

Title	The surface-associated exopolysaccharide of Bifidobacterium longum 35624 plays an essential role in dampening host proinflammatory responses and repressing local TH17 responses
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- 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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19 ABSTRACT

20	The immune modulating properties of certain bifidobacterial strains, such as Bifidobacterium
21	<i>longum</i> subsp. <i>longum</i> 35624TM (<i>B. longum</i> 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 <i>B. longum</i> 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 ^{neg}) induced vastly more
28	cytokines, including IL-17, which was reversed when exopolysaccharide production was
29	restored in sEPS ^{neg} by genetic complementation. Administration of <i>B. longum</i> 35624 to the T
30	cell transfer colitis model prevented disease symptoms, whereas sEPS ^{neg} 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 ^{neg} 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 $T_H 17$ responses.
37	IMPORTANCE

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- 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 be2

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.

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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 TM (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+ 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⁺ T cells into Foxp3⁺ 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⁺ 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,

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86 designated as eps_{624} , 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.

MATERIALS AND METHODS Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in

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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 (Cm; 10 μ g ml⁻¹ for *E. coli* and 5 μ g ml⁻¹ for *B. longum* **35624**), erythromycin (Em; 100 μ g 103 ml⁻¹ for *E. coli*), tetracycline (Tet; 10 μ g ml⁻¹ for *E. coli* and 10 μ g ml⁻¹ for *B. longum* **35624**), 104 ampicillin (Amp; 100 µg ml⁻¹ for *E. coli*) or kanamycin (Km; 50 µg ml⁻¹ for *E. coli*). All 105 106 antibiotics were obtained from Sigma Aldrich, Dorset, England). The commercially available B. longum 35624[™] culture was provided by Alimentary Health limited (Cork, Ireland). 107 108 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⁻¹) 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 $^\circ C$ 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 <i>B. longum</i> 35624 or sEPS ^{neg} 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_{600} had reached 0.3-0.4, after which cells were
128	harvested by centrifugation (6,500 rpm, 10 min, and 4 $^{\circ}$ 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 $^{\circ}$ C) was included to concentrate the competent
131	cells. For electroporation 5 μ l of plasmid DNA was mixed with 50 μ l of competent cells,
132	transferred into an electroporation cuvette with 0.2 cm inter-electrode distance and pulsed at
133	$2.5~kV, 25~\mu F$ and 200 Ω using a Gene Pulser II Electroporation System (Biorad, Hercules,
134	California USA). For recovery, 800 μ l of RCM supplemented with 0.05 % L-cysteine
135	hydrochloride were added to bacteria and incubated anaerobically for 2.5 h at 37 $^{\circ}$ 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	$^{\circ}\text{C}.$ The correct orientation and integrity of all constructs was verified by PCR and subsequent
139	DNA sequencing, which was performed at Eurofins (Ebersberg, Germany).

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 ⁺ RepA- integration plasmid (26), using the unique <i>HindIII</i> and
144	Xbal 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- β -D-galactopyranoside) (40
148	μ g ml ⁻¹) and IPTG (isopropyl- β -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 pORI19tet(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 <i>B. longum</i> 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 (isoschizomer of M.EcoRII) was
157	confirmed by their observed resistance to EcoRII restriction. Plasmid preparations of
158	methylated pORI19 <i>tet(W)_pgt</i> were introduced by electroporation into <i>B. longum</i> 35624 with
159	subsequent selection on RCA plates supplemented with Tet.
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Construction of sEPS^{comp}. For the construction of plasmid pBC1.2_ pgt_{624} +BI0343, a 160

Construction of sEPS^{neg}. An internal 583 bp fragments of the *pgt*₆₂₄ gene was

- 161 DNA fragment encompassing pgt₆₂₄ 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 ^{neg} by electroporation with
170	subsequent selection of transformants on RCA supplemented with Tet and Cm. The resultant
171	sEPS ^{neg} strain harboring pBC1.2 $pgt+BI0343$ was designated sEPS ^{comp} .
172	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in
172 173	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being
172 173 174	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2
 172 173 174 175 	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES
 172 173 174 175 176 	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double
 172 173 174 175 176 177 	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double distilled water (2 min each wash) before dehydration through an ethanol series (50, 70, 96,
 172 173 174 175 176 177 178 	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double distilled water (2 min each wash) before dehydration through an ethanol series (50, 70, 96, and 100 %) for 5 min each. All fixation and washing steps were carried out at room
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 172 173 174 175 176 177 178 179 180 	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double distilled water (2 min each wash) before dehydration through an ethanol series (50, 70, 96, and 100 %) for 5 min each. All fixation and washing steps were carried out at room temperature. Following dehydration, the samples were critically point dried in a POLARON E3100 critical point drier (Agar Scientific, Stansted, UK), and coated with 10 nm of
172 173 174 175 176 177 178 179 180 181	Electron Microscopy. After culture in MRS medium, bacteria were gently rinsed in Piperazine-N,N-bis-2-ethane sulphonic acid (PIPES) buffer (0.1 M, pH 7.4) before being fixed in 2.5 % glutaraldehyde in PIPES buffer for 5 min. The samples were rinsed twice (2 min each time) in PIPES buffer and post-fixed with 1 % osmium tetroxide in 0.1 M PIPES buffer (pH 6.8), for 60 min in the dark. The samples were rinsed three times in double distilled water (2 min each wash) before dehydration through an ethanol series (50, 70, 96, and 100 %) for 5 min each. All fixation and washing steps were carried out at room temperature. Following dehydration, the samples were critically point dried in a POLARON E3100 critical point drier (Agar Scientific, Stansted, UK), and coated with 10 nm of 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 μ A) and images captured with
- 184 Quartz PCI (Quartz Imaging Corporation, Vancouver, Canada).
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agar plates to minimize carryover of media components, an exopolysaccharide solution was
generated by agitating the cells in PBS. The harvested exopolysaccharide solution was mixed
with ethanol and the exopolysaccharide aggregated at the center of the surface of the ethanol
solution, which facilitated harvesting of the exopolysaccharide without the need for
centrifugation. The exopolysaccharide aggregations were taken with a spatula, resuspended in
water and dialysed against water to remove contaminants and residual ethanol. The
exopolysaccharide was applied 2 times on Bakerbond SPE C18 columns (Avantor, Deventer,
The Netherlands) as indicated by the manufacturer using a HyperSep-96 [™] vacuum manifold
(Thermo Scientific, Waltham, USA). The flow-through fraction was collected and filtered
through 0.45 μ m syringe filters. Quantification of total carbohydrate levels was performed as
previously described (28) using a phenol-sulphuric acid method in microplate format. The
absence of contaminating proteins was confirmed by measuring the total soluble protein
content of the exopolysaccharide preparation using the BCA protein quantification kit
(Thermo Scientific) according to manufacturer's instructions. Bovine serum albumin was
used for generation of standards. Lipopolysaccharide contamination was monitored using the
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

Exopolysaccharide isolation. The exopolysaccharide was isolated as previously

described (23). Briefly, following harvesting of B. longum 35624 cells, which were grown on

209	mononuclear cens (PBMCs) were isolated from heating donors using density gradient
210	centrifugation. Human monocyte-derived dendritic cells (MDDCs) were differentiated with
211	1000 IU ml ⁻¹ GM-CSF (Peprotech, London, UK) and 1000 IU ml ⁻¹ IL-4 (Novartis, Basel,
212	Switzerland) from purified CD14 ⁺ cells using MACS separation (Miltenyi Biotec, Bergisch
213	Gladbach, Germany). Bacterial strains for in vitro assays were cultured in MRS medium
214	supplemented with 0.05 % L-cysteine for 48 hours under anaerobic conditions at 37°C. Cells
215	were harvested and washed once with sterile PBS by centrifugation at 6,500 rpm for 10
216	minutes. Bacterial cell number was determined by microscopy using a Petroff-Hausser
217	chamber and bacteria were diluted as appropriate in PBS for incubation with human cells.
218	PBMCs and MDDCs were stimulated for 24 h at 37°C, 5 % CO_2 with bacterial strains at a
219	concentration of 50 bacterial cells to 1 PBMC or MDDC. Human and bacterial cell co-
220	cultures were performed in complete RPMI-1640 (cRPMI) medium (Sigma, Buchs,
221	Switzerland) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 Units ml ⁻¹)
222	and streptomycin (0.1 mg ml ⁻¹) (Sigma). Purified exopolysaccharide from <i>B. longum</i> 35624
223	was also added (final concentration 100 μ g/ml) to PBMC cultures (in duplicate) stimulated
224	with sEPS ^{neg} . Cytokine concentrations were measured using the Bio-Plex Multiplex System
225	(Biorad). For human dendritic cells staining, the following anitibodies were used: PE-Cy7
226	anti-human CD274 (PD-L1), APC anti-human CD273 (PD-L2) and Pacific Blue anti-human
227	CD11c (eBioscience, Vienna, Austria). THP-1-Blue TM NF-кВ monocyte cell line (Invivogen,
228	San Diego, USA) was maintained and sub-cultured in cRPMI medium (Sigma) in presence of
229	200μ g/ml Zeocin (Invivogen). For the co-culture experiment with bacteria, 10^5 cells/well
230	were seeded in a 96 well-plate in a total volume of 200 μ l/well of cRPMI medium. The cells
231	were stimulated over a range of different bacterial concentrations for 24 h and activation of
232	NF- κ B/AP-1 pathway was evaluated by Quanti-Blue TM assay according to the manufacturer's
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233	instructions. In addition, MDDCs were stimulated with different bacterial strains and NF- κB
234	phosphorylation measured over a time course. MDDCs were lysed using Bio-Plex Pro cell
235	signaling reagent kit (Biorad) and cell lysates were stored at -80°C until analysis. Protein
236	concentration was determined using Bio-Rad's DC^{TM} protein assay and equal amounts of
237	protein were used to measure NF-κB p65 (Ser ⁵³⁶) in the Bio-Plex Pro [™] Magnetic Cell
238	Signaling Assay (Biorad). Results are expressed as MFI (mean fluorescence intensity).
239	T cell transfer colitis model. C.B-17 severe combined immunodeficient (SCID and
240	BALB/c female mice (8-12 weeks of age) were obtained from Charles River (Sulzfeld,
241	Germany) and maintained under specific pathogen free conditions. The animals were housed
242	at the AO Research Institute, Davos, Switzerland, in individually ventilated cages for the
243	duration of the study, and all experimental procedures were carried out in accordance with
244	Swiss law (Permit number: 2013_32). Colitogenic CD4 ⁺ CD25 ⁻ CD45RB ^{hi} cells were isolated
245	from BALB/c donor mouse spleens using the MACS Miltenyi system (depletion of
246	CD4+CD25+ cells followed by positive selection of CD45RB FITC-labeled cells). At day 0,
247	colitis was induced by intraperitoneal transfer of 4×10^5 cells per C.B-17 SCID mouse (8
248	mice per group). Bacterial cells were prepared as described above and counted using
249	microscopy (Petroff-Hausser chamber) prior to dilution in sterile PBS. 1 x 10 ⁹ B. longum
250	35624 cells, or its isogenic derivatives, were administered to each mouse by intragastric
251	gavage (total volume of 200 μ l). Bacteria were gavaged from the beginning of the study (day
252	0) and continued to be gavaged every second day until animals were euthanized at the end of
253	the study. Sixteen days after study initiation, disease severity scores were recorded, while
254	animal weights were monitored every day. Disease severity scores included feces condition (
255	1 - wet; 2 - diarrhea; 3 - bloody diarrhea or rectal prolapse), activity (1 - isolated, abnormal

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260	software (http://simgene.com/Primer3). The primers, designated 2420t (Forward: CAG TGG
261	GGT GCG ACT ACA; Reverse: GCG CGA ACC AGA AGA TGT) generated a 494 bp
262	amplicon. Bacterial DNA from fecal samples was extracted using QIAamp DNA Stool Mini
263	Kit (Qiagen). DNA was quantified using Nanodrop (Thermo Scientific) and 100 ng of total
264	DNA was assayed using SYBR Green PCR Master Mix (Biorad). The thermal cycling
265	conditions consisted of an initial denaturation step of 15 min at 95°C, followed by 30 cycles
266	of denaturation at 94°C for 45 sec, annealing for 45 sec at 56°C, and extension at 72°C for 45
267	sec. B. longum 35624 DNA concentrations were quantified using the absolute quantitation
268	protocol of the ABI 7900 Fast real-Time PCR system (Applied Biosystem, CA, USA). In a
269	parallel experiment, BALB/c healthy mice (6 mice per group) were gavaged with B. longum
270	35624 or its isogenic derivative for 3 weeks, as described above for the colitis study.
271	Following euthanasia on day 26, mesenteric lymph nodes were isolated in order to
272	obtain single cell suspensions. Lymph nodes were mechanically disrupted using a syringe
273	plunger to grind the nodes on a nylon cell strainer (70 μ m). The strainer was washed with
274	PBS and the single cell suspensions were centrifuged at 300 g for 10 minutes. Cell pellets
275	were resuspended in 1 ml of cRPMI medium (Sigma) and cells were counted using a Scepter
276	Cell Counter (Millipore, Billerica, MA, USA). Cells were diluted to a final density of
277	1x10 ⁶ cells ml ⁻¹ in cRPMI and cells were dispensed in propylene tubes to perform FACS
278	staining. Lamina propria mononuclear cells were isolated as described previously (29)
279	following removal of epithelial cells and collagenase VIII/DNaseI (Roche) digestion of the 13

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

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tissue. At the end of the process, cells were counted using the Scepter Cell Counter (Millipore) and diluted to a final concentration of 1×10^6 cells ml⁻¹ in cRPMI in propylene

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 µg of ovalbumin (OVA, Grade V>98%, Sigma) 289 emulsified in Imject[™] 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. On days 19, 25 and 27, mice received *B. longum* **35624** or sEPS^{neg} intra-nasally (~1x10⁹ 291 292 bacteria per dose in a total volume of 50 µl 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 µl 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 1×10^6 cells/ml 300 in complete RPMI (Sigma) and stimulated ex vivo with 50 µg/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 ⁻¹ and 500 ng ml ⁻¹ ,
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-γ 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^{neg}**, 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 <i>B. longum</i> 35624 involved the heterologous expression of a <i>B. longum</i>
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 <i>eps</i> ₆₂₄ cluster, i.e. <i>pgt</i> ₆₂₄ , encoding the
337	predicted priming glycosyl transferase (pGT), resulting in an isogenic derivative of B. longum
338	35624 , which was designated sEPS ^{neg} .
339	In order to assess if the sEPS ^{neg} 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 ^{neg} , thus confirming its exopolysaccharide-deficient phenotype (Fig. 1). Furthermore,
343	and in contrast to the parent strain <i>B. longum</i> 35624 , the EPS ^{neg} strain exhibits a so-called
344	dropping phenotype when grown in liquid medium (i.e. the EPS ^{neg} 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_{624} , the adjacent and co-transcribed genes pgt_{624} and $BI0343$ 16

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

357 In vitro responses to B. longum 35624, sEPS^{neg} and sEPS^{comp}. The wild-type strain 358 B. longum 35624 and its derivatives sEPS^{neg} or sEPS^{comp} 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^{neg} strain was shown to induce higher levels of IL-12p70, IFN-y and IL-17 secretion, with comparable induction of IL-10 (Fig. 2A). The 361 sEPS^{comp} strain induced similar levels of cytokine secretion as *B. longum* **35624**, confirming 362 363 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 significantly reduced IL-12p70 and IFN- γ secretion in response to the sEPS^{neg} strain, but did 365 366 not alter IL-17 or IL-10 responses to the sEPS^{neg} strain (Fig. 2B).

367 Similarly to PBMCs, human MDDCs were co-incubated with *B. longum* 35624 or
368 sEPS^{neg} strains, and cytokine secretion was measured after a 24 hour exposure. Secreted IL369 17, IL-6 and TNF-α levels, but not IL-10, were all shown to be significantly higher for the
370 sEPS^{neg}-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^{neg} –induced expression of
372 the MDDC inhibitory molecules programmed death-ligand 1 (PD-L1) and PD-L2, which bind
17

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

375 Activation of the transcription factor NF-κB is critical for the induction of

376 inflammatory genes, including cytokines. Thus, we measured NF-κB activation in the

377 monocyte cell line THP-1, containing a SEAP reporter for NF-κB and AP-1 activation. The

378 sEPS^{neg} strain was shown to induce higher levels of NF- κ B/AP-1 activation, compared to *B*.

379 longum 35624-stimulated THP-1 cells (Fig. 3C). To confirm this result, NF-κB

380 phosphorylation was measured in MDDCs over time, following exposure to bacteria. Both B.

381 *longum* **35624** and sEPS^{neg} strains induced similar levels of NF-κB phosphorylation at early

382 time points (Fig. 3D). However, sustained high levels of phosphorylated NF-κB were

383 observed at later time points for the sEPS^{neg}-stimulated MDDCs, which were not observed for

384 *B. longum* **35624**-stimulated cells.

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

387 The sEPS^{neg} strain does not protect against colitis development. Colitis was

induced in SCID mice by adoptively transferring CD4⁺CD25⁻CD45RB^{hi} lymphocytes. Mice
were administered *B. longum* 35624, sEPS^{neg} or sEPS^{comp} daily by oral gavage. As previously
described, *B. longum* 35624 treatment prevented weight loss and disease symptoms in this
model (34). However, mice treated with the sEPS^{neg} strain exhibited significant weight loss
and severe disease symptoms, while restoration of EPS production in the sEPS^{comp} strain
promoted a similar response as to *B. longum* 35624 (Fig. 4A). Following euthanasia, the

colon:body weight ratio was significantly higher in animals administered the sEPS^{neg} strain,

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394

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^+
398	lymphocytes in animals administered the sEPS ^{neg} , with a trend towards increased numbers of
399	IFN- γ^+ lymphocytes, which was not statistically significant (Fig. 4C). No significant
400	difference in IL-10 ⁺ lymphocytes was observed. No differences in the gastrointestinal transit
401	of <i>B. longum</i> 35624 or the sEPS ^{neg} derivative was observed (Supplementary Fig. S2).
402	The administration of the sEPS ^{neg} strain to healthy immunocompetent animals did not result
403	in gastrointestinal inflammation, indicating that the sEPS ^{neg} mutant did not induce colitis in
404	healthy animals. However, administration of sEPS ^{neg} did provoke a trend in an increased
405	percentage of IL-17 ⁺ and IFN- γ^+ lymphocytes, associated with an increase in CCR9 ⁺ 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 ^{neg} to exert its $T_{\rm H}17$ -
409	enhancing effects.
410	sEPS ^{neg} exacerbates IL-17 responses within the lung. In order to further assess the
411	ability of sEPS ^{neg} 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	$T_{\rm H}17$ responses within the lungs of challenged animals (35). Either the <i>B. longum</i> 35624 or its
414	isogenic derivative sEPS ^{neg} 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 ⁺ lymphocytes within lung tissue, compared to control animals
417	(Fig. 5A). Exposure to the <i>B. longum</i> 35624 strain did not influence the percentage of IL- 17^+

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while macroscopically the colons of these mice appeared severely inflamed with visible

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418	lymphocytes within the lung, however, exposure to sEPS ^{neg} significantly increased the
419	percentage of IL- 17^+ 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 ^{neg} 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, <i>ex vivo</i> secretion of IL-17 and the percentage of IL- 17^+ cells within lung tissue correlate
430	with the highest levels for both assay systems being observed for sEPS ^{neg} -treated animals.
431	OVA sensitization and challenge resulted in an increased percentage of IFN- γ^+
422	hymphosytes within lung tissue, compared to control primels (Fig. 5A). Exposure to the P
432	lymphocytes within lung ussue, compared to control annuals (Fig. 5A). Exposure to the B.
433	<i>longum</i> 35624 strain prevented the increase in the percentage of IFN- γ^+ lymphocytes within
434	the lung, which was not observed following exposure to the sEPS ^{neg} 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- γ 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 II -10 ⁺
130	
439	lymphocytes within lung tissue and exposure to <i>B. longum</i> 35624 or the sEPS ^{neg} strains
440	further increased the percentage of $IL-10^+$ lymphocytes within the lung (Fig. 5C). Re-

These findings suggest that the absence of the exopolysaccharide on *B. longum* 35624
promotes T_H17 responses in the inflamed lung, similar to the effects described above for the
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 <i>B. longum</i> 35624 modulates cytokine secretion and NF-κB
455	activation in vitro, while in murine models exposure to a B. longum 35624 derivative unable
456	to synthesize exopolysaccharide promotes $T_{\rm H}17$ 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
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2	463	contribute to strain-specific traits due to the expected structural and therefore functional
2	464	diversity of such molecules. Of note, pathogen-associated exopolysaccharides have long been
2	465	known to be critical in host-microbe interactions, where they facilitate adherence and
4	466	colonization within the human host, with additional immunomodulatory effects (40, 41).
2	467	Exopolysaccharides can also mediate the beneficial immune effects associated with certain
2	468	commensal microbes. As already mentioned, a strong modulator of intestinal immune
2	469	responses is PSA from <i>B. fragilis</i> , which is well described to influence lymphocyte
2	470	polarization and PSA suppresses IL-17 production by intestinal immune cells (42-44). In line
2	471	with data presented in this manuscript, exopolysaccharide gene knockout mutants of
2	472	Lactobacillus casei Shirota induced significantly more pro-inflammatory cytokine secretion
2	473	from a mouse macrophage cell line, compared to wild-type cells (45). In addition, the
2	474	cytokine response of PBMCs to two isogenic strains of <i>B. animalis</i> subsp. <i>lactis</i> that differ
4	475	only in their exopolysaccharide-producing phenotype suggest that the mucoid strain could
4	476	have higher anti-inflammatory activity (31). The data presented in this manuscript are in
4	477	agreement with these previous reports and further supports the concept that
4	478	exopolysaccharides from bifidobacteria may elicit immune-modulatory activities.
2	479	Interestingly, the induction of PD-L1 and PD-L2 on dendritic cells was similar for the
2	480	wild type <i>B. longum</i> 35624 strain and its isogenic derivative sEPS ^{neg} . Similarly, the induction
2	481	of IL-10 was not negatively impacted by the loss of exopolysaccharide from the bacterium.
2	482	This suggests that not all immune-regulatory effects induced by <i>B. longum</i> 35624 are
2	483	mediated solely by exopolysaccharide. The bifidobacterial cell wall is a complex arrangement
2	484	of macromolecules, consisting of a thick peptidoglycan layer that surrounds the cytoplasmic
2	485	membrane, which is decorated with other glycopolymers, such as (lipo)teichoic acids,
2	486	polysaccharides and proteins, all of which may influence the immune response (46, 47) A few 22

487	examples include the cell wall-associated proteins p40 and p75 from L. casei ssp. rhamnosus
488	GG, the S-layer protein from <i>L. acidophilus</i> , or the STp peptide from <i>L. plantarum</i> (48-50).
489	$T_{\rm H}17$ cells are a subset of CD4+ 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 $T_{\rm H}17$ cells occurs following T-cell antigen receptor
492	recognition of an MHC class II-bound peptide in the presence of cytokines including TGF-β1,
493	IL-6 or IL-1 β (52, 53). While T _H 17 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 _H 17 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 $T_H 17$ 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 ^{neg} . 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 $T_{\rm H}17$ development. The
505	observation that addition of purified exopolysaccharide to sEPS ^{neg} -stimulated PBMCs
506	suppresses the exaggerated IL-12p70 and IFN- γ 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 _H 17 responses by binding to host receptors,

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509 or if the exopolysaccharide is simply masking T_H17-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_H17 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.

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529

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712 FIGURE LEGENDS

Figure 1. B. longum 35624 electron microscopy

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- 714 Representative scanning electron microscopy (SEM) images for the B. longum 35624 parent 715 strain (A, upper panel) and its isogenic derivatives, sEPS^{neg} mutant (B, middle panel) and sEPS^{comp} mutant (C, bottom panel) are illustrated. Arrows indicate the 'stringy' layer of 716 extracellular polysaccharide visible on the B. longum 35624 parent strain and sEPS^{comp} strain . 717 718 Scale bars are indicated at the bottom right of each panel. 719 720 Figure 2. PBMC cytokine response to bacterial strains (A) PBMCs from 6 healthy donors were stimulated with B. longum 35624 or its isogenic 721 derivatives sEPS^{neg} or sEPS^{comp} (50 bacteria:1 PBMC) for 24 hours and cytokine secretion 722 723 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 adding isolated exopolysaccharide on sEPS^{neg} strain-induced PBMC secretion of IL-12p70, 726
- 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^{neg} 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^{neg} (50 bacteria:1 MDDC) for 24 hours. Cytokine secretion into
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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 <i>B. longum</i> 35624 versus the sEPS ^{neg} strain). (C) THP-1 NF- κ B activation
737	following exposure to increasing concentrations of B. longum 35624 or its isogenic derivative
738	sEPS ^{neg} (n=4 experimental replicates). (D) Activation of NF- κ B in MDDCs exposed to B.
739	longum 35624 or its isogenic derivative sEPS ^{neg} (n=3, 50 bacteria:1 MDDC). Statistical
740	significance was determined using two-way ANOVA (*p<0.05 <i>B. longum</i> 35624 versus the
741	sEPS ^{neg} strain).
742	
743	Figure 4. sEPS ^{neg} is not protective in a T cell transfer colitis model

744 Following receipt of CD4⁺CD25⁻CD45RB^{hi} T cells, C.B-17 SCID mice were orally

745 administered *B. longum* **35624** (n=8), sEPS^{comp} (n=8) or sEPS^{neg} (n=8) strains. (A) Weight

746 loss and disease activity were monitored over time. Statistical significance was determined

via 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^{neg} –treated

animals is provided. (C) The percentage of IL-17⁺, IFN- γ^+ and IL-10⁺ 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^{neg} strain versus the other strains).

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Figure 5. sEPS^{neg} promotes T_H17 responses in the lung 755

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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^{neg} (OVA & sEPS^{neg}, n=8).
- (A) The percentage of IL-17⁺ CD3⁺CD4⁺ 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- γ^+ and IL-10⁺ CD3⁺CD4⁺ T lymphocytes and *ex vivo* IFN- γ 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
- 765 was determined using the Kruskal-Wallis test and Dunn's multiple comparison test (*p<0.05

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compared to the OVA group).

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

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli EC101	Cloning host, repA ⁻ , Km ^r	Law et al., 1995 (26)
E. coli XL1blue	Cloning host, , Tet ^r	Stratagene
E. coli EC101 pNZ-M.1185	E. coli EC101 harbouring pNZ-M.1185	This study
B. longum 35624	Parent strain	Alimentary Health
sEPS ^{neg}	<i>B. longum</i> 35624 harbouring insertion mutation in priming glycosyl transferase encoding gene, pgt_{624}	This study
sEPS ^{comp}	sEPS ^{neg} harbouring pBC1.2_pgt ₆₂₄ +BI0343	This study
Plasmids		
pNZEm	Gene expression vector, Em ^r	Margolles et al., 2006 (56)
pORI19	Em ^r , repA ⁻ , ori ⁺ , cloning vector	Law et al., 1995 (25)
pORI19 <i>tet</i> (w)_pgt	pORI19 harbouring internal fragment of <i>BI0342</i> (<i>pgt</i>) and tetW gene	This study
pBC1.2	E. coli –Bifidobacterial shuttle vector	Álvarez-Martín <i>et al.</i> , 200 (27)
pBC1.2_ <i>pgt</i> ₆₂₄ + <i>BI0343</i>	pBC1.2 harbouring the cotranscribed genes $pgt_{624}+BI0343$ under the control of their native promoter	This study
pNZ-M.1185	<i>B11185</i> (<i>M.1185</i> , isoschizomer of M.EcoRII) cloned with its own promoter in pNZEM	This study

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

Purpose	Primer	Sequence ^a
Cloning of M 1185 in	BI1185F PstI	GACTECACCCCACTAGGTAACCAAACG
pNZEm	biiioji_i su	GACIGCAGOCCCACIAGOTAACCAAACG
pi vezeni	BI1185R_SpeI	GCGCACTAGTCTAGAGCAAAGCCAGTATAG
Cloning of internal 583 bp	BI0342F HindIII	GATAAGCTTGCGTCGGCAACTCAACTACC
fragment of <i>pgt</i> in pORI19	_	
	BI0342R_XbaI	GAT TCTAGA CGTCGGCGTTCACTACCATC
Cloning of <i>pgt</i> ₆₂₄ +BI0343	BI0342FSalI	GACGTCGACACTCCACTCTCGCTGATCG
in pBC1.2	BI0343EcoRI	GGC GAATTC TAATCAACCAAGGGGGGTCTG

^a Restriction sites incorporated into oligonucleotide primer sequences are indicated in bold

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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^{neg} mutant (B, middle panel) and sEPS^{comp} mutant (C, bottom panel) are illustrated. Arrows indicate the 'stringy' layer of extracellular polysaccharide visible on the *B. longum* **35624** parent strain and sEPS^{comp} 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^{neg} or sEPS^{comp} (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^{neg} 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^{neg} strain alone).





MDDCs were generated from 4 healthy donors and were stimulated with *B. longum* **35624** or its isogenic derivative sEPS^{neg} (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^{neg} strain). (C) THP-1 NF-kB activation following exposure to increasing concentrations of *B. longum* **35624** or its isogenic derivative sEPS^{neg} (n=4 experimental replicates). (D) Activation of NF-kB in MDDCs exposed to *B. longum* **35624** or its isogenic derivative sEPS^{neg} (n=3, 50 bacteria: 1 MDDC). Statistical significance was determined using two-way ANOVA (*p<0.05 *B. longum* **35624** versus the sEPS^{neg} strain).



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Figure 4. sEPSneg is not protective in a T cell transfer colitis model

Following receipt of CD4+CD25-CD45RBhiT cells, C.B-17 SCID mice were orally administered B. longum 35624 (n=8), sEPScomp (n=8) or sEPSneg (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 sEPSneg-treated animals is provided. (C) The percentage of IL-17+, IFN-g+ and IL-10⁺ 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^{neg} strain versus the other strains).

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Figure 5



Figure 5. sEPS^{neg} promotes $T_H 17$ 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 *B. longum* **35624** (OVA & 35624, n=8), or intranasally administered sEPS^{neg} (OVA & sEPS^{neg}, n=8). (A) The percentage of IL-17⁺ CD3⁺CD4⁺ 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-g⁺ and IL-10⁺ CD3⁺CD4⁺ T lymphocytes and *ex vivo* IFN-g 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).