Isolation and characterization of a high molecular mass β-glucan from *Lactobacillus fermentum* Lf2 and evaluation of its immunomodulatory activity.

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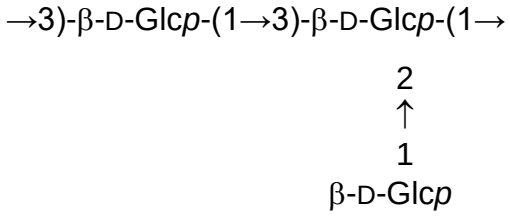
ABSTRACT

When grown in a semi-defined medium, *L. fermentum Lf2* synthesizes significant quantities (~2 g/L) of two exopolysaccharides (EPS). The two EPS were separated by preparative size exclusion chromatography to give a high molecular mass β-glucan (1.23×10^6 Da) and a medium molecular mass heteroglycan (8.8×10^4 Da). The structure of the high molecular mass β-glucan was determined using a combination of NMR spectroscopy, monomer and linkage analysis. The EPS has the following structure:



The immunomodulatory activity of the high molecular mass EPS was studied in peripheral blood mononuclear cells (PBMC). Exposure of PBMC to an aqueous solution of the EPS for 24 h led to increased cell proliferation, changes in expression of the cytokines CD14 and TLR2, and to an increase in production of TNF-α compared to controls. In contrast, when cells that had been treated with EPS for 24 h and from which the EPS had been removed, were subsequently exposed to the bacterial antigen LPS very low levels of TNF-α production were observed. This result indicates that the EPS imparts immunotolerance in PBMC. An ability to modulate the release of the proinflammatory mediators, such as TNF-α, is an important goal in the development of therapies for the treatment of diseases, such as Crohn's disease and ulcerative colitis, associated with excessive release of inflammatory mediators.

1. Introduction

In the last ten years, significant advances have been made in determining the role that the microflora resident in the human gastrointestinal tract (GIT) play in maintaining the health of their hosts [1-4]. A growing number of diseases, including obesity [5, 6], liver disease [7, 8], cancer [9, 10], inflammatory bowel dieases [11-13] and neurodegenerative diseases [14-16], have been linked to changes in the populations of bacteria in the gut. The molecular mechanisms through which the gut microbiota interact with their mammalian hosts is the topic of current research and much of this work focuses on activities occurring at the gastrointestinal (GI) barrier [17, 18]. The GI barrier separates the intestinal gut microflora from the systemic blood and organs of the body and is composed of a layer of epithelial cells coated in mucin. In a correctly functioning barrier, the presence of highly viscous mucin and a tightly packed layer of epithelial cells prevents the bacteria passing through to the underlying organs. The barrier is not a passive organ and active communication occurs between the commensal bacteria and the professional immune cells that live just below the barrier [17]. Whilst a number of different methods of communication exist, one of the best understood is the signalling initiated by the lipopolysaccharide (LPS) of Gram-negative bacteria [19]. LPS binds to the pattern recognition receptors, TLR 2 and 4 [20], which are present on the surface of epithelial cells. Binding of LPS by TLR4 occurs in combination with CD14 and gives rise to a signalling cascade that releases proinflammatory mediators, including TNF-α, into the lamina propria below the barrier [19]. The release of TNF-α ultimately leads to a range of pro-inflammatory responses [21] which provide protection against pathogenic bacteria but can also initiate overactive inflammation which is central to the aetiology of a range of pathologies such as inflammatory bowel disease.

It has been reported that exopolysaccharides (EPS) [22, 23] [24, 25] secreted by Gram-positive bacteria, can influence the release of both pro- and anti-inflammatory mediators. It has also been shown that specific EPS can modulate the immune response generated by other bacterial antigens and, in doing so, provide a degree of immunotolerance [24]. It is likely that immunotolerance has evolved as a way of preventing damage that can occur through too robust an immune response e.g. as occurs in autoimmune diseases [26]. One of the potential molecular mechanisms for inducing immunotolerance is through the interaction of bacterial antigens, including exopolysaccharides, with pattern recognition receptors present on the host cell surface [27-29]. Unfortunately, progress in understanding the molecular mechanisms by which EPS can induce immunotolerance has been restricted by the limited availability of high purity EPS that can be used in cell culture studies and it is this area of work the current paper addresses.

L. fermentum Lf2 is a lactic acid bacteria (LAB) that was isolated as a contaminant culture during cheese manufacture [30]. Under optimized conditions, the strain produces significant quantities of a mixture of EPS (~2g/L). It has previously been demonstrated that the EPS mixture has immunomodulatory activity [31]. The aim of the current study was purity the polysaccharides, to characterize the high molecular mass EPS and to investigate its immunomodulatory activity with peripheral blood mononuclear cells (PBMC). These cells are easily isolated from whole blood and although there are some small differences in the composition and activation status compared with the immune cells of the gastrointestinal tract, they share certain key phenotypic characteristics [32] and have previously been used to study the behaviour of mucosal immunity of the GI-tract [33].

2. Results and Discussion

2.1 Composition of the crude EPS and isolation of a high molecular mass EPS.

SEC-MALLS analysis of the crude EPS produced by *L. fermentum* Lf2 identified that a mixture of macromolecules was being secreted into the surrounding medium during fermentation, including a high molecular mass EPS (HMw-EPS), a medium molecular mass EPS and a UV absorbing species with a low molecular mass (data not shown). After preparative size exclusion chromatography a pure sample of the HMw-EPS was obtained in the early eluting fractions. The SEC-MALLS trace for the purified HMw-EPS contained a single peak (RT= 26 mins) with a weight average molecular mass of 1.23×10^6 Da and a polydispersity value of 1.104. The molecular mass and polydispersity are similar to those recorded for EPS isolated from other LAB [34]. The next fractions that eluted from the SEC included a combination of the medium molecular mass polysaccharide and the UV absorbing species, believed to be small amounts of cell wall material, that could not be separated.

2.2 Characterization of the HMw-EPS.

Monomer analysis and determination of the absolute configuration of the monosaccharides identified that the HMw-EPS was composed entirely of D-glucose confirming that the material was a glucan. Linkage analysis, using permethylated alditol acetates, confirmed the presence of a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (corresponding to a terminal glucopyranose), a 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol (corresponding to a 1,3-linked glucopyranose) and a 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylglucitol (corresponding to a 1,2,3-linked glucopyranose) in an approximately 1:1:1 ratio.

The ^{1}H-NMR spectrum (Fig 1, top, F2-axis) had two distinct resonances in the anomeric region (4.893 & 4.821 ppm) with an integral ratio of 1:1.9 which is consistent with the EPS having a trisaccharide repeating unit. Each of the anomeric signals had

large $^{3}J_{H1,H2}$-coupling constants (7.7 & 7.4 Hz) which is indicative of β-linked hexoses [35].

Figure 1.

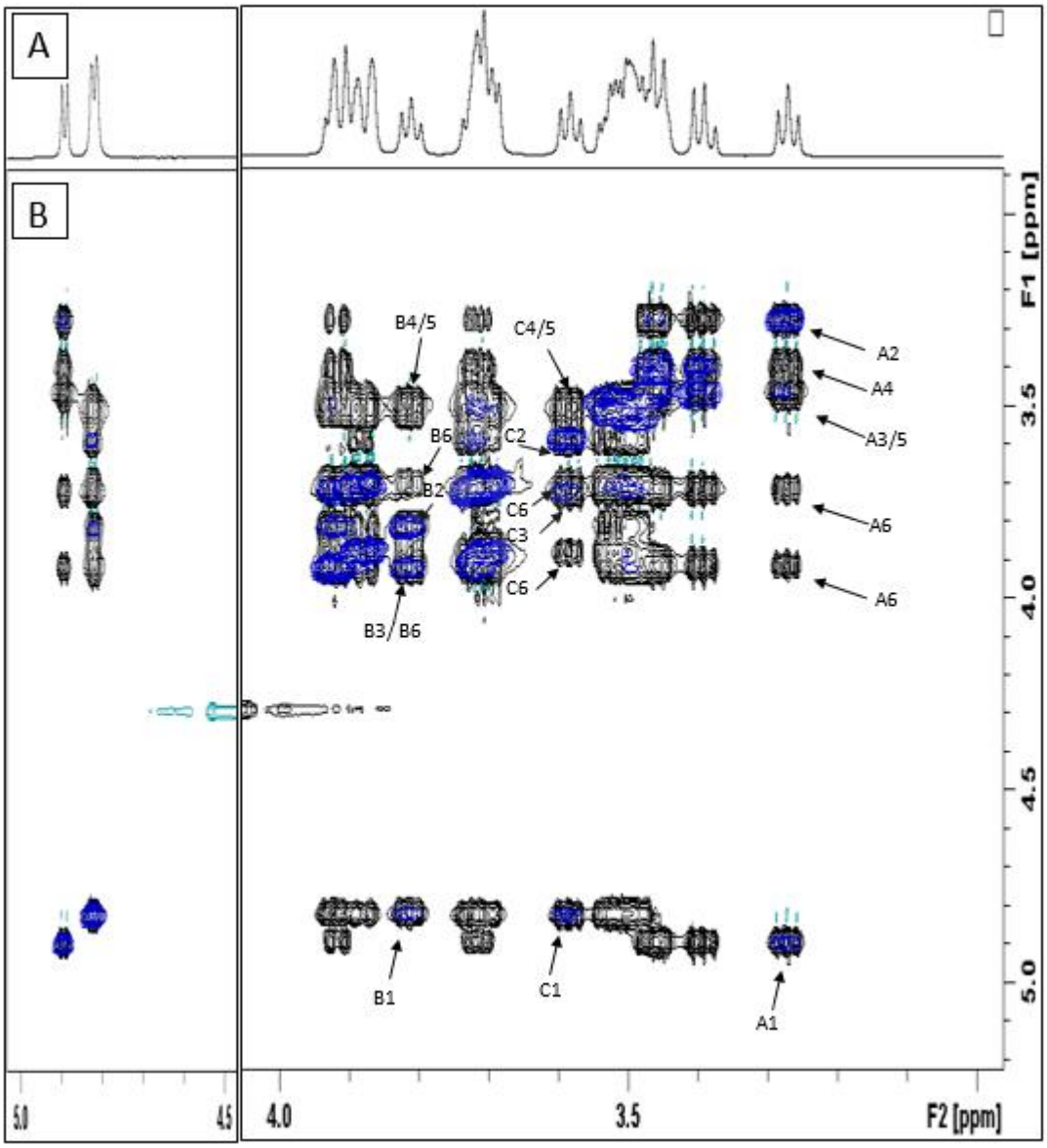


Fig 1. (A) Top (F2-axis) selected regions of the ^{1}H NMR spectrum for the HMw-EPS recorded at 70 ^{o}C on a Bruker 600 MHz spectrometer; (B) Blue Contours: ^{1}H-^{1}H COSY spectrum for the HMw-EPS recorded at 70 ^{o}C; (B) Black contours: ^{1}H-^{1}H-TOCSY spectrum recorded at 70 ^{o}C. Labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective ring protons.

The location of the H2 protons was identified through inspection of a ^{1}H-^{1}H-COSY spectrum (Fig 1, blue contours) and identification of the H1-H2 cross peaks. Fortunately, each H2 appears in a clear part of the spectrum and each has a unique chemical shift; these were labelled **A**2 (3.273 ppm), **B**2 (3.813 ppm) and **C**2 (3.585 ppm). The positions of the remaining ring protons were determined by following the transmission of scalar coupling (H2 through to H6) on the ^{1}H-^{1}H-TOCSY spectrum (Fig 1, black contours). A combination of an edited ^{1}H-^{13}C-HSQC spectrum (not shown) and a ^{1}H-^{13}C-HSQC-TOCSY spectrum (Fig 2) was used to identify the location of the carbon resonances.

Figure 2.

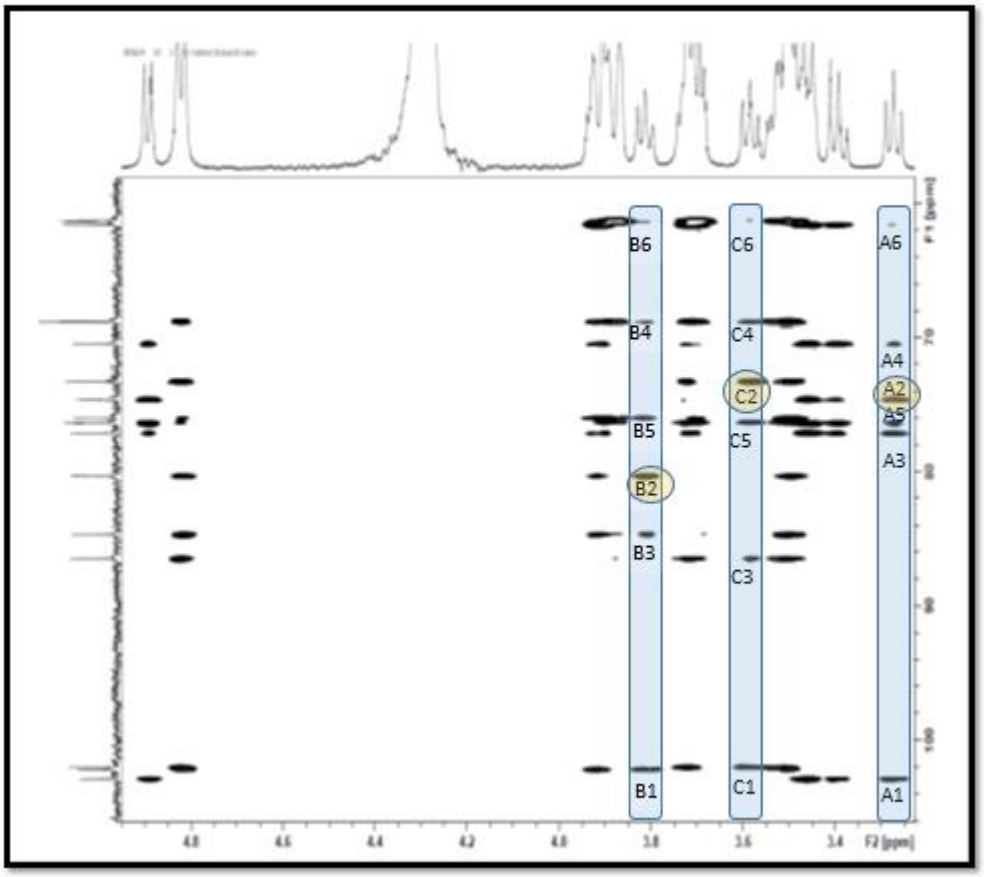


Fig. 2. ^{13}C-^{1}H HSQC-TOCSY spectrum for the HMw-EPS recorded in solution in D_2O (5-10 mg in 0.65 mL) at 70 ^{o}C on a Bruker 400MHz spectrometer, labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective ring

carbons; resonances highlighted in circles correspond to the three HSQC cross peaks used to establish correlations.

The chemical shifts for the individual proton and carbons are listed in Table 1. The chemical shifts for the protons and carbons of residue **A** are consistent with **A** being a terminal β-linked glucopyranose monomer [36]. The low field shift of both the C2 and C3 signals in residue **B** are consistent with **B** being a 1,2,3-linked glucopyranose Similarly, the low field shift of **C** C3 identifies **C** as a 1,3-linked glucopyranose. The latter combination of linkages is consistent with the structure of the repeat unit of the HMw-EPS being:

C **B**

→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→
2
↑
1
β-D-Glc*p*

A

The linkage residue **A** to the 2-position of **B** was confirmed by examination of a ROESY spectrum (data not shown) on which an inter-residue NOEs was observed between **A** H1 and **B** H2.

Table 1.

Sugar		1	2	3	4	5	6
β-Glc A	^{1}H	4.893	3.273	3.455	3.392	3.474	3.915/3.713
	^{13}C	102.85	74.59	77.08	70.43	76.31	61.55
-2,3)-β-Glc B	^{1}H	4.821	3.813	3.927	3.513	3.481	3.877/3.703
	^{13}C	102.14	80.27	84.64	68.84	75.98	61.33
-3)-β-Glc C	^{1}H	4.821	3.585	3.717	3.513	3.479	3.882/3.705
	^{13}C	101.97	73.24	86.42	68.84	76.31	61.22

Table 1.Proton and carbon chemical shifts (ppm) for the Lf2 HM_W-EPS recorded in D_2O at 70 ^oC. Chemical shifts are referenced to external acetone 2.225 ppm for ^1H and 31.55 ppm for ^{13}C. Labels (A-C) identify the different monosaccharides and the numbers (1-6) identify the respective carbons/ protons.

A number of microorganisms, including fungi and bacteria, generate β-glucans that have main chains composed of β-(1,3)-linked glucoses [37, 38] but only a small number include 1,2-linked branches. Three bacterial species generate EPS that are identical to that reported here and these are *Oenococcus oeni* I4 [39], *Pediococcus damnosus* 2.6 [40] and one of the two EPSs produced by *Lactobacillus spp. G-77* [40, 41]. Whilst *Streptococcus pneumoniae* Type 37 produces a CPS having a main chain of β-(1,3)-glucoses where each glucose in the main chain has a terminal glucose, connected via a β-link, at C2 [42].

2.3 PBMC activation and cell culture.

It has previously been reported that the crude EPS extract from *L. fermentum* Lf2 has biological activity: the EPS mixture protected mice against *Salmonella* infection and moderate immunomodulatory activity was demonstrated [31]. In order to determine if the biological activity was associated with the HM_W-EPS, a range of experiments were undertaken to study the response of the exposure of peripheral blood mononuclear cells (PBMC) to the HM_W-EPS in the absence or presence of the stimulants lipopolysaccharide (LPS) and phytohaemagglutinin (PHA).

In the first experiments, PBMC isolated from different donors were exposed to two concentrations of the purified HMw-EPS. In all donors, the metabolic activity of the cells increased after treatment with the HMw-EPS ($p < 0.001$ - 0.0001) (Fig 3A).

Figure 3.

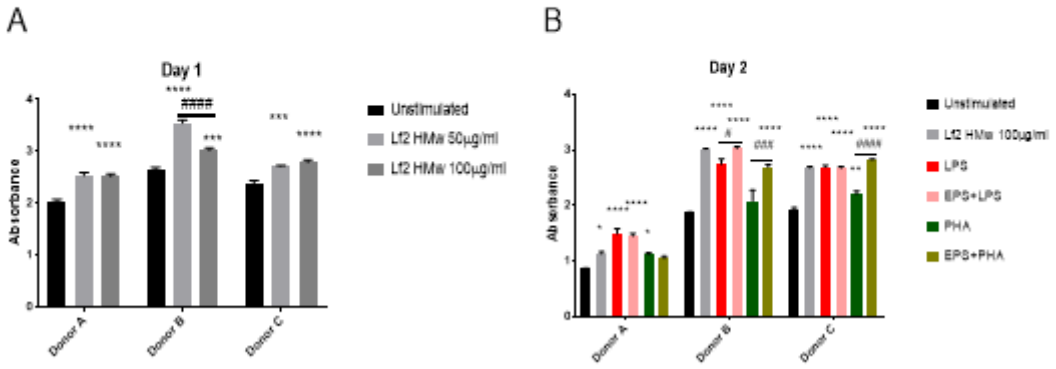


Fig. 3. Non-radioactive MTS assay was used for measuring metabolic activity of the cells under different culture conditions as measured after **A**. Experiment 1, Day 1 and **B**. Experiment 2, Day 2. **A** For Experiment 1, Unstimulated conditions were left untreated while Lf2 HMw 50µg/ml and Lf2 HMw 100µg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37˚C, 5% CO_2. Following 24h incubation, cells were harvested and $1x10^5$ cells/well were transferred into 96-well plates for MTS assay performed following the supplier's instructions. **B** For Experiment 2, Day 2, Unstimulated condition was left untreated on Day 0 then washed and left unstimulated on Day 1. Lf2 HMw 100µg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, washed and stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively on Day 1. Following 24h incubation, cells were harvested and $1x10^5$ cells/well were transferred into 96-well plates for MTS assay performed following the supplier's instructions. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ when compared between two indicated conditions.

The levels of activation were comparable for both EPS concentrations used (50 µg/mL and 100 µg/mL), except for Donor B where higher levels were obtained for 50 µg/mL EPS ($p < 0.0001$). The results suggest that the EPS did not have a negative impact on the viability of the cells. At the concentrations employed, the EPS was able to activate PBMC in culture compared to controls, indicating a functional interaction between the two. Interestingly, this activation persisted for 24 h after the EPS had been removed from solution (see discussion below). This is in agreement with previous reports where cell wall extracts and purified EPS stimulated cell proliferation of different immune cell types: including BALB/c mice splenocytes and mesenteric lymphocytes [43] using EPS from LAB, and mouse lymphocytes by EPS from *Paenibacillus jamilae* CP-7 [44]. In contrast, EPS from bifidobacteria has been reported to have anti-proliferative activity on rat PBMC and gut associated lymphoid tissue cells [45]. Whilst other authors have reported bifidobacteria EPS stimulates proliferation of the intestinal cell line Caco-2 but not HT-29 [46] the EPS from *Lactobacillus gasseri* strains G10 and H15 had no effect on the proliferation of the cervical cancer cell line HeLa [47]. These differences are not surprising since it has previously been demonstrated that EPS can exert a differential effect on the metabolic activity of different cell types [48].

In the next set of experiments, PBMC were initially exposed to the HM_W-EPS for 24 h after which time the EPS was removed. The cells were then incubated for a further 24 h either without any additional treatment or by exposing the cells to one of the stimulants LPS or PHA. Appropriate duplicate controls, in which cells were not treated with EPS, were run for each of the experiments. In this second group of experiments, the metabolic activity of the cells remained elevated in the EPS treated conditions (Fig 3B), regardless of the type of subsequent stimulation ($p < 0.05$-

0.0001). Treatment with LPS alone (red bars) also showed higher metabolic activity as measured by the MTS assay, and this was true regardless of whether or not they had been pre-treated with EPS ($p < 0.0001$).

Apparent anomalies in the MTS results were observed for PHA treatment, where although a statistically significant activation was observed for two out of three donors tested ($p < 0.05$-0.01) the recorded levels did not correspond to the high level of cell proliferation observed on microscopic images for this condition (Fig 4). It is possible that the conditions used for PHA stimulation interfered with the results of the MTS assay such that the level of metabolic activity that this assay measured did not accurately correspond to the level of proliferation exhibited after PHA stimulation [49, 50] . Microscopic inspection of the cells (Fig 4B) confirmed the high levels of proliferation and clumping of cells after PHA stimulation when compared to the unstimulated and untreated condition (Fig 4A) and the degree of cell aggregation was attenuated by previous treatment with EPS (Fig 4C).

Figure 4.

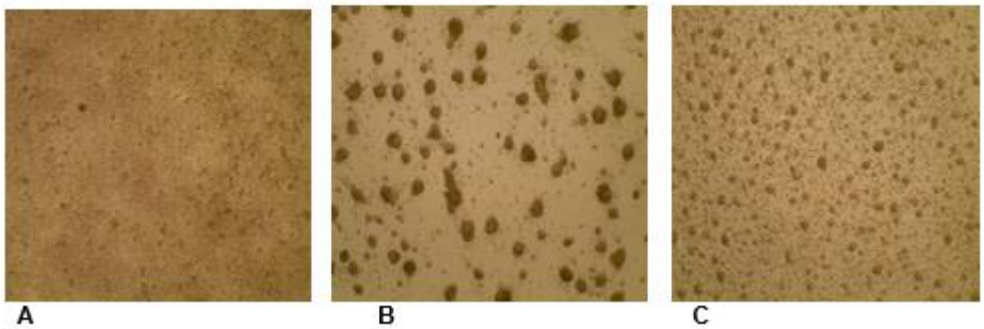


Fig. 4. Representative microscopic images showing the effect of PHA with and without EPS pre-treatment on PBMC proliferation as seen at Day 2 of experiment 2. **A**. Untreated at Day 0, then washed and unstimulated at Day 1; **B**. Untreated at Day 0, then washed and stimulated with 10µg/ml PHA at Day 1; **C**. Pre-treated with 100µg/ml Lf2 HMw EPS at Day 0, then washed and stimulated with 10µg/ml PHA at Day 1.

The MTS assays gave results in agreement with previous reports, where the optimal PHA concentration for cell stimulation was 2.5 µg and where only modest increases in metabolic activity was reported for cultures stimulated with 10 µg/ml PHA [50]. From inspection of the microscope images it is clear that pre-treatment with the HMw-EPS partially reversed the effect of PHA by reducing the amount of cell aggregation and suggests that binding of the plant lectin or the resulting agglutination of the lectin bound cells is blocked. It is important to note that this reduced agglutination is taking place 24 h after the HMw-EPS has been removed which suggests that the initial exposure to the β-glucan has changed the expression of lectin binding receptors on the surface of the PBMC.

*2.4 Flow cytometric assessment of pattern recognition receptors CD14 and TLR-2***.**

In an attempt to measure changes in expression of surface receptors, in the next experiments, levels of expression of the pattern recognition receptors CD14 and TLR-2 were studied. After exposure of PBMC cells to the HMw-EPS for 24 h, in the three donors the average levels of expression CD14 were either marginally increased (Donor 1) or lower (Donor 2 and 3) compared to the untreated condition ($p < 0.0001$, Fig 5). Small decreases in CD14 levels in PBMC after 24 h stimulation with LPS has been previously reported [51]. Downregulation of CD14 expression after LPS stimulation was also observed in alveolar macrophages [52]. The results of the first experiments are in contrast to what was observed in the next set of experiments in which PBMC were treated with HMw-EPS for 24 h at which point the EPS was removed and then the cells were exposed to either no stimulant or to one of the stimulants LPS or PHA. Now prior-treatment with HMw-EPS led to a robust increase in the CD14 expression (Fig 5B). This was true for all conditions: untreated unstimulated

vs EPS pre-treated unstimulated (grey bars versus black bars); untreated LPS stimulated vs EPS pre-treated and LPS stimulated (red versus pink bars, with $p < 0.0001$ in all cases), and in Donor B only, untreated PHA stimulated vs EPS pre-treated and PHA stimulated (green versus olive bars $p < 0.01$).

Figure 5.

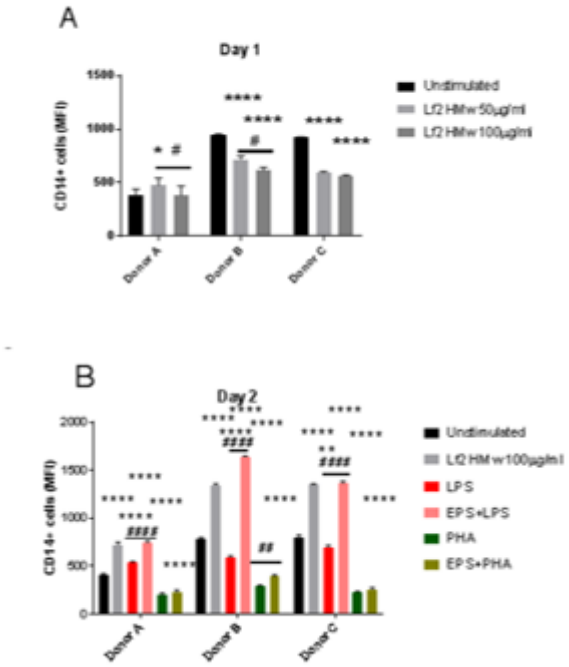


Fig 5. Changes in CD14 expression on PBMC following different conditions presented as median fluorescence intensity (MFI) of $CD14^+$ cells after **A**. Day 1 and **B**. Day 2 experiment. **A** For Day 1, Unstimulated conditions were left untreated while Lf2 HMw 50µg/ml and Lf2 HMw 100µg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37˚C, 5% CO_2. Following 24h incubation, cells were harvested and stained with anti-CD14 antibody and analysed by flow cytometry. **B** For Day 2 experiment, Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100µg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2

HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively on Day 1. Following 24h incubation, cells were harvested and stained with anti-CD14 antibody and analysed by flow cytometry. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ when compared between two indicated conditions. Gates were set on single cells and debris was excluded using FSC vs SSC plots. Gating was performed using FMO (Fluorescence Minus One) controls.

The results suggest that the presence of β-glucan does not immediately lead to a significant increase in production of CD14. Binding of the β-glucan to CD14 may well result in internalisation of the receptor and the observation of reduced levels, as was seen in the first experiment for donors 2 and 3. In contrast, once the β-glucan has been removed, and after a further incubation for 24 h, the cells now respond by significantly increasing the amount of the CD14 receptor present. As CD14 is a known receptor for bacterial antigens this might be expected to increase the signalling pathways leading to the release of proinflammatory mediators normally activated by antigens such as LPS but the reverse was observed (see discussion describing TNF-α expression below).

LPS stimulation, without EPS pretreatment (red bars, Fig 5B) led to a moderate decrease in CD 14 expression in two out of three donors ($p < 0.0001$ for Donor B and $p < 0.01$ for Donor C) whilst the levels of CD14 markers were marginally higher in Donor A ($p < 0.0001$). The latter results are identical to what was observed for the β-glucan in the first experiment and this suggests that the molecular mechanisms by which LPS and EPS stimulate PBMC may be the same. PHA is known to attenuate and down regulate expression of CD14 and this was observed in the second set of

experiments ($p < 0.0001$ for all three donors). Pre-treatment with the β-glucan only marginally reduced the decrease in expression caused by PHA, as seen in Donor B ($p < 0.01$).

The effect of PBMC stimulation on TLR-2 expression was less consistent and the only result of note is that there was a clear decrease, in the second experiment, in TLR-2 expression after β-glucan pre-treatment, followed by LPS stimulation, compared to the LPS stimulated alone ($p < 0.05$ for Donor C and $p < 0.0001$ for Donors A and B, red versus pink bars Fig 6). For cells treated with PHA the data was also mixed with a stimulation of TLR-2 expression occurring in one donor and a reduction in one, in all cases pre-treatment with β-glucan had no effect.

Figure 6.

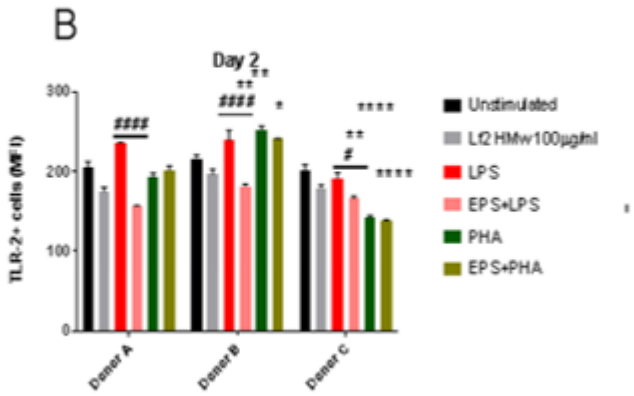


Fig. 6. Changes in TLR-2 expression on PBMC following different conditions presented as median fluorescence intensity (MFI) of TLR-2^+ cells for the Day 2 experiment. Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100µg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively on Day 1.

Following 24h incubation, cells were harvested and stained with anti-TLR-2 antibody and analysed by flow cytometry. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ when compared to the untreated, unstimulated condition; # $p < 0.05$, #### $p < 0.0001$ when compared between two indicated conditions. Gates were set on single cells and debris was excluded using FSC vs SSC plots. Gating was performed using FMO (Fluorescence Minus One) controls.

2.5 Stimulation of TNF-α secretion.

Treatment of PBMC with HMw-EPS gave rise to a very large increase in TNF-α secretion, for both concentrations of the polysaccharide (Fig 7A). TNF-α is a proinflammatory mediator and is produced when LPS binds to CD14 activating the signalling pathway [19], the current results are consistent with a similar activation pathway being present for the HMw-EPS: binding of the glucan to CD14 leading to the release of TNF-α. As was the case in the previous studies, in the next series of experiments PBMC cells were treated with HMw-EPS for 24 h, the β-glucan was removed before adding either no stimulant or one of the stimulants LPS or PHA and incubating the cells for a further 24 h. When the PBMC cells were left unstimulated in the second part of these experiments the levels of TNF-α returned to values similar to the controls. On first inspection, this result is not surprising: the β-glucan is no longer present to bind to CD-14 and, as such, the signalling pathway should no longer be activated. However, results with LPS suggest an alternative explanation involving desensitization of the signalling pathway could also explain this result.

When LPS was added as a stimulant to cells that had been exposed to the HMw-EPS, a very significant reduction in TNF-α production was observed compared to the control in which the PBMC cells had not been exposed to the HMw-EPS where a large

377 increase in TNF-α production was observed (Fig 7B red versus very small pink bars).
378 The failure to observe TNF-α secretion in cells which had been treated with HMw-EPS
379 and then exposed to LPS could be thought of as unexpected. However, this type of
380 desensitization is observed in other signalling pathways, a number of activation
381 cascades include steps in which there is a transformation of a binding component e.g.
382 by phosphorylation, which desensitizes a cell surface receptor such that the signal can
383 be rapidly stopped [53]. This would have an advantage during the release of
384 proinflammatory mediators where it would prevent an excessive inflammatory
385 response. Before the signal can be reinstated, the receptor needs to be recycled so it
386 can be reactivated. Incubation of PBMC cells with PHA produced a robust stimulation
387 of TNF-α confirming that the cells used here were immune competent and although
388 less dramatic than after LPS stimulation pre-treatment with the β-glucan also reduced
389 the amount of TNF-α secreted.

390 The key findings on the role of the HMw-EPS on the immune stimulation of PBMC are
391 that when the cells were pretreated with the β-glucan and subsequently stimulated
392 with the bacterial antigen LPS the secretion of TNF-α was virtually abolished. This
393 effect is at least in part mediated by a reduction in the TLR-2, which is a target for
394 LPS. When the PMBC that had been pretreated with the β-glucan were stimulated with
395 PHA there was also a reduction in the secretion of TNF-α but this was much less
396 marked. These data suggest that this β-glucan which of bacterial origin has a greater
397 role in reducing the inflammation produced by LPS which is produced by enteric
398 bacteria. However, the PHA data indicate that the β-glucan can play a more general
399 role in the reduction of inflammation.

Figure 7.

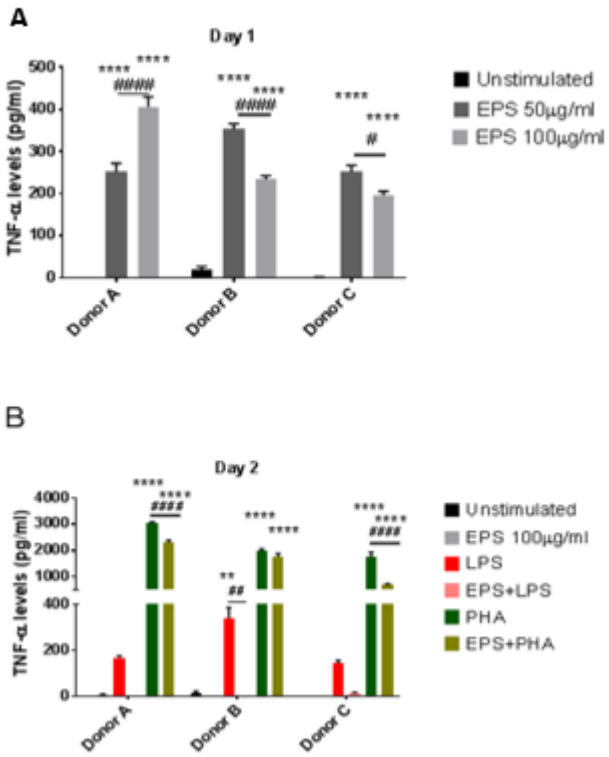


Fig. 7. Changes in TNF-α levels in cell culture supernatants following different conditions after **A**. Day 1 and **B**. Day 2 experiment. **A** For Day 1, Unstimulated conditions were left untreated while Lf2 HMw 50µg/ml and Lf2 HMw 100µg/ml were treated with EPS at indicated concentrations on Day 0 and incubated at 37˚C, 5% CO_2. Following 24h incubation, supernatants were stored at -80˚C until subsequent analysis for TNF-α levels by ELISA. **B** For Day 2 experiment, Unstimulated condition was left untreated on Day 0, then washed and left unstimulated on Day 1. Lf2 HMw 100µg/ml were pre-treated at Day 0 with indicated concentration of EPS, then washed and left unstimulated on Day 1. LPS and PHA alone are conditions that were left untreated on Day 0, then washed and subsequently stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively. EPS + LPS and EPS + PHA represent the conditions that were pre-treated with EPS Lf2 HMw on Day 0, then washed and stimulated with 100ng/ml LPS and 10µg/ml PHA, respectively on Day 1. Following 24h incubation, supernatants were stored at -80˚C until subsequent analysis for TNF-α levels by ELISA. Two-Way ANOVA was used to compare the conditions for each donor. Data are presented as Mean ± SEM. ** $p < 0.01$, **** $p < 0.0001$ when compared to the

417 untreated, unstimulated condition; # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ when
418 compared between two indicated conditions

419

420 TNF-α is a key proinflammatory cytokine, the blocking of its release by the HMw-EPS
421 could produce an immune tolerant phenotype, with potentially important implications
422 given TNF-α has a central role in the aetiology of inflammatory bowel diseases such
423 as Crohn's disease and ulcerative colitis. Further work is needed to determine the
424 mechanism by which the HM_W-EPS blocks TNF-α production.

425 **3. Experimental Section**

426 *3.1 Materials*

427 Unless otherwise stated, reagents were purchased from Sigma-Aldrich Company
428 Ltd. (Poole, Dorset UK) and were used as supplied.

429 *3.2 EPS production and purification***.**

430 *L. fermentum* Lf2 was grown at the Instituto de Lactologia Industrial (Santa Fe,
431 Argentina). The growth conditions, as well as the crude EPS extraction and purification
432 methods have been reported [31, 54]. Size Exclusion Chromatography coupled with
433 Multi-Angle Laser Light Scattering (SEC-MALLS-Wyatt technology, Santa Barbara, CA,
434 USA) was used to determine the composition of the crude EPS. EPS samples (1 mg/mL)
435 were prepared in aq. $NaNO_3$ (0.1M) and stirred for 16 h to ensure the EPS was
436 completely dissolved. Samples (100 µL) were injected, in triplicate, into a SEC-MALLS
437 system (three columns connected in series: PL Aquagel-OH 40, 50 and 60 (8 µm, 30
438 cm x 7.5 mm, Agilent, Cheadle, UK) with a flow rate of 0.7 mL/min. A differential
439 refractometer (Optilab rEX, Wyatt technology, Santa Barbara, CA, USA) was used to
440 determine the concentration of the polysaccharide and a Dawn-EOS MALLS detector

441 (laser operating at 690 nm) was used to determine the weight average molecular mass
442 of the polysaccharide. An in-line UV detector (Shimadzu, Milton Keynes, UK) was used
443 for the detection of proteins and nucleic acids. ASTRA version 6.0.1 software (Wyatt
444 technology, Santa Barbara, CA, USA) was used for the data analysis.

445 The crude EPS was purified by preparative size exclusion chromatography on a
446 Sephacryl S-500 HR column (XK26/60-GE Healthcare, Fisher Scientific, UK) eluting
447 with ultrapure water at a flow rate of 5.0 mL min^{-1} using a FPLC kit (AKTA PRIME-
448 Amersham Pharmacia, Biotech, GE Healthcare Life Sciences, Buckinghamshire, UK)
449 with a set wavelength of 254 nm. In total, sixty 5 mL fractions were collected and the
450 location of polysaccharides in the different fractions was identified by determining the
451 carbohydrate content of each fraction using the Dubois method [55, 56]. Fractions
452 containing EPS were pooled and freeze-dried.

453 *3.3 NMR analysis of the high molecular mass EPS***.**

454 NMR spectra of the EPS were recorded in solution in D_2O (5-10 mg in 0.65 mL)
455 and were run at 70 °C on Bruker NMR spectrometers at either 400, 500 or 600 MHz
456 (Bruker-biospin, Coventry, UK) using Bruker's TOPSPIN 4.0.1 software for analysis.
457 Chemical shifts are expressed in ppm relative to internal acetone, 2.225 for ^{1}H and
458 31.55 for ^{13}C. A series of 2D-spectra were recorded including: a 2D gradient-selected
459 double quantum filtered correlation spectrum (gs-DQF-COSY) recorded in magnitude
460 mode; a total correlation spectroscopy (TOCSY) experiment recorded with a mixing
461 times of 120 ms; ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra
462 (decoupled and coupled); and finally, a rotating frame nuclear Overhauser effect
463 spectrum (ROESY, mixing time of 200 ms). The 2D spectra were recorded with 256
464 experiments of 1024 data points. For the majority of spectra, time-domain data were

multiplied by phase-shifted (squared-) sine-bell functions. After applying zero filling and Fourier transformation, data sets of 1024-1024 points were obtained.

*3.4 Composition of the high molecular weight polysaccharide***.**

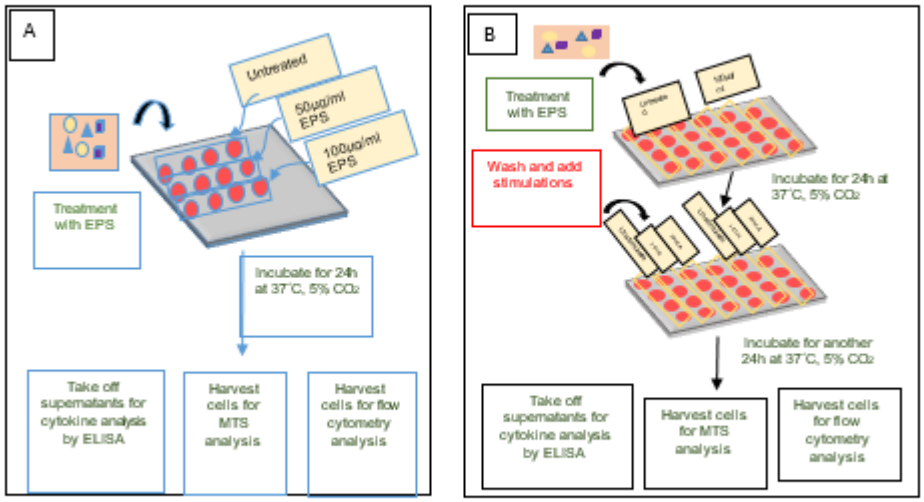
The monosaccharides present were determined after acid hydrolysis either directly using HPAEC-PAD analysis or as their alditol acetates, as previously described [24]. The absolute configuration of the sugars was determined by preparation of their respective 2-(*S*)-butylglycosides using Gerwig's method [57]. For linkage analysis, the samples were permethylated using the procedures described by Stellner [58].

3.5 PBMC isolation and cell culture.

Venous blood was collected after obtaining informed consent from three healthy volunteers in blood collecting tubes containing citrate-dextrose solution (ACD) at a whole blood to anticoagulant ratio of 9:1. The study was approved by the Local Ethics Committee (School of Applied Sciences, University of Huddersfield, UK). Blood samples were layered over Histopaque®-1077 and PBMC isolated using a standard density gradient centrifugation protocol. Enriched PBMC were suspended in RPMI 1640 medium (Gibco, Life Technologies, UK) supplemented with 4 mM L-glutamine, 50 IU/ml/50 µg/ml/ penicillin/streptomycin and 20 mM HEPES (all from Gibco, Life Technologies, UK) and 10% heat-inactivated fetal bovine serum (FBS) and $2x10^6$ cells/well were added to 24-well plates (Starlab, UK). The cells were then challenged as shown in Figure 8. For the Day 1 experiment (Fig 8A), cells were treated with two different concentrations of EPS (50 µg/mL and 100µg/mL) or left untreated, four replicates were set up for each condition. For the Day 2 experiment (Fig 8B), at Day 0, half of the wells were treated with 100 µg/mL EPS while the other half was left

489 untreated. Cells were incubated for 24 h, at 37 ˚C, 5% CO_2 after which they were
490 washed with supplemented RPMI 1640 and stimulated with either LPS
491 (Lipopolysaccharides from *Escherichia coli* O111:B4) at a final concentration of 100
492 ng/mL or PHA-M (Lectin from *Phaseolus vulgaris* (red kidney bean)) at 10 µg/ml, or
493 left unstimulated (Fig 8B) (final incubation of 24 h, at 37 ºC, 5% CO_2). Each condition
494 was set up in four replicates.

Figure 8.



495

496 **Fig. 8.** Schematic representation of the cell culture protocol completed on **A**.
497 Experiment 1 and **B**. Experiment 2. **A**. On Day 0, cells were treated with two different
498 Lf2 HMw EPS concentrations (50µg/ml and 100µg/ml) or left untreated at 37˚C, 5%
499 CO_2. Following 24h incubation, supernatants were taken off and stored at -80˚C for
500 subsequent analysis by ELISA. Cells were harvested and $1x10^5$ cells/well were
501 transferred into 96-well plates for MTS assay performed following the supplier's
502 instructions. The remaining cells were stained with antibodies for CD14 and TLR-2
503 and analysed for the expression of these markers by flow cytometry. **B**. on Day 0 cells
504 were pre-treated with 100µg/ml EPS or left untreated and incubated for 24h at 37˚C,
505 5% CO_2. On Day 1, cells were washed to remove EPS and were stimulated with LPS
506 (100ng/ml) or PHA (10µg/ml) or left unstimulated and incubated for another 24h at
507 37˚C, 5% CO_2. On Day 2, supernatants were then stored at -80˚C for subsequent

cytokine analysis by ELISA. As before $1x10^5$ of harvested cells/well were transferred into 96-well plate for MTS assay while remaining cells were stained for flow cytometric analysis of CD14 and TLR-2 expression.

3.6 MTS assay.

In order to measure the metabolic activity of the cell cultures under different conditions, the CellTiter 96® AQueous One Solution Cell Proliferation Assay, MTS assay (Promega, UK) was used as per the manufacturer's instructions. At the end of each experiment $1x10^5$ cells from individual wells were transferred into separate wells of a 96-well plate and tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulfate; PES) were added and the absorbance at 490nm was measured.

*3.7 Flow cytometry***.**

To monitor CD14 and TLR-2 expression the remaining PBMC were washed with FACS buffer (PBS, Lonza, UK, 1% Bovine serum albumin, BSA, 0.1% sodium azide, Sigma Aldrich, UK), and fixed with 1% paraformaldehyde (PFA, Sigma Aldrich, UK). Cells were then washed with permeabilisation solution (Perm Wash, BD Biosciences, UK) and stained with anti-CD14 and anti-TLR-2 antibodies (Biolegend, UK). Following the incubation, cells were washed with Perm Wash buffer and resuspended in 1% PFA until acquisition using The Guava® easyCyte flow cytometer (Merck Milipore, UK). Data were acquired using guavaSoft 2.7 software for Windows and analysed using its 3.1.1. version. Data were presented as median fluorescence intensity indicating the level of marker's expression (MFI of $CD14^+$ and MFI of $TLR\text{-}2^+$ cells). Doublet and debris exclusion were performed and gating was set based on Fluorescence Minus One (FMO) controls.

3.8 Measurement of TNF-α levels by ELISA.

Stored supernatants were used for the analysis of TNF-α concentration by ELISA (TNF alpha (Total) Human ELISA Kit, Invitrogen, UK) following the manufacturer's instructions. Briefly, the plate was coated with TNF-α specific capture antigen and left overnight. Following subsequent washes, non-specific binding was blocked using blocking buffer after which samples were added and incubated for 2 h at room temperature. Plates were then washed and biotinylated detection antibody was added, after which avidin horseradish peroxidase (avidin-HRP) was used to bind to the biotin on the detection antibody. Tetramethylbenzidine was then added as the substrate for the HRP enzyme, and the reaction was stopped with the addition of 1M sulfuric acid. Absorbance was read at 495nm using FLUOstar Optima plate reader (BMG Labtech). The data were analysed using MARS Data Analysis Software, and a 4-parameter logistic regression curve was used as the best fit for the data.

3.9 Statistical analyses.

Due to the variability between the donors, each was treated as a separate group and Two-Way analysis of variance (ANOVA) followed by Tukey's posthoc test for multiple comparison were used to examine the differences between the conditions. For each donor, data are presented as mean ± SEM (standard error of the mean) of the replicates of the conditions set up in experiment.

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