed out from the femur and tibia and washed.  $3 \times$   
135  $10^5$  cells ml<sup>-1</sup> bone marrow cells were seeded into 10 cm Petri dishes in 10 ml RPMI 1640 (Sigma-  
136 Aldrich, St. Louis, MO, USA) containing 10% (v/v) heat inactivated fetal calf serum supplemented  
137 with penicillin (100 U ml<sup>-1</sup>), streptomycin (100 mg ml<sup>-1</sup>), glutamine (4 mM), 50 mM 2-  
138 mercaptoethanol (all purchased from Cambrex Bio Whittaker) and 15 ng ml<sup>-1</sup> murine GM-CSF  
139 (harvested from a GM-CSF transfected Ag8.653 myeloma cell line). The cells were incubated for 8  
140 days at 37 °C in 5% CO<sub>2</sub> humidified atmosphere. On day 3, 10 ml of complete medium containing  
141 15 ng/ml GM-CSF was added. On day 6, 10 ml were removed and replaced by fresh medium. Non-  
142 adherent, immature DCs were harvested on day 8.

143 **Stimulation of DCs with bacterial cells, bacterial molecules and beads.** Immature DCs ( $2 \times 10^6$   
144 cells ml<sup>-1</sup>) were resuspended in fresh medium supplemented with 10 ng ml<sup>-1</sup> GM-CSF, and 500 µl well<sup>-1</sup>  
145 of DCs suspension were seeded in 48-well tissue culture plates (Nunc, Roskilde, Denmark).  
146 *Lactobacillus helveticus* MIMLh5 was tested at multiplicity of infection (MOI) values of 5 and 50. S-  
147 layer protein from *L. helveticus* MIMLh5 was used at 10 µg ml<sup>-1</sup>. Lipopolysaccharide (LPS) from

148 *Escherichia coli* (Sigma-Aldrich) was used in all experiments as internal control at 1  $\mu\text{g ml}^{-1}$  (not  
149 shown). Uncoated beads and S-layer-coated beads were used at corresponding MOIs of 5 and 50 for  
150 ELISA experiments. Cytochalasin D (Sigma-Aldrich) was added at a concentration of 0.5  $\mu\text{g ml}^{-1}$  1 h  
151 prior to the incubation of DCs with bacteria. In the MAPK inhibition experiments, DCs ( $2 \times 10^6$  cells  
152  $\text{ml}^{-1}$ ) were pre-incubated for 1 h with (i) SP600125 (final concentration 25  $\mu\text{M}$ ), a specific inhibitor of  
153 JNK1/2 (Invivogen, San Diego, CA, USA), (ii) SB203580 (final concentration 10  $\mu\text{M}$ ), a specific  
154 inhibitor of p38 MAPK (Invivogen), and (iii) the MEK1/2 inhibitor U0126 (final concentration 10  $\mu\text{M}$ )  
155 which blocks MEK1/2 and thereby phosphorylation of the target ERK1/2 (Cell Signaling, MA, USA).  
156 In all the conditions described, DCs and stimuli were incubated at 37 °C in 5%  $\text{CO}_2$ . For time course  
157 experiments, DCs were harvested for RNA extraction after 2, 4, 6, and 10 h; the supernatant for ELISA  
158 analysis was collected after 4, 6, 10, and 20 h. In the other experiments, DCs were harvested for RNA  
159 extraction after 4 or 6 h, and the supernatant for ELISA analysis after 20 h.

160 **Cytokine quantification in DCs supernatant.** The concentration of IL-12(p70), IL-10, TNF- $\alpha$   
161 and IL-1 $\beta$  was analyzed by using commercially available ELISA Antibody pairs (R&D systems,  
162 Minneapolis, MN, USA) and the concentration of IFN- $\beta$  by an ELISA kit from PBL Assay Science  
163 (Piscataway, NJ, USA) according to the manufacturers' instructions.

164 **RNA extraction.** Murine bone marrow-derived DCs were harvested and total RNA was  
165 extracted using the MagMAX sample separation system (Applied Biosystems, Foster City, CA,  
166 USA), including a DNase treatment step for genomic DNA removal. RNA concentration was  
167 determined by Nanodrop (Thermo, Wilmington, DE, USA).

168 **Reverse transcription and qPCR reaction (RT-qPCR).** Five hundred nanograms of total RNA  
169 was reverse-transcribed by the TaqMan Reverse Transcription Reagent kit (Applied Biosystems,  
170 Foster City, CA, USA) using random hexamer primers according to the manufacturer's instructions.  
171 The obtained cDNA was stored in aliquots at -80 °C. Primers and probes were obtained and sequenced  
172 as described previously (Boye et al., 2016; Weiss et al., 2013, 2011). qPCR amplifications were carried  
173 out in a total volume of 10  $\mu\text{l}$  containing 1 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems),



174 forward and reverse primers, TaqMan MGB probe, and the purified target cDNA (6 ng). Cycling was  
175 initiated for 20 s at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C using an ABI Prism  
176 7500 (Applied Biosystems). Amplification reactions were performed in triplicate, and DNA  
177 contamination controls were included. The amplifications were normalized to the expression of the  
178 beta-actin encoding gene. Relative transcript levels were calculated applying the  $2^{-\Delta\Delta C(T)}$  method  
179 (Livak & Schmittgen, 2001).

180 **Preparation of fluorescence-labelled bacteria.** For endocytosis experiments, untreated or S-  
181 layer-depleted *L. helveticus* MIMLh5 cells were fluorescently labelled using Alexa Fluor-conjugated  
182 succinimidyl-esters (SE-AF647; Alexa Fluor 647, Molecular Probes, Eugene, OR). Bacterial cells in  
183 Dulbecco's PBS (DPBS) were centrifuged for 5 min at 13,000 g in 1.5 ml Eppendorf tubes and  
184 resuspended in 750 µl of sodium carbonate buffer (pH 8.5); then SE-AF647 was added (10 µl for  
185 approximately  $2 \times 10^9$  bacterial cells ml<sup>-1</sup>). Bacteria were incubated at room temperature with agitation  
186 for 1 h in the dark, washed three times in sodium carbonate buffer, and finally resuspended in the  
187 original volume of DPBS.

188 **Preparation of FITC-conjugated S-layer.** The purified S-layer from *L. helveticus* MIMLh5 was  
189 dissolved in 5 M LiCl to obtain a 2 mg ml<sup>-1</sup> protein solution, and the pH was adjusted to 9 by adding  
190 diluted NaOH. One ml of the protein solution was treated with 0.05 ml of a 1 mg ml<sup>-1</sup> FITC solution in  
191 DMSO. The reaction was carried out overnight at 4 °C and stopped by adding 9 mL of 5 M urea in  
192 water. The FITC-conjugated protein solution was concentrated to 1 ml – while exchanging buffer to 5  
193 M urea – by using an Amicon® Ultra-15 centrifugal filter unit (MWCO 10 kDa, Merck Millipore Ltd.,  
194 Cork, Ireland). The FITC-labeled protein was stored at 4 °C.

195 **S-layer adsorption on polystyrene nanoparticles (NPs).** Fifty micrograms of Nile Red  
196 fluorescent (RF) polystyrene particles (NP) with an average size of 0.84 µm (Kisker Biotech, Steinfurt,  
197 Germany) were added to 1 ml of a 0.2 mg ml<sup>-1</sup> FITC-labeled S-layer solution in 5M urea, and gently  
198 stirred at 4 °C for 2 hours. Then, the NP suspension was slowly diluted to a final volume of 10 ml by  
199 progressive addition of water over a several hours. The preparation was kept overnight at 4 °C under

200 stirring, and centrifuged at 10000 g for 30 m at 4 °C. The precipitated NPs were washed tree times  
201 with 5 M urea to remove unbound proteins, and the S-layer-coated NPs were suspended in 1 ml of  
202 ultrapure water.

### 203 **Evaluation of *L. helveticus* MIMLh5 and S-layer-coated beads (SI-beads) uptake by DCs.**

204 Non-adherent immature DCs were harvested and resuspended in complete medium to a concentration  
205 of  $2 \times 10^6$  cells ml<sup>-1</sup>. Pretreatment of cells with cytochalasin D (Sigma-Aldrich) at a final concentration  
206 of 0.5 µg ml<sup>-1</sup> was performed in flasks for 1 h at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere before  
207 the addition of stimuli. After that, 150 µl of DCs were seeded ( $3 \times 10^5$  cells well<sup>-1</sup>) in 96-well U-  
208 bottom tissue culture plates (NUNC) and incubated for 30 min at 37 °C and 5% CO<sub>2</sub> in a humidified  
209 atmosphere with either fluorescent beads (uncoated or S-layer coated), or with fluorescently labelled *L.*  
210 *helveticus* MIMLh5 (with and without a S-layer protein coating). Bacteria were used at a MOI of 5,  
211 and beads were tested at both MOI 5 and 50. All conditions were tested in triplicate, in at least three  
212 different experiments. Before each experiment, beads were treated in an ultrasound bath for at least 2  
213 min to gently ensure size uniformity. All the stimuli were tested in absence and presence of  
214 cytochalasin D. Controls included untreated cells and cells incubated only with cytochalasin D without  
215 any stimulus. After incubation with beads and bacteria, DCs were spun down ( $1200 g \times 5$  min at 4 °C),  
216 washed twice with cold PBS containing 1% FCS (washing buffer), and then fixed with 1%  
217 formaldehyde in washing buffer. Cells were analyzed by flow cytometry on a FacsCantoII (BD  
218 Biosciences). Unless otherwise stated, data are from at least three independent experiments. Data  
219 analysis was performed using the FLOWJO version 10 software (Ashland, OR).

220 **Statistical analysis.** Statistical calculations were performed using the software program GraphPad  
221 Prism 5. The significance of the results was analyzed by unpaired heteroscedastic Student's t test with  
222 two-tailed distribution. Differences of  $P < 0.05$  were considered significant.

223 **Ethics statement.** All animals used as a source of bone marrow cells were housed under  
224 conditions approved by the Danish Animal Experiments Inspectorate (Forsøgdyrstilsynet), Ministry  
225 of Justice, Denmark, and experiments were carried out in accordance with the guidelines 'The

226 Council of Europe Convention European Treaty Series 123 for the Protection of Vertebrate Animals  
227 used for Experimental and other Scientific Purposes'. Since the animals were employed as sources  
228 of cells, and no live animals were used in experiments, no specific approval was required for this  
229 study. Hence, the animals used for this study are included in the general facility approval for the  
230 faculty of Health and Medical Sciences, University of Copenhagen.

231

## 232 RESULTS

### 233 Depletion of S-layer from *L. helveticus* MIMLh5 reduces INF- $\beta$ , IL-12 and IL-10

234 **production by DCs.** To study the role of S-layer protein in the *L. helveticus* MIMLh5-mediated  
235 induction of a Th1 activating response in DCs, we compared the levels of different cytokines  
236 produced by DCs upon stimulation with *L. helveticus* MIMLh5 or n-MIMLh5. SDS-PAGE  
237 confirmed that the protein was efficiently removed from bacterial surface, as evidenced by the  
238 strong reduction of the 45 kDa band corresponding to MIMLh5 S-layer protein, while leaving  
239 apparently unaltered the other proteins (**Fig. 1**, lanes 4-6). Then, the expression of *Ifn $\beta$* , *Il12*, *Il10*,  
240 *Tnfa* and *Il1 $\beta$*  genes in DCs at 2, 4, 6 and 10 h was analyzed by RT-qPCR following stimulation  
241 with bacteria and the S-layer protein; furthermore, the concentrations of the corresponding  
242 cytokines was assessed by ELISA in DC supernatant collected after 10 h of incubation.

243 Removal of S-layer protein from the surface of MIMLh5 influenced the ability of the bacterium  
244 to induce *Ifn $\beta$* , *Il12*, *Il10*, *Tnfa* and *Il1 $\beta$*  (**Fig. 2A**). ELISA data evidenced that the levels of IFN- $\beta$ ,  
245 IL12 and IL10 were significantly lowered in the supernatant of DCs stimulated with n-MIMLh5  
246 compared to intact MIMLh5 (**Fig. 2B**). The purified S-layer protein did not induce the expression of  
247 *Ifn $\beta$*  or *Il12* at any of the considered time points (**Fig. 2A**), as also confirmed at protein level by  
248 ELISA at 10 h (**Fig. 2B**). In contrast, S-layer protein induced the expression of *Il10* and the pro-  
249 inflammatory cytokines *Tnfa* and *Il1 $\beta$*  (**Fig. 2A**), as also confirmed by corresponding cytokine  
250 quantification in DCs supernatant (**Fig. 2B**). Overall, n-MIMLh5 induced the same cytokine  
251 expression profile as MIMLh5, but to a lower extent (**Fig. 2A**). The cytokine concentrations in the

252 supernatant harvested after 10 h reflected the expression profiles of each gene, with a significant  
253 difference between n-MIMLh5 and MIMLh5 regarding IFN- $\beta$ , IL-12 and IL-10 concentration (**Fig.**  
254 **2B**).

255 **The effect of MAPK-inhibition on IL-12 production does not differ between MIMLh5 and**  
256 **n-MIMLh5.** To test whether *L. helveticus* MIMLh5 S-layer protein influences the signaling  
257 pathways that initiate IL-12 production by DCs, we investigated the effect of inhibiting specific  
258 mediators of the Mitogen Activated Protein (MAP) kinase cascade (Kaji et al., 2010), namely,  
259 JNK1/2, p38, and ERK 1/2. These pathways have previously been shown to be involved in *L.*  
260 *acidophilus* NCFM-dependent induction of IL-12 production in DCs (Weiss et al., 2011, 2012). The  
261 production of IL-12 was quantified by ELISA upon addition of MAPK inhibitors before bacterial  
262 stimulation. JNK1/2 inhibition resulted in a 26% and 39% reduction of IL-12 upon stimulation with  
263 *L. helveticus* MIMLh5 and n-MIMLh5, respectively (**Fig. 3**). Inhibition of p38 lowered IL-12  
264 production by 24% (MIMLh5) and 66% (n-MIMLh5), whereas blocking ERK 1/2 caused an  
265 increase in IL-12 of 62% (MIMLh5) and 32% (n-MIMLh5) (**Fig. 3**).

266 **Inhibition of bacterial endocytosis lowers IL-12 production in DCs stimulated with intact**  
267 **but not S-layer-depleted MIMLh5 cells.** To test whether the S-layer protein affects endocytosis of  
268 *L. helveticus* MIMLh5 by DCs, we quantified IL-12, IL-10 and TNF- $\alpha$  by ELISA after stimulation  
269 of DCs with either MIMLh5 or n-MIMLh5 in the presence of cytochalasin D, an inhibitor of actin-  
270 dependent cytoskeleton rearrangement (Cooper, 1987). We found that the presence of cytochalasin  
271 D significantly lowered IL-12 production (by 27%) when DCs were stimulated with MIMLh5,  
272 whereas it did not significantly affect the IL-12 levels when DCs were stimulated with n-MIMLh5  
273 (**Fig. 4A**). In addition, pre-treatment with cytochalasin D increased IL-10 production induced by  
274 MIMLh5 and n-MIMLh5 (**Fig. 4B**), whereas TNF- $\alpha$  levels were not significantly affected (**Fig.**  
275 **4C**).

276 **Endocytosis of *L. helveticus* MIMLh5 is partly dependent on the presence of S-layer**  
277 **protein on the bacterial surface.** To study the endocytosis of *L. helveticus* MIMLh5 in DCs, we

278 prepared Alexa-Fluor 647-labelled cells of MIMLh5 and n-MIMLh5. Labeling was not equally  
279 efficient for the two bacteria and flow cytometry data thus not directly comparable. When DCs were  
280 pre-treated with cytochalasin D before addition of both bacterial preparations, we observed a major  
281 reduction in the number of fluorescent DCs (*i.e.* DCs that internalized fluorescence-labeled  
282 bacteria); nonetheless, the difference in the number of DCs positive for endocytosed bacteria  
283 between cytochalasin D-treated and untreated DCs was greater for the intact MIMLh5 than for n-  
284 MIMLh5, indicating a more pronounced endocytosis of MIMLh5 (**Fig. 5**).

285 To directly demonstrate the role of the S-layer in endocytosis, we coated fluorescent beads of a  
286 size resembling bacterial cells dimension (~ 800 nm) with the isolated MIMLh5 S-layer protein (SI-  
287 beads). The quantity of beads employed to prepare SI-beads to be used in comparative/chasing  
288 experiments was estimated on the basis of the bead average mass and of the volume of individual  
289 beads. In these experiments, we decided to use the beads at the same MOIs used for bacterial cells  
290 (5 and 50), even though it was not possible to assume that the amounts of S-layer protein on beads  
291 and bacterial surface were comparable. The presence of the S-layer protein with the fluorescent  
292 beads was demonstrated by SDS-PAGE (**Fig. 6A**, lanes 2-5), which shows bands at around 45 kDa.  
293 The slightly higher apparent size of proteins detached from SI-beads (**Fig. 6A**, lanes 6-9) may relate  
294 to the extensive unfolding associated with non-covalent interaction of proteins with polystyrene  
295 nanoparticles (Barbiroli et al., 2015; Miriani et al., 2014).

296 SI-beads and plain beads (*i.e.* fluorescence-labelled beads without any protein coating) were  
297 added to DCs, and the proportion of cells taking up beads was evaluated by flow cytometry (DCs  
298 positive of endocytosed beads; **Fig. 6B**). When DCs were incubated with plain beads at MOI 50,  
299 about 28% of them endocytosed the beads, and addition of cytochalasin D only marginally reduced  
300 this number (from 28% to 22%; **Fig. 6B, C**), indicating that the majority of the beads were stuck on  
301 the DCs surface. Conversely, incubation with SI-beads at MOI 50 gave a higher percentage of  
302 positive DCs compared to incubation with plain beads (53% positive), an effect that was also  
303 evident at MOI 5 (13% positive DCs in presence of SI-beads vs 5% in presence of plain fluorescent

304 beads; **Fig. 6 B, D**). The addition of cytochalasin D prior to the addition of SI-beads reduced the  
305 proportion of positive DCs from 13 to 8% at MOI 5, and from 53 to 35% with MOI 50 (**Fig. 6B, C,**  
306 **D**), indicating decreased internalization of coated beads compared to the plain ones.

307

## 308 **DISCUSSION**

309 Here we have demonstrated that depletion of S-layer from *L. helveticus* MIMLh5 significantly  
310 reduced the bacteria's capability to induce IFN- $\beta$ , IL-12 and IL-10 in bone marrow derived  
311 dendritic cells. By contrast, no major reduction in the innate pro-inflammatory cytokines TNF- $\alpha$   
312 and IL-1 $\beta$  was seen. S-layer, as isolated molecule, was a poor immune stimulator, only inducing a  
313 weak expression of the cytokines IL-10, TNF- $\alpha$  and IL-1 $\beta$ , and was unable to activate the  
314 expression of IFN- $\beta$  and IL-12, even if we calculated that the amount of purified S-layer used was  
315 approximately 100 times more than the S-layer present on the bacterial surface of the amount of  
316 cells used in the same experiment. As we have previously demonstrated that induction of INF- $\beta$  by  
317 lactobacilli only takes place in endosomes upon endocytosis of the intact bacteria (Weiss et al.,  
318 2010a, 2011), we hypothesized that S-layer plays a key role in the endocytosis of the bacteria. We  
319 have previously observed that lactobacilli can induce IL-12 by at least two distinct signaling  
320 pathways. One depends on the induction of IFN- $\beta$  through a MAPK pathway inducing c-jun/ATF2  
321 activation of AP-1, which is fully dependent on the MAPK JNK1/2 and, to much lesser degree on  
322 p38, while the MAPK ERK does not seem to be involved (Weiss et al., 2010b, 2011). The other  
323 pathway leads to direct induction of IL-12 and seems to depend on p38 (Lu et al. 1999; Weiss et al.,  
324 2011). Accordingly, we investigated how MAPK inhibitors of JNK1/2, p38 and ERK1/2 (*via*  
325 MEK) affected the IL-12 response upon stimulation with MIMLh5 or n-MIMLh5. The two bacterial  
326 stimulations demonstrated comparable effects as for sensitivity to JNK1/2 inhibition. By contrast,  
327 inhibition of p38 resulted in a marked IL-12 decrease after n-MIMLh5 stimulation, whereas the  
328 highest sensitivity to IL-12 inhibition by ERK1/2 was observed after stimulation with native,  
329 untreated MIMLh5. This let us to conclude that the S-layer depletion from MIMLh5 lowers its

330 ability to promote IFN- $\beta$ -mediated IL-12 production. We hypothesized that this may be due to  
331 impaired endocytosis of the S-layer-depleted bacteria. This is supported by experiments with  
332 cytochalasin D-treated DCs that are unable to endocytose bacteria: the response induced by  
333 MIMLh5 was significantly reduced by cytochalasin D pre-treatment at contrast to n-MIMLh5.  
334 Likewise, we found a higher  $\beta$ -actin-dependent uptake of fluorescence-labelled MIMLh5 than of  
335 fluorescence-labelled n-MIMLh5. As we have previously demonstrated that another S-layer coated  
336 bacterium, *L. acidophilus* NCFM, is endocytosed partly by phagocytosis and partly by  
337 macropinocytosis in murine DCs (Boye et al., 2016), the difference between MIMLh5 and n-  
338 MIMLh5 in the endocytosis by DCs in presence of cytochalasin D may indicate that the S-layer-  
339 depleted bacteria are restricted in uptake by one of these mechanisms, most probably by  
340 phagocytosis. A comparison of the cytochalasin D effects on endocytosis and IL-12 production  
341 indicates that only some of the produced IL-12 is dependent on endocytosis of the bacteria. Along  
342 the same lines, the decrease of INF- $\beta$  in DCs stimulated with S-layer-depleted MIMLh5 was not  
343 complete, which may indicate that some bacteria are still endocytosed by the constitutive  
344 micropinocytosis, that takes place independently from the cell wall structures present on the  
345 bacterial cells. To this end, we have previously shown that only a proportion of endocytosed *L.*  
346 *acidophilus* NCFM is taken up by phagocytosis while the rest was taken up by macropinocytosis  
347 (Boye et al., 2016; Fuglsang et al., 2017). The relative relevance of each event will depend on the  
348 properties of the bacteria, and most notably on the bacterial surface, as well as on ceramide  
349 formation on plasma membrane of DCs (Boye et al., 2016; Fuglsang et al, 2017; Abdel Shakor et  
350 al., 2004).

351 We tried to coat fluorescent beads of a size comparable to bacteria with the isolated S-layer  
352 protein, in order to investigate whether this would facilitate endocytosis of the beads. We found a  
353 significantly higher number of bead-positive DCs when S-layer-coated beads were added, compared  
354 to the addition of plain fluorescent beads. This supports a role of the S-layer in facilitating  
355 endocytosis. The naked beads are readily dispersed in water solutions with medium ionic strength

356 but at physiological  $pI$ , as used for this study, they may show some tendency to aggregate.  
357 Association with S-layer proteins are likely to change this property towards more readily dispersed  
358 particles at the physiologic ionic strength. From this study, we cannot establish whether the higher  
359 uptake of S-layer-associated beads is due to the binding to a specific receptor, to a stronger non-  
360 specific attraction to the negatively charged cell surface, or to a higher dispensability. However,  
361 held together with the results from studying the effect of the S-layer-depleted MIMLh5, these data  
362 support a role of S-layer in the endocytosis of MIMLh5. In summary, we have provided evidence  
363 that the S-layer of *L. helveticus* MIMLh5 is involved in endocytosis of the bacterium which is of  
364 importance for a strong Th1-inducing cytokine production. Moreover, this kind of knowledge can  
365 be of help in the selection of probiotic strains for specific purposes, e. g. in cases of exacerbated IgE  
366 production, allergies, and atopy where favoring a Th1 response would be of benefit.

367

## 368 **COMPLIANCE WITH ETHICAL STANDARDS**

### 369 **Conflict of interest**

370 The authors declare that they have no conflict of interest.

371

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375

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- 488

489 **LEGENDS**

490 **Fig. 1.** SDS-PAGE profile of crude cell extract obtained by boiling *L. helveticus* MIMLh5 cells  
491 before and after LiCl treatment. M, molecular weight marker. Lanes 1-3, extracts from  $2.5 \times 10^8$ ,  
492  $3.75 \times 10^8$  and  $5 \times 10^8$  intact MIMLh5 cells, respectively; lanes 4-6, extracts from  $2.5 \times 10^8$ ,  $3.75 \times$   
493  $10^8$  and  $5 \times 10^8$  LiCl-treated MIMLh5 cells, respectively.

494 **Fig. 2.** Cytokine profile elicited in bone marrow-derived dendritic cells (DCs) by *L. helveticus*  
495 MIMLh5 cells with and without (n-MIMLh5) S-layer and by purified S-layer protein. Expression of  
496 cytokines *Ifn* $\beta$ , *Il12*, *Il10*, *Tnfa* and *Il1* $\beta$  was determined by RT-qPCR after 2, 4, 6 and 10 h  
497 incubation (A). Expression profiles are indicated as the fold change of induction (FOI) relative to  
498 the control (unstimulated DCs) which was set at a value of 1 (A). Asterisks indicate statistically  
499 significant differences between MIMLh5 and nMIMLh5 (\*\*:  $P < 0.01$ ; \*:  $P < 0.05$ ) according to  
500 two-way ANOVA analysis along the time-course experiment (A). Protein levels of IFN- $\beta$ , IL-12,  
501 IL-10, TNF- $\alpha$  and IL-1 $\beta$  were measured in the supernatants of DCs by ELISA after 10 h of  
502 incubation (B). Slay: S-layer protein from *L. helveticus* MIMLh5 was used at a concentration of 10  
503  $\mu\text{g ml}^{-1}$ . MIMLh5 cells and S-layer depleted MIMLh5 cells (n-MIMLh5) were both used at a  
504 multiplicity of infection (MOI) of 50. C: unstimulated DCs. Data represent mean of measurements  
505 from triplicates  $\pm$  standard deviation. Asterisks indicate statistically significant differences between  
506 MIMLh5 and n-MIMLh5 (\*:  $P < 0.05$ ) according to unpaired t-test.

507 **Fig. 3.** Stimulation of DCs with *L. helveticus* MIMLh5, S-layer-depleted MIMLh5 cells (n-  
508 MIMLh5), and the purified S-layer protein after pre-incubation with inhibitors for JNK 1/2, p38 and  
509 MEK 1/2. Protein levels of the cytokines IL-12 were measured in the supernatants of DCs by  
510 ELISA after 20 h. MIMLh5 and n-MIMLh5 were used at a MOI of 50. Slay: S-layer protein was  
511 tested at a concentration of  $10 \mu\text{g ml}^{-1}$ . C: control (unstimulated DCs). MAPK inhib: DCs  
512 stimulated only with respective MAPK inhibitors JNK, p38 and MEK. Asterisks indicate  
513 statistically significant differences (\*\*\*:  $P < 0.001$ ; \*\*:  $P < 0.01$ ) according to unpaired t-test. Data  
514 represent mean of measurements from triplicate cultures  $\pm$  standard deviation.

515 **Fig. 4.** Cytokine production in DCs upon stimulation with *L. helveticus* MIMLh5 and S-layer-  
516 depleted MIMLh5 (n-MIMLh5) in presence of cytochalasin D. DCs were prestimulated for 1 h with  
517 cytochalasin D ( $0.5 \mu\text{g ml}^{-1}$ ) before addition of bacterial cells. Protein levels of the cytokines IL-12  
518 (A), IL-10 (B) and TNF- $\alpha$  (C) were measured in the supernatants of DCs by ELISA after 20 h.  
519 MIMLh5 LiCl-treated and untreated cells were used at a MOI of 5. N-MIMLh5: MIMLh5 cells  
520 after removal of the S-layer protein by LiCl-extraction. C: control (unstimulated DCs). Cyt D: DCs  
521 stimulated only with the cytochalasin D. Asterisks indicate statistically significant differences (\*\*:  
522  $P < 0.01$ ; \*:  $P < 0.05$ ) according to unpaired t-test. Data represent mean of measurements from  
523 triplicate cultures  $\pm$  standard deviation.

524 **Fig. 5.** DCs were pretreated with cytochalasin D or media (no bacteria) for 1 h before  
525 stimulation with Alexa Fluor 647-labelled *L. helveticus* MIMLh5 for 30 min followed by flow  
526 cytometry analysis. Data in the histograms (A) are reported as fold of decrease of the number of  
527 DCs positive for endocytosed MIMLh5 and upon treatment with cytochalasin D compared to the  
528 untreated DCs. Fluorescent untreated MIMLh5 (B) and n-MIMLh5 cells (C) were used at a MOI of  
529 5. n-MIMLh5: MIMLh5 cells after removal of the S-layer protein by LiCl-extraction. APC positive  
530 population: DCs uptaking bacteria (B, C). Asterisks indicate statistically significant differences  
531 (\*\*\*:  $P < 0.001$ ) according to unpaired t-test. Dot plots are based on 50.000 cells counted on FACS  
532 CantoII and single cell gating by the use of FSC-A/FSC-H. Means and SD are based on technical  
533 replicates.

534 **Fig. 6.** The presence of S-layer protein from MIMLh5 on polystyrene beads preparation,  
535 revealed by SDS-PAGE with Coomassie blue staining, affects endocytosis in DCs. In lanes 2 to 5  
536 of the SDS-gel the following estimated MOIs of S-layer-coated beads (Sl-beads) were loaded: 20-  
537 30-40-50. In lanes 6 to 9 the following quantity of S-layer protein have been loaded: 5-10-15-20  $\mu\text{g}$ .  
538 (A). DCs were pretreated with cytochalasin D or media (no beads) for 1 h before stimulation with  
539 fluorescent beads prolonged for 1 h, followed by flow cytometry analysis (B, C, D). The percentage  
540 of DCs that have endocytosed beads by macropinocytosis is indicated as percentage of DCs positive

541 of endocytosed beads (B). Fluorescent plain beads (B, C) and fluorescent beads coated with FITC-  
542 S-layer protein (S1-beads) (B, D) were used at a corresponding MOI of 5 and 50. PE positive  
543 population: DCs uptaking beads (C, D). Asterisks indicate statistically significant differences (\*\*\*:  
544  $P < 0.001$ ; \*:  $P < 0.05$ ) according to unpaired t-test. Dot plots are based on 50.000 cells counted on  
545 FACS CantoII and single cell gating by the use of FSC-A/FSC-H. Means and SD are based on  
546 technical replicates.



Fig. 1

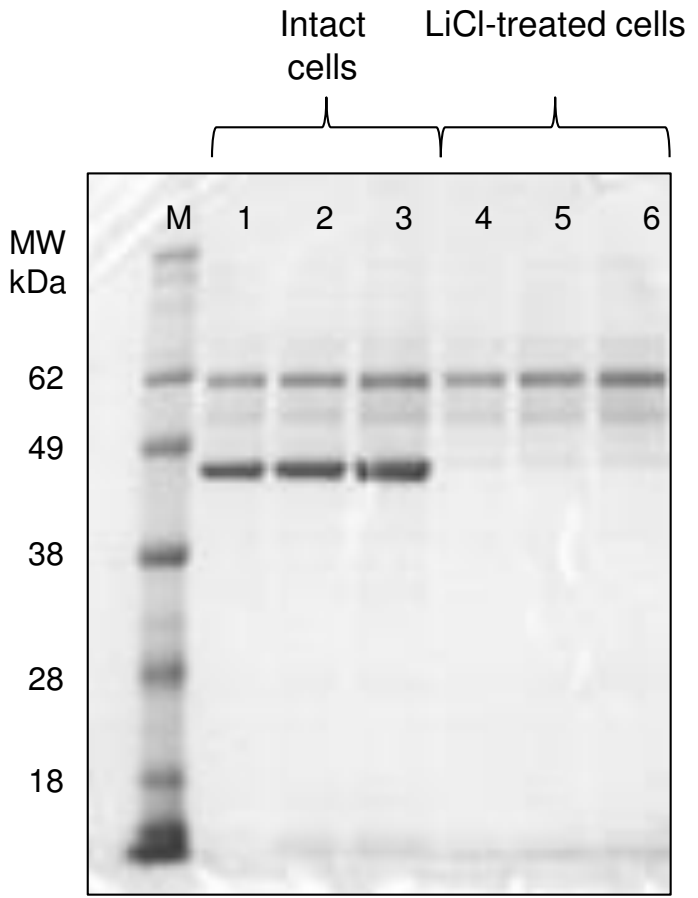


Fig. 2

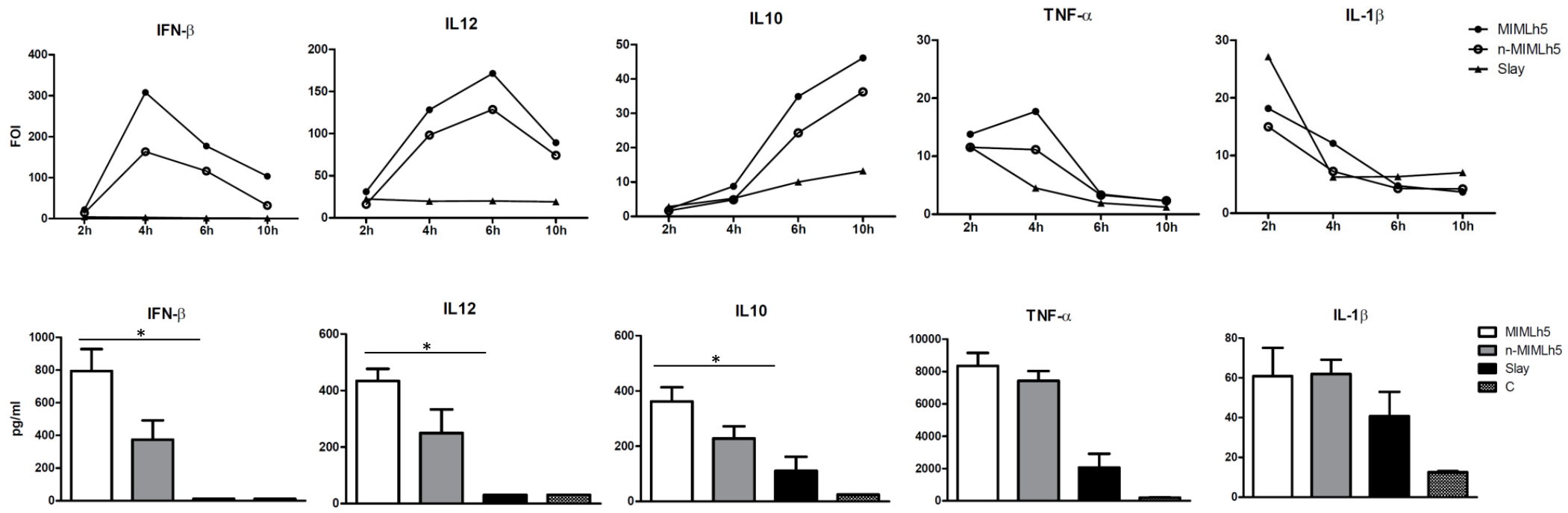


Fig. 3

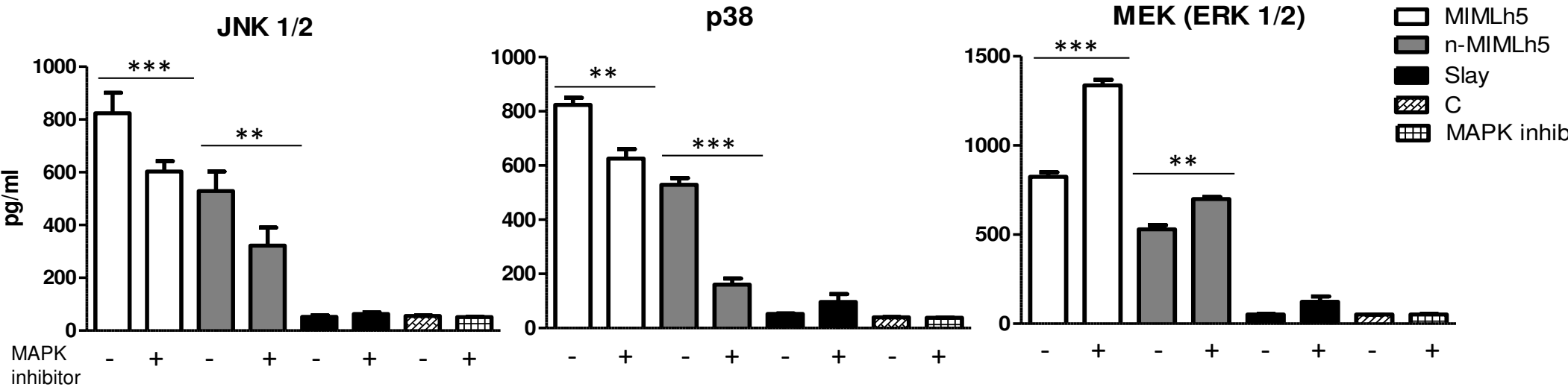


Fig. 4

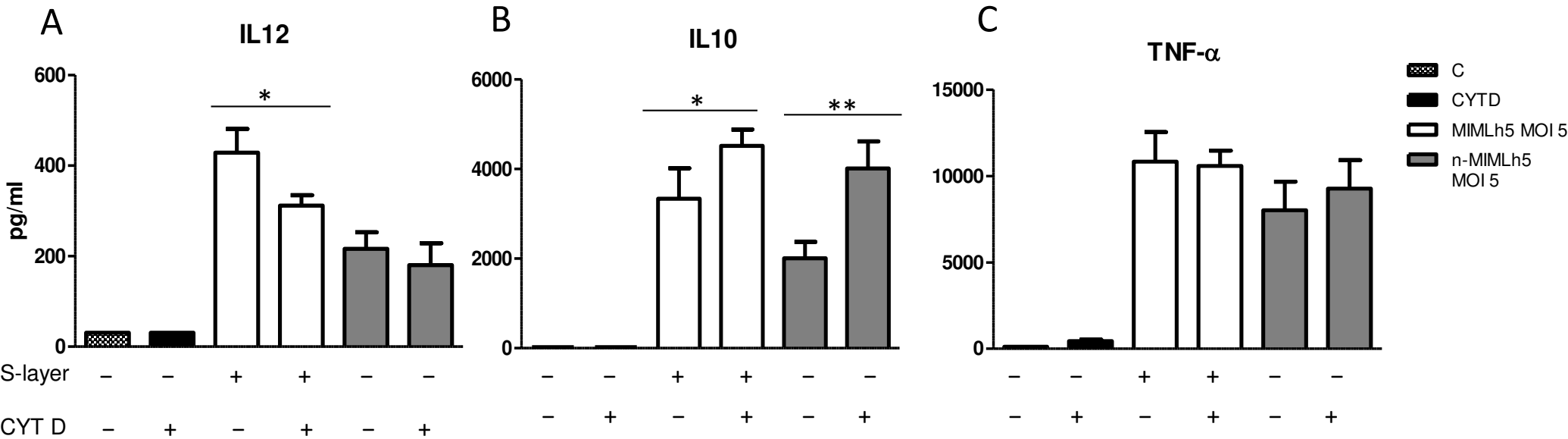
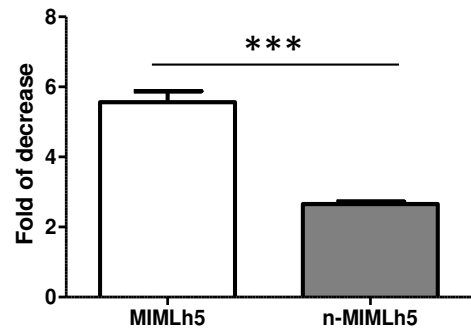
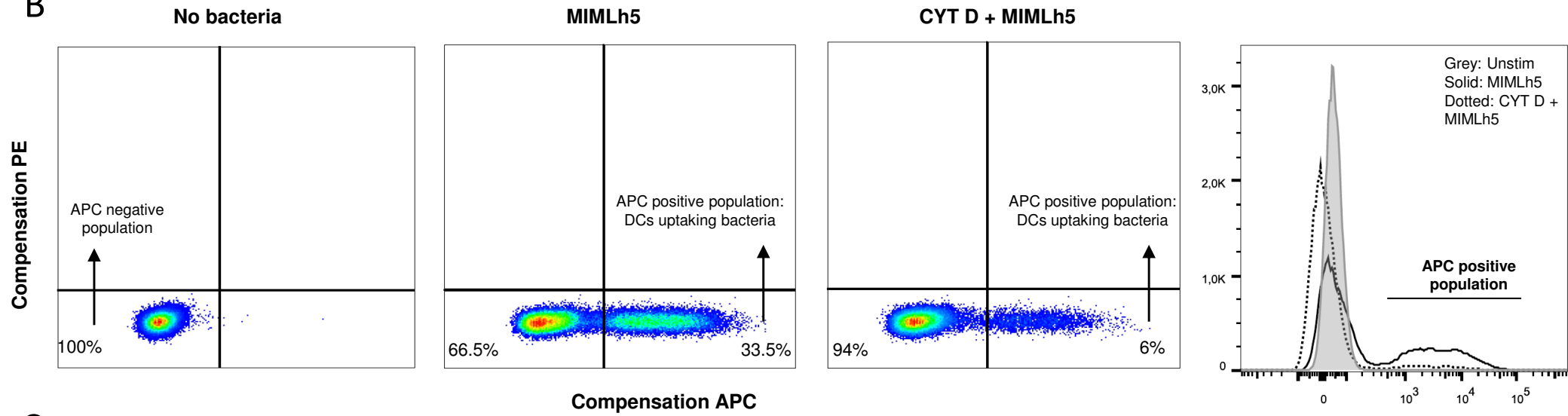


Fig. 5

A



B



C

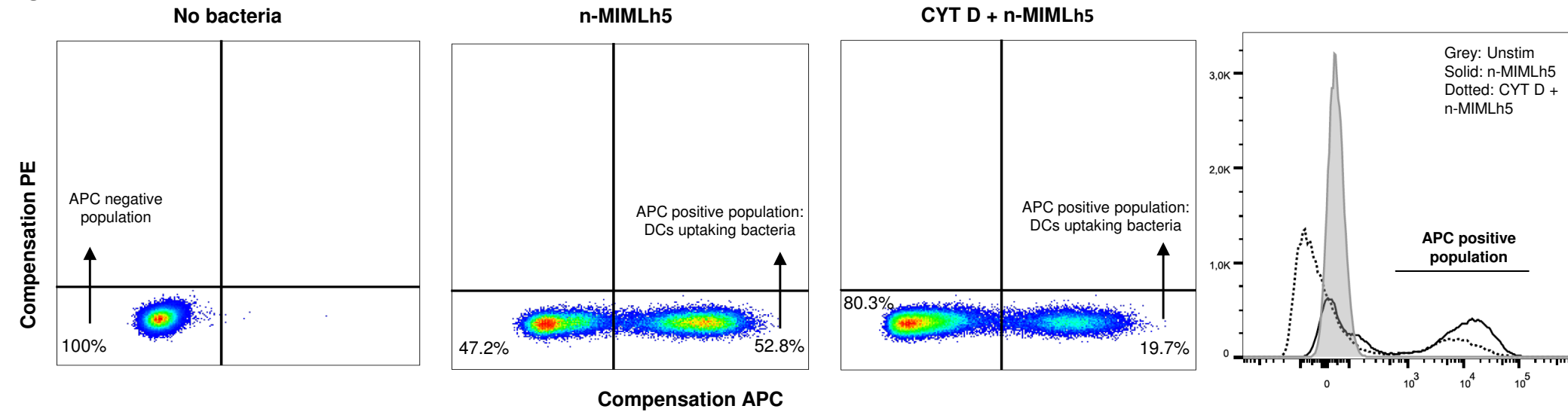


Fig. 6

