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Coláiste na hOllscoile Corcaigh

1 The surface associated exopolysaccharide of *Bifidobacterium longum* 35624 plays an essential role in  
2 dampening host pro-inflammatory responses and in repressing local TH17 responses

3 **Running title:** *B. longum* exopolysaccharide modulates TH17 responses

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13 Running Head: Immunoregulation by bifidobacterial surface polysaccharide

14

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19 **ABSTRACT**

20 The immune modulating properties of certain bifidobacterial strains, such as *Bifidobacterium*  
21 *longum* subsp. *longum* **35624**<sup>TM</sup> (*B. longum* 35624), have been well described, although the  
22 strain-specific molecular characteristics associated with such immune regulatory activity are  
23 not well defined. It has previously been demonstrated that *B. longum* **35624** produces a cell  
24 surface exopolysaccharide and in this study we investigated the role played by this  
25 exopolysaccharide in influencing the host immune response. *B. longum* **35624** induced  
26 relatively low levels of cytokine secretion from human dendritic cells, whereas an isogenic  
27 exopolysaccharide-negative mutant derivative (termed sEPS<sup>neg</sup>) induced vastly more  
28 cytokines, including IL-17, which was reversed when exopolysaccharide production was  
29 restored in sEPS<sup>neg</sup> by genetic complementation. Administration of *B. longum* **35624** to the T  
30 cell transfer colitis model prevented disease symptoms, whereas sEPS<sup>neg</sup> did not protect  
31 against the development of colitis, with associated enhanced recruitment of IL-17+  
32 lymphocytes to the gut. Moreover, intra-nasal administration of sEPS<sup>neg</sup> also resulted in  
33 enhanced recruitment of IL-17+ lymphocytes to the murine lung. These data demonstrate that  
34 the particular exopolysaccharide produced by *B. longum* **35624** plays an essential role in  
35 dampening pro-inflammatory host responses to the strain and that loss of exopolysaccharide  
36 production results in the induction of local T<sub>H</sub>17 responses.

37 **IMPORTANCE**

38 Particular gut commensals, such as *B. longum* **35624**, are known to contribute positively to  
39 the development of mucosal immune cells, resulting in protection from inflammatory  
40 diseases. However, the molecular basis and mechanisms for these commensal-host  
41 interactions are poorly described. In this report, an exopolysaccharide was shown to be

42 decisive in influencing the immune response to the bacterium. We generated an isogenic  
43 mutant unable to produce exopolysaccharide, and observed that this mutation caused a  
44 dramatic change in the response of human immune cells *in vitro*. In addition, mouse models  
45 confirmed that lack of exopolysaccharide production induces inflammatory responses to the  
46 bacterium. These results implicate the surface-associated exopolysaccharide of the *B. longum*  
47 **35624** cell envelope in the prevention of aberrant inflammatory responses.

48

49 **INTRODUCTION**

50           The gut microbiota contributes significantly to host health via multiple mechanisms,  
51 including the digestion of foods, competitive exclusion of pathogens, enhancement of  
52 epithelial cell differentiation and promotion of mucosa-associated lymphoid tissue  
53 proliferation (1, 2). Furthermore, accumulating evidence suggests that the composition and  
54 metabolic activity of the gut microbiota has profound effects on proinflammatory activity and  
55 the induction of immune tolerance within mucosal tissue (3-5). Certain microbes induce  
56 regulatory responses, while others induce effector responses, resulting in the case of healthy  
57 individuals in a balanced homeostatic immunological state, which protects against infection  
58 and controls aberrant, tissue-damaging inflammatory responses (6).

59           One bacterial strain, which is known to induce tolerogenic responses within the gut, is  
60 *Bifidobacterium longum* subsp. *longum* **35624**<sup>TM</sup> (7). Induction of T regulatory (Treg) cells by  
61 the *B. longum* **35624** strain in mice is associated with protection against colitis, arthritis,  
62 allergic responses and pathogen-associated inflammation (8-12). Administration of this  
63 bacterium to humans increases Foxp3<sup>+</sup> lymphocytes in peripheral blood, enhances IL-10  
64 secretion *ex vivo*, and reduces the level of circulating proinflammatory biomarkers in a wide  
65 range of patient groups (13, 14). A number of host mechanisms have been described, which  
66 contribute to the anti-inflammatory activity of this microbe, including Toll-like receptor 2  
67 (TLR-2) and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-  
68 integrin (DC-SIGN) recognition, and retinoic acid release by dendritic cells (13, 15-17).  
69 However, the bacterial strain-specific structural and/or metabolic factors that contribute to  
70 these protective immune responses have as yet remained elusive.

71 A number of different exopolysaccharides from gut microbes have been shown to  
72 induce immune-modulatory effects. Polysaccharide A (PSA) from *Bacteroides fragilis*  
73 mediates the conversion of naïve CD4<sup>+</sup> T cells into Foxp3<sup>+</sup> Treg cells that produce IL-10  
74 during commensal colonization. Functional Treg cells are also induced by PSA during  
75 intestinal inflammation, which requires TLR-2 signaling (18). Further studies have reported  
76 that PSA interacts directly with mouse plasmacytoid dendritic cells via TLR-2 and that PSA-  
77 exposed plasmacytoid dendritic cells express molecules involved in protection against colitis  
78 and stimulate CD4<sup>+</sup> T cells to secrete IL-10 (19). An exopolysaccharide from *Bacillus subtilis*  
79 prevents gut inflammation stimulated by *Citrobacter rodentium*, which is dependent on TLR-  
80 4 and MyD88 signaling (20). Similarly, protection against *C. rodentium* infection by  
81 *Bifidobacterium breve* UCC2003 was dependent on the presence of its exopolysaccharide  
82 (21). Furthermore, it was described that an extracellular polymeric matrix, isolated from  
83 *Faecalibacterium prausnitzii*, displayed anti-inflammatory activity in the mouse dextran  
84 sodium sulphate colitis model (22).

85 We recently described that the *B. longum* **35624** strain-specific EPS gene cluster,  
86 designated as *eps<sub>624</sub>*, is responsible for the production of a cell surface-associated  
87 exopolysaccharide, composed of a branched hexasaccharide repeating unit with two  
88 galactoses, two glucoses, galacturonic acid and the unusual sugar 6-deoxytalose (23). The  
89 overall aim of the current study was to determine if the exopolysaccharide produced by *B.*  
90 *longum* **35624** is related with the immunoregulatory effects of this microorganism. To address  
91 this aim, we investigated if an isogenic derivative of *B. longum* **35624**, which does not  
92 produce exopolysaccharide, is able to exert similar immunological effects to its parent strain  
93 *in vitro* and in colitis and asthma mouse models.

94

95 **MATERIALS AND METHODS**

96 **Bacterial strains, plasmids and culture conditions.** Bacterial strains and plasmids used in  
97 this study are detailed in Table 1. Bifidobacteria were routinely cultured in either de Man  
98 Rogosa and Sharpe medium (MRS; Oxoid Ltd., Basingstoke, Hampshire, United Kingdom)  
99 supplemented with 0.05 % cysteine-HCl or reinforced clostridial medium (RCM; Oxoid Ltd.).  
100 Bifidobacterial cultures were incubated at 37 °C under anaerobic conditions in a Don Whitley  
101 anaerobic Chamber. *Escherichia coli* strains were cultured in Lysogeny broth (LB; Oxoid  
102 Ltd) at 37 °C with agitation. Where appropriate, growth media contained chloramphenicol  
103 (Cm; 10 µg ml<sup>-1</sup> for *E. coli* and 5 µg ml<sup>-1</sup> for *B. longum* **35624**), erythromycin (Em; 100 µg  
104 ml<sup>-1</sup> for *E. coli*), tetracycline (Tet; 10 µg ml<sup>-1</sup> for *E. coli* and 10 µg ml<sup>-1</sup> for *B. longum* **35624**),  
105 ampicillin (Amp; 100 µg ml<sup>-1</sup> for *E. coli* ) or kanamycin (Km; 50 µg ml<sup>-1</sup> for *E. coli*). All  
106 antibiotics were obtained from Sigma Aldrich, Dorset, England). The commercially available  
107 *B. longum* **35624**<sup>TM</sup> culture was provided by Alimentary Health limited (Cork, Ireland).

108 **DNA manipulations.** Chromosomal DNA was isolated from bifidobacteria as  
109 previously described (24). Minipreparation of plasmid DNA from *E. coli* or *B. longum* **35624**  
110 was achieved using the Qiaprep spin plasmid miniprep kit (Qiagen GmbH, Hilden,  
111 Germany). For *B. longum* **35624** an initial lysis step was incorporated into the plasmid  
112 isolation procedure, cells were resuspended in lysis buffer supplemented with lysozyme (30  
113 mg ml<sup>-1</sup>) and incubated at 37 °C for 30 min. Restriction enzymes and T4 DNA ligase were  
114 used according to the supplier's instructions (Roche Diagnostics, Bell Lane, East Sussex,  
115 UK). Synthetic single stranded oligonucleotide primers used in this study were obtained from  
116 Eurofins (Ebersberg, Germany) and are detailed in Table 2. Standard PCRs were performed

6

117 using TaqPCR mastermix (Qiagen), while high fidelity PCR was achieved using *PfuII*  
118 polymerase (Agilent, Santa Clara, California). *B. longum* **35624** colony PCRs were performed  
119 according to standard procedures with the addition of an initial incubation step of 95 °C for 5  
120 minutes to perform cell lysis. PCR fragments were purified using the Qiagen PCR purification  
121 kit. Following electroporation of plasmid DNA into *E.coli* strain EC101,  
122 electrotransformation of *B. longum* **35624** or sEPS<sup>neg</sup> was performed essentially as described  
123 by Maze *et al.* (25) with the following modifications. An overnight culture of *B. longum*  
124 **35624** was sub-cultured twice (first using a 2 % inoculum and then a 1 % inoculum) in MRS  
125 supplemented with 0.05 % cysteine-HCl and 0.2 M sucrose prior to inoculating (4%)  
126 modified Rogosa medium supplemented with 0.05 % cysteine-HCl, 1% (w/v) glucose and 0.2  
127 M sucrose. Bacteria were grown until the OD<sub>600</sub> had reached 0.3-0.4, after which cells were  
128 harvested by centrifugation (6,500 rpm, 10 min, and 4 °C) and washed twice using 1 mM  
129 ammonium citrate buffer (pH 6.0) supplemented with 0.5 M sucrose. An additional  
130 centrifugation step (9,800 \*g, 10 min, and 4 °C) was included to concentrate the competent  
131 cells. For electroporation 5 µl of plasmid DNA was mixed with 50 µl of competent cells,  
132 transferred into an electroporation cuvette with 0.2 cm inter-electrode distance and pulsed at  
133 2.5 kV, 25 µF and 200 Ω using a Gene Pulser II Electroporation System (Biorad, Hercules,  
134 California USA). For recovery, 800 µl of RCM supplemented with 0.05 % L-cysteine  
135 hydrochloride were added to bacteria and incubated anaerobically for 2.5 h at 37 °C.  
136 Transformations were plated on reinforced clostridial agar (RCA) plates supplemented with  
137 appropriate concentrations of relevant antibiotics and incubated 2-3 days anaerobically at 37  
138 °C. The correct orientation and integrity of all constructs was verified by PCR and subsequent  
139 DNA sequencing, which was performed at Eurofins (Ebersberg, Germany).

140           **Construction of sEPS<sup>neg</sup>**. An internal 583 bp fragments of the *pgt*<sub>624</sub> gene was  
141 amplified by PCR using *B. longum* **35624** chromosomal DNA as a template and the  
142 oligonucleotide primers *BI0342F\_HindIII* and *BI0342R\_XbaI*. The PCR product generated  
143 was ligated to pORI19, an Ori<sup>+</sup> RepA- integration plasmid (26), using the unique *HindIII* and  
144 *XbaI* restriction sites that were incorporated into the primers for the *pgt* fragment-  
145 encompassing amplicon, and introduced into *E. coli* EC101 by electroporation. Recombinant  
146 *E. coli* EC101 derivatives containing pORI19 constructs were selected on LB agar containing  
147 Em, and supplemented with X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) (40  
148  $\mu\text{g ml}^{-1}$ ) and IPTG (isopropyl- $\beta$ -D galactopyranoside) (1 mM). The expected genetic structure  
149 of the recombinant plasmid, designated pORI19-*pgt*, was confirmed by restriction mapping  
150 prior to subcloning of the Tet resistance antibiotic cassette, *tet(W)*, from pAM5 (27) as a *SacI*  
151 fragment into the unique *SacI* site on pORI19-*pgt*. The expected structure of a single  
152 representative of the resulting plasmid, designated pORI19*tet(W)*-*pgt*, was confirmed by  
153 restriction analysis. The plasmid was introduced into *E. coli* EC101 harbouring pNZ-M.1185  
154 (this is a plasmid expressing a *B. longum* **35624**-encoded methylase) by electroporation, and  
155 transformants were selected based on Em and Tet resistance. Methylation of the resulting  
156 plasmid complement of such transformants by the *M.1185* (isochizomer of *M.EcoRII*) was  
157 confirmed by their observed resistance to *EcoRII* restriction. Plasmid preparations of  
158 methylated pORI19*tet(W)*-*pgt* were introduced by electroporation into *B. longum* **35624** with  
159 subsequent selection on RCA plates supplemented with Tet.

160           **Construction of sEPS<sup>comp</sup>**. For the construction of plasmid pBC1.2-*pgt*<sub>624</sub> + *BI0343*, a  
161 DNA fragment encompassing *pgt*<sub>624</sub> plus the downstream located gene with locus tag *BI0343*  
162 and the presumed promoter region was generated by PCR amplification from chromosomal

163 DNA of *B. longum* **35624** using *PfuII* polymerase and primer combinations BI0342FSalI and  
164 BI0343EcoRI, where *SalI* or *EcoRI* restriction sites were incorporated at the 5' ends of the  
165 forward primer, and reverse primer, respectively. Amplicons were digested with *SalI*, and  
166 *EcoRI*, and ligated into similarly digested pBC1.2 prior to introduction into *E. coli* XL1blue  
167 by electroporation and subsequent selection of transformants on LB agar supplemented with  
168 Tet and Amp. The integrity of positive clones was confirmed by sequencing and one selected  
169 clone designated pBC1.2 *pgt+BI0343* was introduced into sEPS<sup>neg</sup> by electroporation with  
170 subsequent selection of transformants on RCA supplemented with Tet and Cm. The resultant  
171 sEPS<sup>neg</sup> strain harboring pBC1.2 *pgt+BI0343* was designated sEPS<sup>comp</sup>.

172 **Electron Microscopy.** After culture in MRS medium, bacteria were gently rinsed in  
173 Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being  
174 fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2  
175 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES  
176 buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double  
177 distilled water (2 min each wash) before dehydration through an ethanol series (50, 70, 96,  
178 and 100 %) for 5 min each. All fixation and washing steps were carried out at room  
179 temperature. Following dehydration, the samples were critically point dried in a POLARON  
180 E3100 critical point drier (Agar Scientific, Stansted, UK), and coated with 10 nm of  
181 gold/palladium (80/20) using a Baltec MED 020 unit (Baltec, Buchs, Liechtenstein). Bacterial  
182 preparations were examined using a Hitachi S-4700 scanning electron microscope (SEM),  
183 operated in secondary electron detection mode (3 kV, 40  $\mu$ A) and images captured with  
184 Quartz PCI (Quartz Imaging Corporation, Vancouver, Canada).

185           **Exopolysaccharide isolation.** The exopolysaccharide was isolated as previously  
186 described (23). Briefly, following harvesting of *B. longum* **35624** cells, which were grown on  
187 agar plates to minimize carryover of media components, an exopolysaccharide solution was  
188 generated by agitating the cells in PBS. The harvested exopolysaccharide solution was mixed  
189 with ethanol and the exopolysaccharide aggregated at the center of the surface of the ethanol  
190 solution, which facilitated harvesting of the exopolysaccharide without the need for  
191 centrifugation. The exopolysaccharide aggregations were taken with a spatula, resuspended in  
192 water and dialysed against water to remove contaminants and residual ethanol. The  
193 exopolysaccharide was applied 2 times on Bakerbond SPE C18 columns (Avantor, Deventer,  
194 The Netherlands) as indicated by the manufacturer using a HyperSep-96™ vacuum manifold  
195 (Thermo Scientific, Waltham, USA). The flow-through fraction was collected and filtered  
196 through 0.45 µm syringe filters. Quantification of total carbohydrate levels was performed as  
197 previously described (28) using a phenol-sulphuric acid method in microplate format. The  
198 absence of contaminating proteins was confirmed by measuring the total soluble protein  
199 content of the exopolysaccharide preparation using the BCA protein quantification kit  
200 (Thermo Scientific) according to manufacturer's instructions. Bovine serum albumin was  
201 used for generation of standards. Lipopolysaccharide contamination was monitored using the  
202 pyrogene recombinant factor C assay (Lonza, Bettlach, Switzerland).

203           **In vitro immune assays.** Human blood was purchased from the Swiss blood bank  
204 (Blutspendezentrum, Basel, Switzerland), which obtains the blood following appropriate  
205 screening and consenting of volunteers. Blood samples were anonymized and coded prior to  
206 leaving the blood bank. Research procedures on human blood were performed in accordance  
207 with Swiss law (ethical approval number KEK Nr. 19/08). All experiments with human  
208 blood-derived cells were conducted under biosafety level 2 conditions. Peripheral blood  
10

mononuclear cells (PBMCs) were isolated from healthy donors using density gradient centrifugation. Human monocyte-derived dendritic cells (MDDCs) were differentiated with 1000 IU ml<sup>-1</sup> GM-CSF (Peprotech, London, UK) and 1000 IU ml<sup>-1</sup> IL-4 (Novartis, Basel, Switzerland) from purified CD14<sup>+</sup> cells using MACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany). Bacterial strains for *in vitro* assays were cultured in MRS medium supplemented with 0.05 % L-cysteine for 48 hours under anaerobic conditions at 37°C. Cells were harvested and washed once with sterile PBS by centrifugation at 6,500 rpm for 10 minutes. Bacterial cell number was determined by microscopy using a Petroff-Hausser chamber and bacteria were diluted as appropriate in PBS for incubation with human cells. PBMCs and MDDCs were stimulated for 24 h at 37°C, 5 % CO<sub>2</sub> with bacterial strains at a concentration of 50 bacterial cells to 1 PBMC or MDDC. Human and bacterial cell co-cultures were performed in complete RPMI-1640 (cRPMI) medium (Sigma, Buchs, Switzerland) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 Units ml<sup>-1</sup>) and streptomycin (0.1 mg ml<sup>-1</sup>) (Sigma). Purified exopolysaccharide from *B. longum* **35624** was also added (final concentration 100 µg/ml) to PBMC cultures (in duplicate) stimulated with sEPS<sup>neg</sup>. Cytokine concentrations were measured using the Bio-Plex Multiplex System (Biorad). For human dendritic cells staining, the following antibodies were used: PE-Cy7 anti-human CD274 (PD-L1), APC anti-human CD273 (PD-L2) and Pacific Blue anti-human CD11c (eBioscience, Vienna, Austria). THP-1-Blue<sup>TM</sup> NF-κB monocyte cell line (Invivogen, San Diego, USA) was maintained and sub-cultured in cRPMI medium (Sigma) in presence of 200µg/ml Zeocin (Invivogen). For the co-culture experiment with bacteria, 10<sup>5</sup> cells/well were seeded in a 96 well-plate in a total volume of 200 µl/well of cRPMI medium. The cells were stimulated over a range of different bacterial concentrations for 24 h and activation of NF-κB/AP-1 pathway was evaluated by Quanti-Blue<sup>TM</sup> assay according to the manufacturer's

instructions. In addition, MDDCs were stimulated with different bacterial strains and NF- $\kappa$ B phosphorylation measured over a time course. MDDCs were lysed using Bio-Plex Pro cell signaling reagent kit (Biorad) and cell lysates were stored at -80°C until analysis. Protein concentration was determined using Bio-Rad's DC<sup>TM</sup> protein assay and equal amounts of protein were used to measure NF- $\kappa$ B p65 (Ser<sup>536</sup>) in the Bio-Plex Pro<sup>TM</sup> Magnetic Cell Signaling Assay (Biorad). Results are expressed as MFI (mean fluorescence intensity).

**T cell transfer colitis model.** C.B-17 severe combined immunodeficient (SCID and BALB/c female mice (8-12 weeks of age) were obtained from Charles River (Sulzfeld, Germany) and maintained under specific pathogen free conditions. The animals were housed at the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the duration of the study, and all experimental procedures were carried out in accordance with Swiss law (Permit number: 2013\_32). Colitogenic CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> cells were isolated from BALB/c donor mouse spleens using the MACS Miltenyi system (depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells followed by positive selection of CD45RB FITC-labeled cells). At day 0, colitis was induced by intraperitoneal transfer of  $4 \times 10^5$  cells per C.B-17 SCID mouse (8 mice per group). Bacterial cells were prepared as described above and counted using microscopy (Petroff-Hausser chamber) prior to dilution in sterile PBS.  $1 \times 10^9$  *B. longum* 35624 cells, or its isogenic derivatives, were administered to each mouse by intragastric gavage (total volume of 200  $\mu$ l). Bacteria were gavaged from the beginning of the study (day 0) and continued to be gavaged every second day until animals were euthanized at the end of the study. Sixteen days after study initiation, disease severity scores were recorded, while animal weights were monitored every day. Disease severity scores included feces condition (1 - wet; 2 - diarrhea; 3 - bloody diarrhea or rectal prolapse), activity (1 - isolated, abnormal

position; 2 - huddled, hypoactive or hyperactive; 3 – unconscious), coordination of movement (1 - slightly uncoordinated; 2 – very uncoordinated; 3 – paralysis) and fur quality (1 – reduced grooming; 2 – disheveled; 3 - hair loss). Gut transit was determined by quantifying fecal *B. longum* **35624** levels by PCR. *B. longum* **35624** specific primers were designed using Primer3 software (<http://simgene.com/Primer3>). The primers, designated 2420t (Forward: CAG TGG GGT GCG ACT ACA; Reverse: GCG CGA ACC AGA AGA TGT) generated a 494 bp amplicon. Bacterial DNA from fecal samples was extracted using QIAamp DNA Stool Mini Kit (Qiagen). DNA was quantified using Nanodrop (Thermo Scientific) and 100 ng of total DNA was assayed using SYBR Green PCR Master Mix (Biorad). The thermal cycling conditions consisted of an initial denaturation step of 15 min at 95°C, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing for 45 sec at 56°C, and extension at 72°C for 45 sec. *B. longum* **35624** DNA concentrations were quantified using the absolute quantitation protocol of the ABI 7900 Fast real-Time PCR system (Applied Biosystem, CA, USA). In a parallel experiment, BALB/c healthy mice (6 mice per group) were gavaged with *B. longum* **35624** or its isogenic derivative for 3 weeks, as described above for the colitis study.

Following euthanasia on day 26, mesenteric lymph nodes were isolated in order to obtain single cell suspensions. Lymph nodes were mechanically disrupted using a syringe plunger to grind the nodes on a nylon cell strainer (70  $\mu$ m). The strainer was washed with PBS and the single cell suspensions were centrifuged at 300 g for 10 minutes. Cell pellets were resuspended in 1 ml of cRPMI medium (Sigma) and cells were counted using a Scepter Cell Counter (Millipore, Billerica, MA, USA). Cells were diluted to a final density of  $1 \times 10^6$  cells  $\text{ml}^{-1}$  in cRPMI and cells were dispensed in propylene tubes to perform FACS staining. Lamina propria mononuclear cells were isolated as described previously (29) following removal of epithelial cells and collagenase VIII/DNaseI (Roche) digestion of the

13

280 tissue. At the end of the process, cells were counted using the Scepter Cell Counter  
281 (Millipore) and diluted to a final concentration of  $1 \times 10^6$  cells  $\text{ml}^{-1}$  in cRPMI in propylene  
282 FACS staining tubes.

283 **OVA respiratory allergy model.** Female BALB/c mice (8-12 weeks of age) were  
284 obtained from Charles River and were maintained under specific pathogen free conditions at  
285 the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the  
286 duration of the study. All experimental procedures were carried out in accordance with Swiss  
287 law (Permit number: 2013\_20). 8 mice per group were used in this model. Three  
288 intraperitoneal immunizations with 50  $\mu\text{g}$  of ovalbumin (OVA, Grade V>98%, Sigma)  
289 emulsified in Inject<sup>TM</sup> Alum Adjuvant (Life Technologies, Carlsbad, California, USA) were  
290 performed on days 0, 14 and 21, followed by OVA aerosol challenges on days 26, 27 and 28.  
291 On days 19, 25 and 27, mice received *B. longum* **35624** or sEPS<sup>neg</sup> intra-nasally ( $\sim 1 \times 10^9$   
292 bacteria per dose in a total volume of 50  $\mu\text{l}$  of PBS). Bacterial cells were prepared as  
293 described above. Control animals received three intraperitoneal injections with Alum adjuvant  
294 (without OVA) on days 0, 14 and 21, followed by OVA aerosol challenges on days 26, 27 and  
295 28. Control animals also received 50  $\mu\text{l}$  of PBS intra-nasally on days 19, 25 and 27. All mice  
296 were sacrificed at day 29 for isolation of lung tissue and flow cytometric staining. Lung-  
297 derived single cell suspensions were obtained using a combination of enzymatic digestion  
298 (lung dissociation kit, Miltenyi) and mechanical dissociation with a gentleMACS Dissociator  
299 (Miltenyi), according to the manufacturer's protocol. Lung cells were plated at  $1 \times 10^6$  cells/ml  
300 in complete RPMI (Sigma) and stimulated *ex vivo* with 50  $\mu\text{g}/\text{ml}$  OVA grade VI (Sigma) or  
301 with 500 ng/ml LPS (Sigma) for 48 hours and cytokine secretion quantified by the Bio-Plex  
302 Multiplex System (Biorad).

303           **Flow cytometry.** All flow cytometry analyses were performed on the Gallios Flow  
304 Cytometer (Beckman Coulter, Brea, USA). Mesenteric lymph node or lung single cell  
305 suspensions were stimulated with PMA and ionomycin at 50 ng ml<sup>-1</sup> and 500 ng ml<sup>-1</sup>,  
306 respectively, for 4 hours in presence of Brefeldin A (eBioscience). Viability dye eFluor780  
307 (eBioscience) and the following surface staining antibodies were used: PE-Cy7 anti-mouse  
308 CD3 and Pacific Blue anti-mouse CD4 (Biolegend, San Diego, California USA). Cells were  
309 stained for intracellular cytokines using PE anti-mouse IL-10, Alexa Fluor488 anti-mouse/rat  
310 IL-17A and PerCP-Cy5.5 anti-mouse IFN- $\gamma$  after fixation and permeabilization (Intracellular  
311 Fixation & Permeabilization Buffer Set, eBioscience). Lamina propria cells were in addition  
312 stained for the gut homing molecule CCR9 using Alexa Fluor647 anti-mouse CD199 (CCR9)  
313 from Biolegend.

314           **Statistical analysis.** Unless otherwise indicated, data are presented as box-and-  
315 whisker plots with the median value and max/min values illustrated. In experiments with  
316 technical replicates, the mean was calculated from the technical replicates for each donor and  
317 only the mean value was used for the statistical analysis. The Mann–Whitney U test was used  
318 for the nonparametric statistical analysis of differences between two groups. For analysis of  
319 more than two groups, statistical significance was determined using the Kruskal–Wallis test  
320 and Dunn’s multiple comparison test. A two-way ANOVA was used to compare groups over  
321 time. A p-value less than 0.05 was considered statistically significant.

322

## 323   **RESULTS**

324   **Generation of an isogenic exopolysaccharide-negative derivative of *B. longum* 35624,**  
325 **designated sEPS<sup>neg</sup>, by insertion mutagenesis.** In order to determine the role, if any, of the  
15

326 exopolysaccharide in the reported immunomodulatory activities of the *B. longum* **35624**  
327 strain, we set out to generate an isogenic derivative of this strain that was unable to produce  
328 this cell surface-associated glycan exopolymer. For this purpose, we employed a mutagenesis  
329 strategy that was based on previously described methods (30). The particular mutagenesis  
330 strategy employed for *B. longum* **35624** involved the heterologous expression of a *B. longum*  
331 **35624**-encoded DNA methylase in *E. coli* strain EC101 so as to methylate any plasmid DNA  
332 in this latter cloning host. When such methylated plasmid DNA is subsequently introduced  
333 into *B. longum* **35624**, it will be protected from digestion by the native restriction-  
334 modification systems encoded by the latter strain and will therefore allow homology-guided,  
335 site-directed mutagenesis as has been described previously (30). Employing this strategy, we  
336 created an insertion mutation in the first gene of the *eps*<sub>624</sub> cluster, i.e. *pgt*<sub>624</sub>, encoding the  
337 predicted priming glycosyl transferase (pGT), resulting in an isogenic derivative of *B. longum*  
338 **35624**, which was designated sEPS<sup>neg</sup>.

339 In order to assess if the sEPS<sup>neg</sup> mutant had, as would be expected, lost its ability to  
340 produce exopolysaccharide we performed electron microscopy analysis, which indeed  
341 revealed that the 'stringy' sEPS layer present on the parent strain *B. longum* **35624** is absent  
342 on EPS<sup>neg</sup>, thus confirming its exopolysaccharide-deficient phenotype (Fig. 1). Furthermore,  
343 and in contrast to the parent strain *B. longum* **35624**, the EPS<sup>neg</sup> strain exhibits a so-called  
344 dropping phenotype when grown in liquid medium (i.e. the EPS<sup>neg</sup> strain was found to  
345 sediment during growth in liquid medium, but the *B. longum* **35624** strain remained in  
346 suspension). A similar phenotype was observed for exopolysaccharide-negative variants of *B.*  
347 *breve* UCC2003 and *Bifidobacterium animalis* subs. *lactis* (21, 31), thereby substantiating the  
348 loss of exopolysaccharide production. To ensure that the observed phenotype is directly  
349 linked to the inactivation of *pgt*<sub>624</sub>, the adjacent and co-transcribed genes *pgt*<sub>624</sub> and *BI0343*

350 were cloned together with the presumed promoter sequence in plasmid pBC1.2, after which  
351 the resultant construct was introduced in the sEPS<sup>neg</sup> strain. The resulting strain, designated  
352 sEPS<sup>comp</sup>, was shown to produce exopolysaccharide (Fig. 1). A similar complementation  
353 approach was previously described (32, 33). The sEPS<sup>neg</sup> and sEPS<sup>comp</sup> mutants grew more  
354 slowly compared to the parent strain *B. longum* **35624**, likely due to the presence of  
355 antibiotics in their culture media, but by 38 hours of culture all bacteria were at similar  
356 numbers and had reached stationary phase (Supplementary Fig. S1).

357 ***In vitro* responses to *B. longum* 35624, sEPS<sup>neg</sup> and sEPS<sup>comp</sup>.** The wild-type strain  
358 *B. longum* **35624** and its derivatives sEPS<sup>neg</sup> or sEPS<sup>comp</sup> were co-incubated with human  
359 PBMCs for 24 hours, followed by analysis of cytokine secretion in cell-free supernatants. As  
360 compared with *B. longum* **35624**, the sEPS<sup>neg</sup> strain was shown to induce higher levels of IL-  
361 12p70, IFN- $\gamma$  and IL-17 secretion, with comparable induction of IL-10 (Fig. 2A). The  
362 sEPS<sup>comp</sup> strain induced similar levels of cytokine secretion as *B. longum* **35624**, confirming  
363 that enhanced pro-inflammatory cytokine secretion is specifically associated with the lack of  
364 exopolysaccharide production. The addition of isolated exopolysaccharide to the co-cultures  
365 significantly reduced IL-12p70 and IFN- $\gamma$  secretion in response to the sEPS<sup>neg</sup> strain, but did  
366 not alter IL-17 or IL-10 responses to the sEPS<sup>neg</sup> strain (Fig. 2B).

367 Similarly to PBMCs, human MDDCs were co-incubated with *B. longum* **35624** or  
368 sEPS<sup>neg</sup> strains, and cytokine secretion was measured after a 24 hour exposure. Secreted IL-  
369 17, IL-6 and TNF- $\alpha$  levels, but not IL-10, were all shown to be significantly higher for the  
370 sEPS<sup>neg</sup>-stimulated MDDCs, compared to *B. longum* **35624** -stimulated MDDCs (Fig. 3A). In  
371 contrast, no differences were found in the *B. longum* **35624** or sEPS<sup>neg</sup> -induced expression of  
372 the MDDC inhibitory molecules programmed death-ligand 1 (PD-L1) and PD-L2, which bind

373 to PD-1 on activated lymphocytes and play an important role in down-regulating the immune  
374 system (Fig. 3B).

375       Activation of the transcription factor NF- $\kappa$ B is critical for the induction of  
376 inflammatory genes, including cytokines. Thus, we measured NF- $\kappa$ B activation in the  
377 monocyte cell line THP-1, containing a SEAP reporter for NF- $\kappa$ B and AP-1 activation. The  
378 sEPS<sup>neg</sup> strain was shown to induce higher levels of NF- $\kappa$ B/AP-1 activation, compared to *B.*  
379 *longum* **35624**-stimulated THP-1 cells (Fig. 3C). To confirm this result, NF- $\kappa$ B  
380 phosphorylation was measured in MDDCs over time, following exposure to bacteria. Both *B.*  
381 *longum* **35624** and sEPS<sup>neg</sup> strains induced similar levels of NF- $\kappa$ B phosphorylation at early  
382 time points (Fig. 3D). However, sustained high levels of phosphorylated NF- $\kappa$ B were  
383 observed at later time points for the sEPS<sup>neg</sup>-stimulated MDDCs, which were not observed for  
384 *B. longum* **35624**-stimulated cells.

385       Taken together, these results suggest a role for this exopolysaccharide in preventing *in*  
386 *vitro* inflammatory responses to *B. longum* **35624**.

387       **The sEPS<sup>neg</sup> strain does not protect against colitis development.** Colitis was  
388 induced in SCID mice by adoptively transferring CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> lymphocytes. Mice  
389 were administered *B. longum* **35624**, sEPS<sup>neg</sup> or sEPS<sup>comp</sup> daily by oral gavage. As previously  
390 described, *B. longum* **35624** treatment prevented weight loss and disease symptoms in this  
391 model (34). However, mice treated with the sEPS<sup>neg</sup> strain exhibited significant weight loss  
392 and severe disease symptoms, while restoration of EPS production in the sEPS<sup>comp</sup> strain  
393 promoted a similar response as to *B. longum* **35624** (Fig. 4A). Following euthanasia, the  
394 colon:body weight ratio was significantly higher in animals administered the sEPS<sup>neg</sup> strain,

395 while macroscopically the colons of these mice appeared severely inflamed with visible  
396 necrotic regions, which was not observed when animals had been administered *B. longum*  
397 **35624** (Fig. 4B). Within the mesenteric lymph nodes, there were significantly more IL-17<sup>+</sup>  
398 lymphocytes in animals administered the sEPS<sup>neg</sup>, with a trend towards increased numbers of  
399 IFN- $\gamma$ <sup>+</sup> lymphocytes, which was not statistically significant (Fig. 4C). No significant  
400 difference in IL-10<sup>+</sup> lymphocytes was observed. No differences in the gastrointestinal transit  
401 of *B. longum* **35624** or the sEPS<sup>neg</sup> derivative was observed (Supplementary Fig. S2).

402 The administration of the sEPS<sup>neg</sup> strain to healthy immunocompetent animals did not result  
403 in gastrointestinal inflammation, indicating that the sEPS<sup>neg</sup> mutant did not induce colitis in  
404 healthy animals. However, administration of sEPS<sup>neg</sup> did provoke a trend in an increased  
405 percentage of IL-17<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> lymphocytes, associated with an increase in CCR9<sup>+</sup> T cells,  
406 within the lamina propria of healthy animals, although these differences did not reach  
407 statistical significance (Supplementary Fig. S3). These data suggest that an inflamed micro-  
408 environment, such as that present in the SCID model, is required for sEPS<sup>neg</sup> to exert its T<sub>H</sub>17-  
409 enhancing effects.

410 **sEPS<sup>neg</sup> exacerbates IL-17 responses within the lung.** In order to further assess the  
411 ability of sEPS<sup>neg</sup> to promote IL-17 responses *in vivo*, we utilized the ovalbumin (OVA)  
412 sensitization and respiratory challenge model, as we and others previously evidenced potent  
413 T<sub>H</sub>17 responses within the lungs of challenged animals (35). Either the *B. longum* **35624** or its  
414 isogenic derivative sEPS<sup>neg</sup> were administered intra-nasally to examine the influence of these  
415 strains on IL-17 responses within the lung. OVA sensitization and challenge resulted in an  
416 increased percentage of IL-17<sup>+</sup> lymphocytes within lung tissue, compared to control animals  
417 (Fig. 5A). Exposure to the *B. longum* **35624** strain did not influence the percentage of IL-17<sup>+</sup>

418 lymphocytes within the lung, however, exposure to sEPS<sup>neg</sup> significantly increased the  
419 percentage of IL-17<sup>+</sup> lymphocytes (Fig. 5A). Single cell suspensions were generated from the  
420 lungs of all animals challenged as indicated above, and these lung cells were re-stimulated *ex*  
421 *vivo* with OVA or LPS to assess IL-17 secretion. OVA sensitized and challenged animals  
422 displayed increased *ex vivo* secretion of IL-17 to both OVA and LPS stimulation, compared to  
423 non-sensitized animals, suggesting that innate TLR-4 responses to LPS and allergen-specific  
424 lymphocyte responses to OVA are increased in the inflamed lungs of allergic animals (Fig.  
425 5A). The *in vivo* exposure to *B. longum* **35624** did not alter the *ex vivo* secretion of IL-17 by  
426 lung cells stimulated with either LPS or OVA. However, if animals had been exposed to  
427 sEPS<sup>neg</sup> *in vivo* previously, the isolated lung cells secreted significantly more IL-17 *ex vivo* in  
428 response to both TLR-4 stimulation with LPS and allergen restimulation with OVA (Fig. 5A).  
429 Thus, *ex vivo* secretion of IL-17 and the percentage of IL-17<sup>+</sup> cells within lung tissue correlate  
430 with the highest levels for both assay systems being observed for sEPS<sup>neg</sup>-treated animals.

431 OVA sensitization and challenge resulted in an increased percentage of IFN- $\gamma$ <sup>+</sup>  
432 lymphocytes within lung tissue, compared to control animals (Fig. 5A). Exposure to the *B.*  
433 *longum* **35624** strain prevented the increase in the percentage of IFN- $\gamma$ <sup>+</sup> lymphocytes within  
434 the lung, which was not observed following exposure to the sEPS<sup>neg</sup> strain (Fig. 5B). Re-  
435 stimulation of lung single cell suspensions *ex vivo* with OVA or LPS did not result in  
436 significant levels of IFN- $\gamma$  being secreted and no statistically significant differences were  
437 observed between the groups (Fig. 5B).

438 OVA sensitization and challenge resulted in an increased percentage of IL-10<sup>+</sup>  
439 lymphocytes within lung tissue and exposure to *B. longum* **35624** or the sEPS<sup>neg</sup> strains  
440 further increased the percentage of IL-10<sup>+</sup> lymphocytes within the lung (Fig. 5C). Re-

441 stimulation *ex vivo* was also associated with increased secretion of IL-10 following *in vivo*  
442 exposure to *B. longum* **35624** or the sEPS<sup>neg</sup> strains (Fig. 5C).

443 These findings suggest that the absence of the exopolysaccharide on *B. longum* **35624**  
444 promotes T<sub>H</sub>17 responses in the inflamed lung, similar to the effects described above for the  
445 inflamed gut.

446

## 447 DISCUSSION

448 In order to avoid immune-mediated destruction of mucosal tissues, the host can activate  
449 regulatory mechanisms that can block proinflammatory responses to commensal microbes  
450 present on mucosal surfaces. Bifidobacteria comprise a significant proportion of the gut  
451 microbiota and many strains are currently used as probiotics. However, the precise  
452 mechanisms by which such bifidobacteria interact with host immune cells are not fully  
453 understood. In this report we describe that the presence of a cell surface-associated  
454 exopolysaccharide produced by *B. longum* **35624** modulates cytokine secretion and NF- $\kappa$ B  
455 activation *in vitro*, while in murine models exposure to a *B. longum* **35624** derivative unable  
456 to synthesize exopolysaccharide promotes T<sub>H</sub>17 responses both within the gut and the lung.

457 Bifidobacterial cell surface-associated exopolysaccharides have previously been  
458 proposed to (i) mediate some of their health-promoting benefits, (ii) contribute to their  
459 tolerance of the harsh conditions within the gut, and (iii) to influence composition of the gut  
460 microbiome through their use as a fermentable substrate by other microbes (36-39). In  
461 general, bacterial exopolysaccharide consists of repeating mono- or oligosaccharide subunits  
462 connected by varying glycosidic linkages, which are structurally very diverse, and which may

463 contribute to strain-specific traits due to the expected structural and therefore functional  
464 diversity of such molecules. Of note, pathogen-associated exopolysaccharides have long been  
465 known to be critical in host–microbe interactions, where they facilitate adherence and  
466 colonization within the human host, with additional immunomodulatory effects (40, 41).  
467 Exopolysaccharides can also mediate the beneficial immune effects associated with certain  
468 commensal microbes. As already mentioned, a strong modulator of intestinal immune  
469 responses is PSA from *B. fragilis*, which is well described to influence lymphocyte  
470 polarization and PSA suppresses IL-17 production by intestinal immune cells (42-44). In line  
471 with data presented in this manuscript, exopolysaccharide gene knockout mutants of  
472 *Lactobacillus casei* Shirota induced significantly more pro-inflammatory cytokine secretion  
473 from a mouse macrophage cell line, compared to wild-type cells (45). In addition, the  
474 cytokine response of PBMCs to two isogenic strains of *B. animalis* subsp. *lactis* that differ  
475 only in their exopolysaccharide-producing phenotype suggest that the mucoid strain could  
476 have higher anti-inflammatory activity (31). The data presented in this manuscript are in  
477 agreement with these previous reports and further supports the concept that  
478 exopolysaccharides from bifidobacteria may elicit immune-modulatory activities.

479         Interestingly, the induction of PD-L1 and PD-L2 on dendritic cells was similar for the  
480 wild type *B. longum* **35624** strain and its isogenic derivative sEPS<sup>neg</sup>. Similarly, the induction  
481 of IL-10 was not negatively impacted by the loss of exopolysaccharide from the bacterium.  
482 This suggests that not all immune-regulatory effects induced by *B. longum* **35624** are  
483 mediated solely by exopolysaccharide. The bifidobacterial cell wall is a complex arrangement  
484 of macromolecules, consisting of a thick peptidoglycan layer that surrounds the cytoplasmic  
485 membrane, which is decorated with other glycopolymers, such as (lipo)teichoic acids,  
486 polysaccharides and proteins, all of which may influence the immune response (46, 47) A few

487 examples include the cell wall-associated proteins p40 and p75 from *L. casei* ssp. *rhamnosus*  
488 GG, the S-layer protein from *L. acidophilus*, or the STp peptide from *L. plantarum* (48-50) .

489  $T_H17$  cells are a subset of CD4<sup>+</sup> T helper cells that mediate protective immunity to  
490 extracellular bacterial and fungal pathogens, predominantly at epithelial surfaces (51).  
491 Polarization of naïve T cells into  $T_H17$  cells occurs following T-cell antigen receptor  
492 recognition of an MHC class II-bound peptide in the presence of cytokines including TGF- $\beta$ 1,  
493 IL-6 or IL-1 $\beta$  (52, 53). While  $T_H17$  cells are required for protective immunity, these cells  
494 massively infiltrate the inflamed intestine of inflammatory bowel disease patients, where they  
495 produce IL-17 and other cytokines, triggering and amplifying the inflammatory process (54).  
496 Our data suggests that the *B. longum* **35624** strain-associated exopolysaccharide prevents the  
497 induction of a  $T_H17$  response to this bacterium. Multiple mechanisms may be involved in this  
498 process. For example the exaggerated induction of cytokines, including IL-6, from dendritic  
499 cells may support  $T_H17$  lymphocyte polarization and development. Support for this  
500 hypothesis can be seen when we restimulate OVA-specific T cells with OVA and we observe  
501 increased secretion of IL-17 when the lungs were previously exposed to sEPS<sup>neg</sup>. These OVA-  
502 specific T cells are not reacting to bifidobacteria-associated antigens, but more IL-17 is  
503 secreted upon OVA challenge suggesting that it is the cytokine microenvironment, provided  
504 by innate cells such as dendritic cells, that is supporting excessive  $T_H17$  development. The  
505 observation that addition of purified exopolysaccharide to sEPS<sup>neg</sup>-stimulated PBMCs  
506 suppresses the exaggerated IL-12p70 and IFN- $\gamma$  secretion, but not IL-17 secretion, also  
507 suggests that multiple mechanisms may be involved. Future studies will determine if it is the  
508 exopolysaccharide itself that can directly inhibit  $T_H17$  responses by binding to host receptors,

509 or if the exopolysaccharide is simply masking T<sub>H</sub>17-promoting molecules on the surface of  
510 this bacterium.

511 In conclusion, we have identified a novel immunoregulatory activity associated with  
512 the presence of a exopolysaccharide in the human commensal *B. longum* **35624** strain. Our  
513 findings suggest that this exopolysaccharide is required to prevent a potent tissue-damaging  
514 T<sub>H</sub>17 response to a commensal bacterium. Accordingly, our data on the *B. longum* 35624-  
515 associated exopolysaccharide corroborates, and expands, the published concept that  
516 exopolysaccharides produced by certain lactic acid bacteria and bifidobacteria may elicit  
517 immune-modulatory activities (55), which are important for appropriate host-microbe  
518 communication.

519

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529

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532

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712 **FIGURE LEGENDS**

713 **Figure 1.** *B. longum* **35624** electron microscopy

714 Representative scanning electron microscopy (SEM) images for the *B. longum* **35624** parent  
715 strain (A, upper panel) and its isogenic derivatives, sEPS<sup>neg</sup> mutant (B, middle panel) and  
716 sEPS<sup>comp</sup> mutant (C, bottom panel) are illustrated. Arrows indicate the ‘stringy’ layer of  
717 extracellular polysaccharide visible on the *B. longum* **35624** parent strain and sEPS<sup>comp</sup> strain.  
718 Scale bars are indicated at the bottom right of each panel.

719

720 **Figure 2.** PBMC cytokine response to bacterial strains

721 (A) PBMCs from 6 healthy donors were stimulated with *B. longum* **35624** or its isogenic  
722 derivatives sEPS<sup>neg</sup> or sEPS<sup>comp</sup> (50 bacteria:1 PBMC) for 24 hours and cytokine secretion  
723 into the culture supernatant was quantified. Data are presented as box-and-whisker plots with  
724 the median value and max/min values illustrated. Statistical significance was determined  
725 using the Kruskal–Wallis test and Dunn’s multiple comparison test (\*p<0.05). (B) Effect of  
726 adding isolated exopolysaccharide on sEPS<sup>neg</sup> strain-induced PBMC secretion of IL-12p70,  
727 IFN-gamma, IL-17 and IL-10. Each line connects the data from the same donor. The Mann–  
728 Whitney U test was used for the statistical analysis (\*p<0.05 versus the sEPS<sup>neg</sup> strain alone).

729

730 **Figure 3.** MDDC response to bacterial strains

731 MDDCs were generated from 4 healthy donors and were stimulated with *B. longum* **35624** or  
732 its isogenic derivative sEPS<sup>neg</sup> (50 bacteria:1 MDDC) for 24 hours. Cytokine secretion into

733 the culture supernatant (A) and cell surface expression of the inhibitory molecules PD-L1 or  
734 PD-L2 (B) were quantified. Data are presented as box-and-whisker plots with the median  
735 value and max/min values illustrated. The Mann–Whitney U test was used for the statistical  
736 analysis (\* $p < 0.05$  *B. longum* **35624** versus the sEPS<sup>neg</sup> strain). (C) THP-1 NF- $\kappa$ B activation  
737 following exposure to increasing concentrations of *B. longum* **35624** or its isogenic derivative  
738 sEPS<sup>neg</sup> (n=4 experimental replicates). (D) Activation of NF- $\kappa$ B in MDDCs exposed to *B.*  
739 *longum* **35624** or its isogenic derivative sEPS<sup>neg</sup> (n=3, 50 bacteria:1 MDDC) . Statistical  
740 significance was determined using two-way ANOVA (\* $p < 0.05$  *B. longum* **35624** versus the  
741 sEPS<sup>neg</sup> strain).

742

743 **Figure 4.** sEPS<sup>neg</sup> is not protective in a T cell transfer colitis model

744 Following receipt of CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> T cells, C.B-17 SCID mice were orally  
745 administered *B. longum* **35624** (n=8), sEPS<sup>comp</sup> (n=8) or sEPS<sup>neg</sup> (n=8) strains. (A) Weight  
746 loss and disease activity were monitored over time. Statistical significance was determined  
747 using two-way ANOVA (\* $p < 0.05$ ). (B) Following euthanasia, the colon:body weight ratio  
748 was determined. A representative picture of colons from *B. longum* **35624** or sEPS<sup>neg</sup> –treated  
749 animals is provided. (C) The percentage of IL-17<sup>+</sup>, IFN- $\gamma$ <sup>+</sup> and IL-10<sup>+</sup> lymphocytes from  
750 mesenteric lymph nodes are illustrated (n=8 per group). Data are presented as box-and-  
751 whisker plots with the median value and max/min values illustrated. Statistical significance  
752 was determined using the Kruskal–Wallis test and Dunn’s multiple comparison test (\* $p < 0.05$   
753 sEPS<sup>neg</sup> strain versus the other strains).

754

755 **Figure 5.** sEPS<sup>neg</sup> promotes T<sub>H</sub>17 responses in the lung

756 Non-sensitized animals received an OVA aerosol challenge and were intranasally

757 administered PBS (Control, n=8). Sensitized animals received an OVA aerosol challenge and

758 were intranasally administered PBS (OVA, n=8), or intranasally administered *B. longum*

759 **35624** (OVA & 35624, n=8), or intranasally administered sEPS<sup>neg</sup> (OVA & sEPS<sup>neg</sup>, n=8).

760 (A) The percentage of IL-17<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes, isolated from lung tissue, and

761 secretion of IL-17 from isolated lung cells re-stimulated *ex vivo* with OVA or LPS. Similarly,

762 IFN- $\gamma$ <sup>+</sup> and IL-10<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes and *ex vivo* IFN- $\gamma$  and IL-10 secretion were

763 quantified using identical methods, (B) and (C) respectively. Data are presented as box-and-

764 whisker plots with the median value and max/min values illustrated. Statistical significance

765 was determined using the Kruskal–Wallis test and Dunn’s multiple comparison test (\*p<0.05

766 compared to the OVA group).

767 **Table 1.** Bacterial strains and plasmids used in this study.

| <i>Strain or plasmid</i>                          | <i>Relevant characteristics</i>                                                                                                      | <i>Reference or source</i>               |
|---------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------|
| <b>Strains</b>                                    |                                                                                                                                      |                                          |
| <i>E. coli</i> EC101                              | Cloning host, repA <sup>-</sup> , Km <sup>r</sup>                                                                                    | Law <i>et al.</i> , 1995 (26)            |
| <i>E. coli</i> XL1blue                            | Cloning host, , Tet <sup>r</sup>                                                                                                     | Stratagene                               |
| <i>E. coli</i> EC101 pNZ-M.1185                   | <i>E. coli</i> EC101 harbouring pNZ-M.1185                                                                                           | This study                               |
| <i>B. longum</i> 35624                            | Parent strain                                                                                                                        | Alimentary Health                        |
| sEPS <sup>neg</sup>                               | <i>B. longum</i> <b>35624</b> harbouring insertion mutation in priming glycosyl transferase encoding gene, <i>pgt</i> <sub>624</sub> | This study                               |
| sEPS <sup>comp</sup>                              | sEPS <sup>neg</sup> harbouring pBC1.2_ <i>pgt</i> <sub>624</sub> + <i>BI0343</i>                                                     | This study                               |
| <b>Plasmids</b>                                   |                                                                                                                                      |                                          |
| pNZEm                                             | Gene expression vector, Em <sup>r</sup>                                                                                              | Margolles <i>et al.</i> , 2006 (56)      |
| pORI19                                            | Em <sup>r</sup> , repA <sup>-</sup> , ori <sup>+</sup> , cloning vector                                                              | Law <i>et al.</i> , 1995 (25)            |
| pORI19tet(w)_ <i>pgt</i>                          | pORI19 harbouring internal fragment of <i>BI0342</i> ( <i>pgt</i> ) and <i>tetW</i> gene                                             | This study                               |
| pBC1.2                                            | <i>E. coli</i> –Bifidobacterial shuttle vector                                                                                       | Álvarez-Martín <i>et al.</i> , 2007 (27) |
| pBC1.2_ <i>pgt</i> <sub>624</sub> + <i>BI0343</i> | pBC1.2 harbouring the cotranscribed genes <i>pgt</i> <sub>624</sub> + <i>BI0343</i> under the control of their native promoter       | This study                               |
| pNZ-M.1185                                        | <i>BI1185</i> ( <i>M.1185</i> , isoschizomer of M.EcoRII) cloned with its own promoter in pNZEM                                      | This study                               |

768

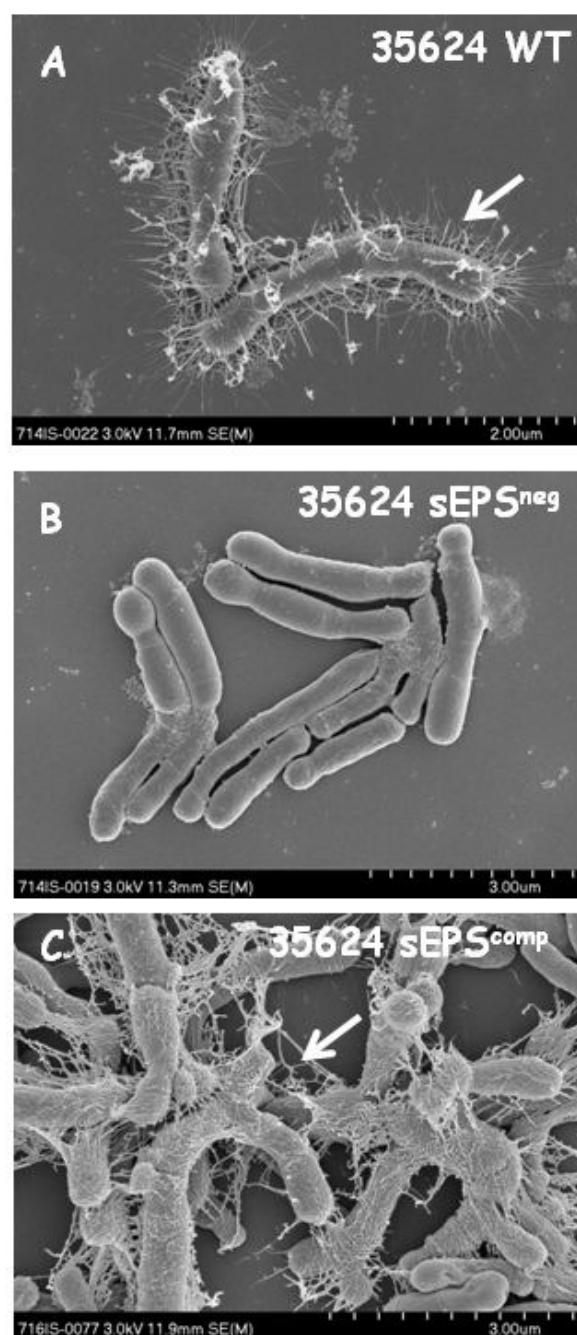
769

770 **Table 2.** Oligonucleotide primers used in this study are described.

| <i>Purpose</i>                                                 | <i>Primer</i>   | <i>Sequence<sup>a</sup></i>              |
|----------------------------------------------------------------|-----------------|------------------------------------------|
| Cloning of M.1185 in<br>pNZEm                                  | BI1185F_PstI    | GACT <b>GCAGGCC</b> CACTAGGTAACCAAACG    |
|                                                                | BI1185R_SpeI    | GCGCACT <b>AGT</b> CTAGAGCAAAGCCAGTATAG  |
| Cloning of internal 583 bp<br>fragment of <i>pgt</i> in pORI19 | BI0342F_HindIII | GATA <b>AAGCTT</b> GCGTCGGCAACTCAACTACC  |
|                                                                | BI0342R_XbaI    | GATT <b>CTAGAC</b> GTCGGCGTTCCTACTACCATC |
| Cloning of <i>pgt</i> <sub>624</sub> +BI0343<br>in pBC1.2      | BI0342FSalI     | GAC <b>GTCGAC</b> ACTCCACTCTCGCTGATCG    |
|                                                                | BI0343EcoRI     | GGC <b>GAATTCT</b> AATCAACCAAGGGGGTCTG   |

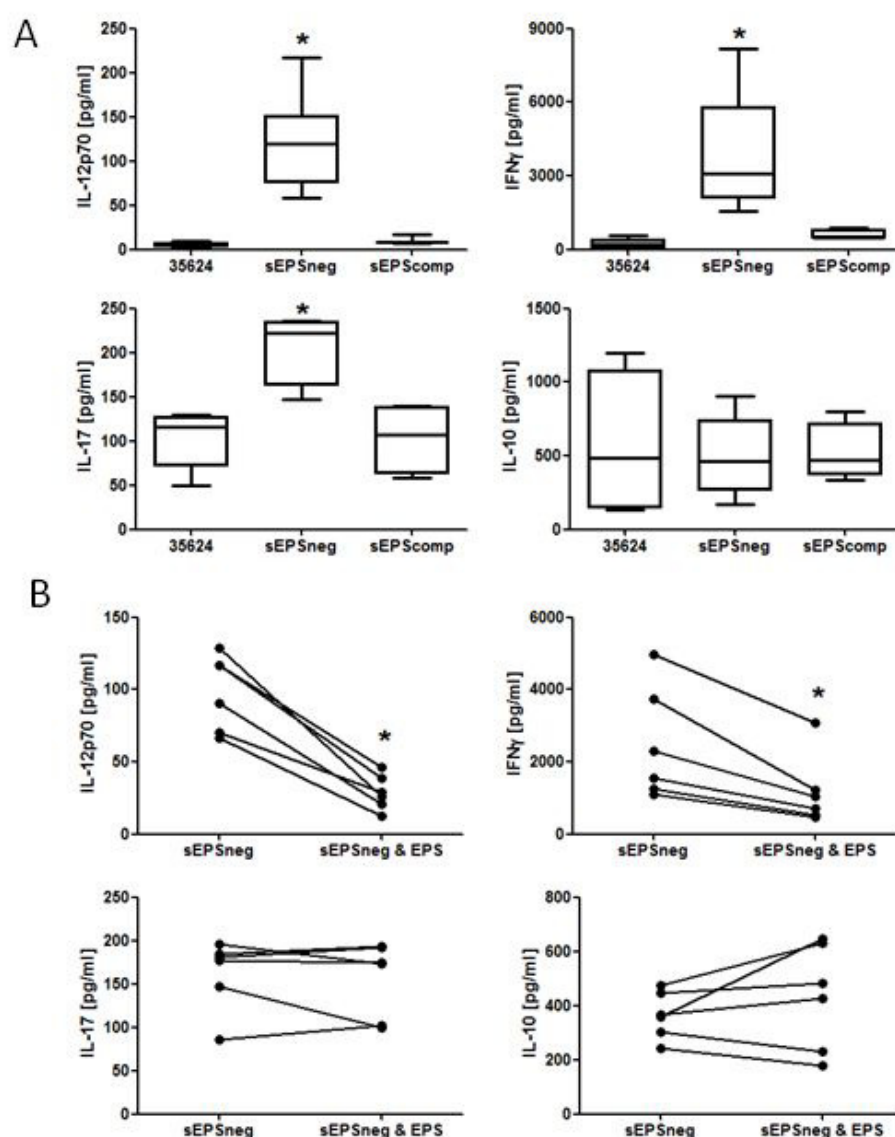
771 <sup>a</sup> Restriction sites incorporated into oligonucleotide primer sequences are indicated in bold

Figure 1

**Figure 1.** *B. longum* 35624 electron microscopy

Representative scanning electron microscopy (SEM) images for the *B. longum* 35624 parent strain (A, upper panel) and its isogenic derivatives, sEPS<sup>neg</sup> mutant (B, middle panel) and sEPS<sup>comp</sup> mutant (C, bottom panel) are illustrated. Arrows indicate the 'stringy' layer of extracellular polysaccharide visible on the *B. longum* 35624 parent strain and sEPS<sup>comp</sup> strain. Scale bars are indicated at the bottom right of each panel.

Figure 2

**Figure 2.** PBMC cytokine response to bacterial strains

(A) PBMCs from 6 healthy donors were stimulated with *B. longum* 35624 or its isogenic derivatives sEPS<sup>neg</sup> or sEPS<sup>comp</sup> (50 bacteria: 1 PBMC) for 24 hours and cytokine secretion into the culture supernatant was quantified. Data are presented as box-and-whisker plots with the median value and max/min values illustrated. Statistical significance was determined using the Kruskal-Wallis test and Dunn's multiple comparison test (\* $p < 0.05$ ). (B) Effect of adding isolated exopolysaccharide on sEPS<sup>neg</sup> strain-induced PBMC secretion of IL-12p70, IFN- $\gamma$ , IL-17 and IL-10. Each line connects the data from the same donor. The Mann-Whitney U test was used for the statistical analysis (\* $p < 0.05$  versus the sEPS<sup>neg</sup> strain alone).

Figure 3

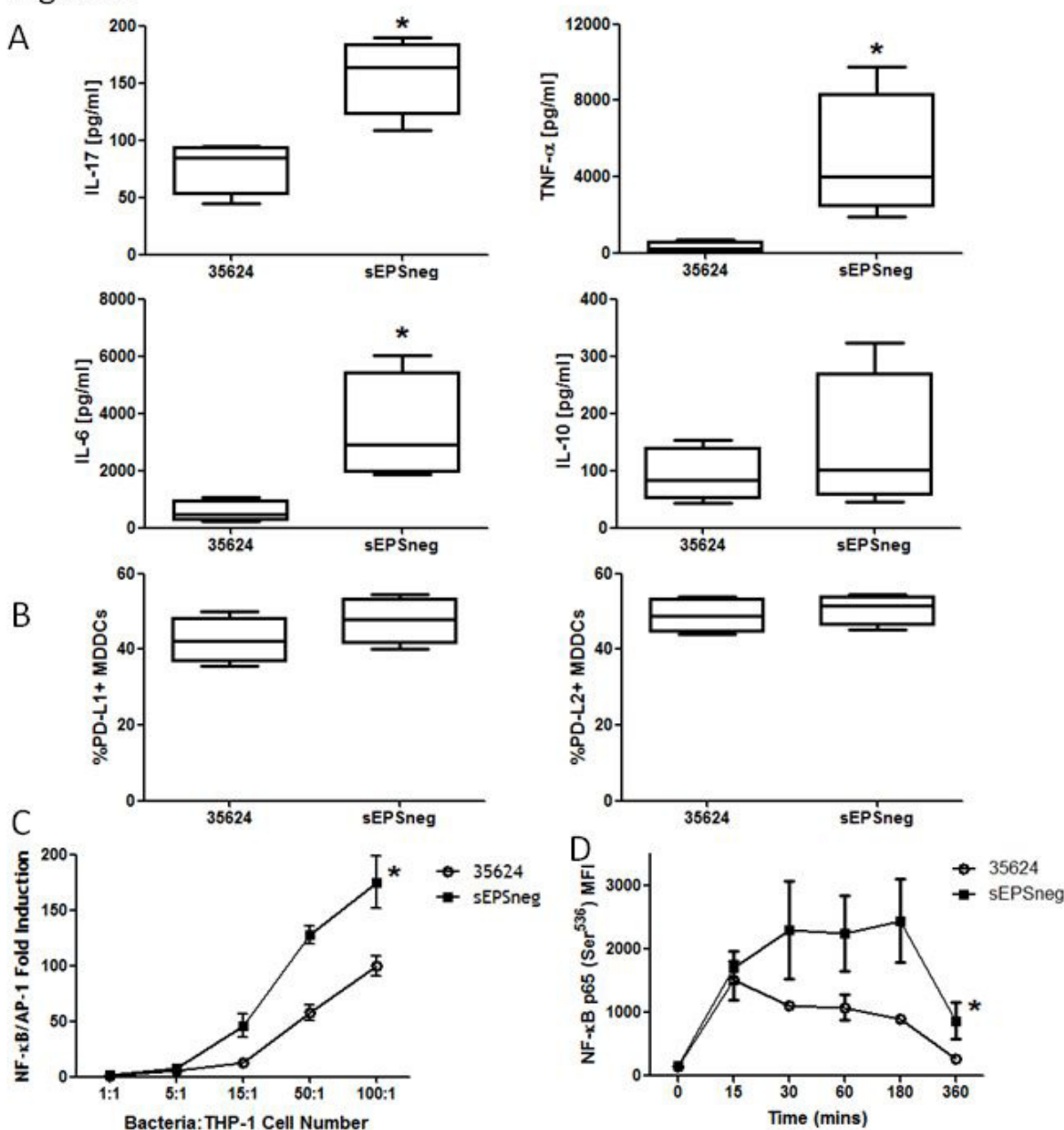


**Figure 3.** MDDC response to bacterial strains

MDDCs were generated from 4 healthy donors and were stimulated with *B. longum* 35624 or its isogenic derivative sEPS<sup>neg</sup> (50 bacteria:1 MDDC) for 24 hours. Cytokine secretion into the culture supernatant (A) and cell surface expression of the inhibitory molecules PD-L1 or PD-L2 (B) were quantified. Data are presented as box-and-whisker plots with the median value and max/min values illustrated. The Mann-Whitney U test was used for the statistical analysis (\* $p < 0.05$  *B. longum* 35624 versus the sEPS<sup>neg</sup> strain). (C) THP-1 NF-κB activation following exposure to increasing concentrations of *B. longum* 35624 or its isogenic derivative sEPS<sup>neg</sup> ( $n = 4$  experimental replicates). (D) Activation of NF-κB in MDDCs exposed to *B. longum* 35624 or its isogenic derivative sEPS<sup>neg</sup> ( $n = 3$ , 50 bacteria:1 MDDC). Statistical significance was determined using two-way ANOVA (\* $p < 0.05$  *B. longum* 35624 versus the sEPS<sup>neg</sup> strain).

Figure 4

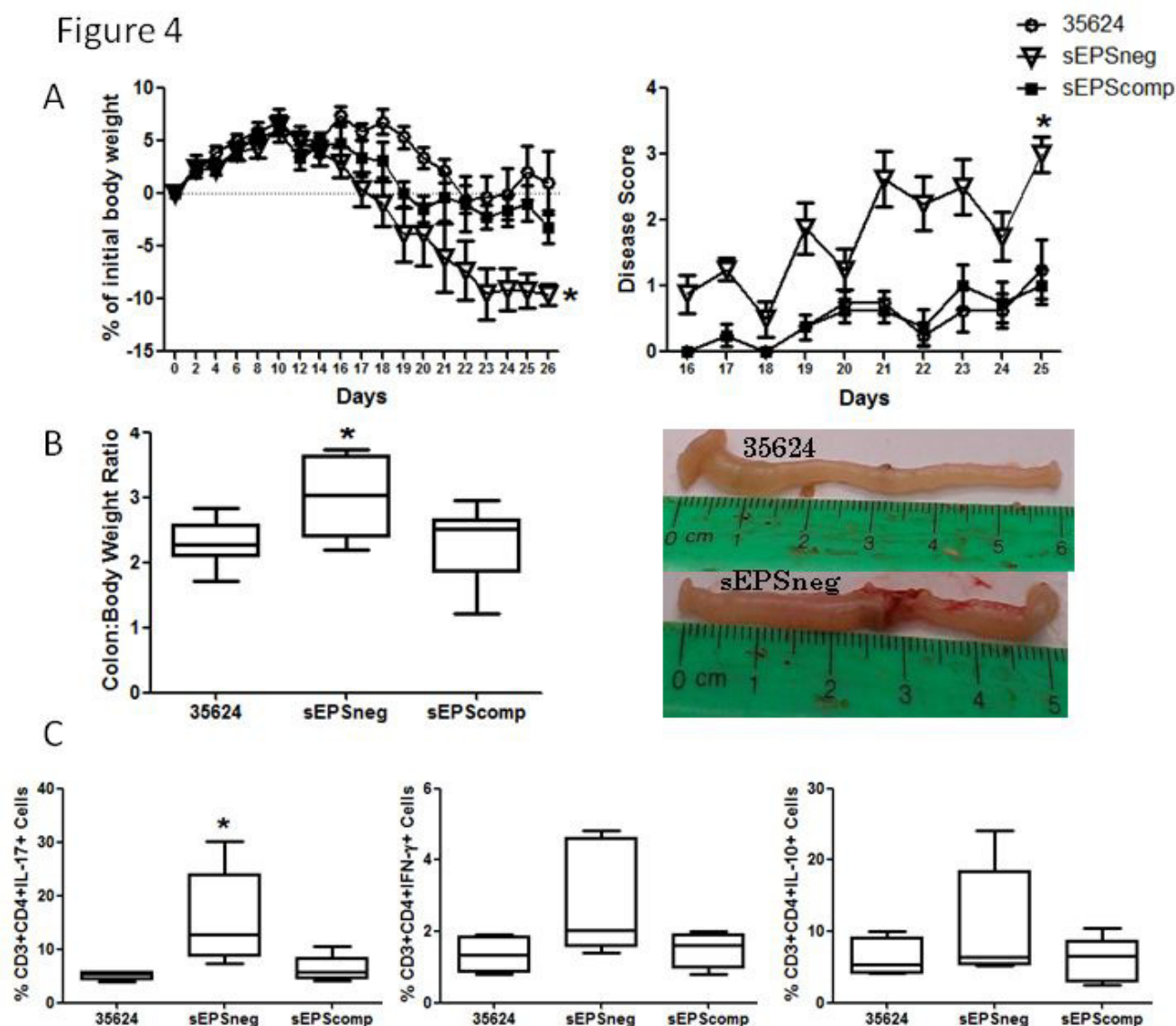
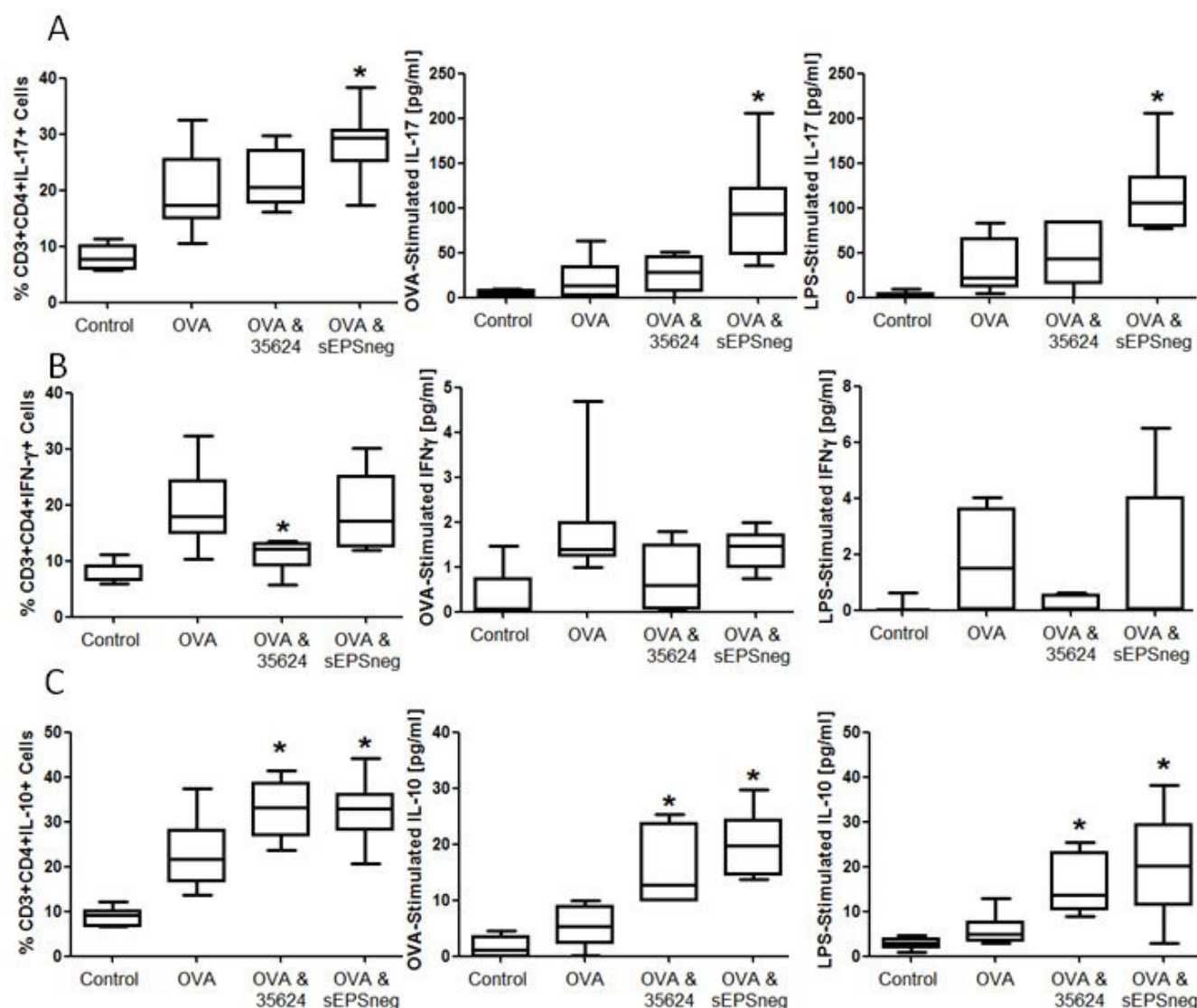


**Figure 4.** sEPS<sup>neg</sup> is not protective in a T cell transfer colitis model

Following receipt of CD4<sup>+</sup>CD25<sup>-</sup>CD45RB<sup>hi</sup> T cells, C.B-17 SCID mice were orally administered *B. longum* 35624 (n=8), sEPS<sup>comp</sup> (n=8) or sEPS<sup>neg</sup> (n=8) strains. (A) Weight loss and disease activity were monitored over time. Statistical significance was determined using two-way ANOVA (\*p<0.05). (B) Following euthanasia, the colon:body weight ratio was determined. A representative picture of colons from *B. longum* 35624 or sEPS<sup>neg</sup>-treated animals is provided. (C) The percentage of IL-17<sup>+</sup>, IFN- $\gamma$ <sup>+</sup> and IL-10<sup>+</sup> lymphocytes from mesenteric lymph nodes are illustrated (n=8 per group). Data are presented as box-and-whisker plots with the median value and max/min values illustrated. Statistical significance was determined using the Kruskal-Wallis test and Dunn's multiple comparison test (\*p<0.05 sEPS<sup>neg</sup> strain versus the other strains).

Figure 5

**Figure 5.** sEPS<sup>neg</sup> promotes T<sub>H</sub>17 responses in the lung

Non-sensitized animals received an OVA aerosol challenge and were intranasally administered PBS (Control, n=8). Sensitized animals received an OVA aerosol challenge and were intranasally administered PBS (OVA, n=8), or intranasally administered *B. longum* 35624 (OVA & 35624, n=8), or intranasally administered sEPS<sup>neg</sup> (OVA & sEPS<sup>neg</sup>, n=8). (A) The percentage of IL-17<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes, isolated from lung tissue, and secretion of IL-17 from isolated lung cells re-stimulated *ex vivo* with OVA or LPS. Similarly, IFN- $\gamma$ <sup>+</sup> and IL-10<sup>+</sup> CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes and *ex vivo* IFN- $\gamma$  and IL-10 secretion were quantified using identical methods, (B) and (C) respectively. Data are presented as box-and-whisker plots with the median value and max/min values illustrated. Statistical significance was determined using the Kruskal-Wallis test and Dunn's multiple comparison test (\*p<0.05 compared to the OVA group).