Bacteroides fragilis signals through Toll-like receptor (TLR) 2 and not through TLR4

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Although it is desirable to identify the interactions between endotoxin/LPS and the innate immune mechanism, it is often not possible to isolate these interactions from other cell wall-related structures of protein or polysaccharide origin. There is no universally accepted method to extract different LPSs from different bacteria, and their natural state will be influenced by their interactions with the associated molecules in the bacterial outer membrane. It is now believed that Toll-like receptor (TLR) 4 is the main signal transducer of classical LPS (i.e. *Escherichia coli* LPS), while TLR2 is used by certain non-classical LPSs. There are contradictory reports as to whether *Bacteroides fragilis* LPS, a non-classical LPS, signals primarily through TLR2 or TLR4. This study was designed to address this problem. Different non-purified and purified *B. fragilis* LPSs extracted by different methods together with different heat-killed, whole-cell populations of *B. fragilis* were used to elucidate the TLR specificity. All of these *B. fragilis* preparations showed a significant signalling specificity for TLR2 but not for TLR4. This indicates that changing the extraction methods, with or without applying a repurification procedure, and varying the cell populations do not alter the TLR specificity of *B. fragilis* LPS.

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INTRODUCTION

Species belonging to the genus *Bacteroides* are the predominant Gram-negative, anaerobic bacteria found in the normal faecal/colonic microbiota. *Bacteroides fragilis* is of great importance as an opportunistic pathogen and is commonly associated with bacteraemia, soft tissue infections, intra-abdominal infections and abscesses (Mancuso *et al.*, 2005) often following trauma to the colon. Other *Bacteroides* species such as *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* are found more commonly than *B. fragilis* in faeces, resulting in the commonly held view that it is not found in high concentrations in the gut; however, in some individuals it is the most common species of *Bacteroides* found attached to the colonic mucosa

(Poxton *et al.*, 1997). In terms of pathogenicity, the LPSs of *B. fragilis* have long been known to be of lower endotoxicity than those of other enteric bacteria such as *Escherichia coli* (Hofstad *et al.*, 1977). However, from studies in our laboratory we showed that the endotoxicity was dependent on the extraction technique used (Delahooke *et al.*, 1995).

B. fragilis produces antigenically distinct and within strain variable large capsules (LCs) and small capsules (SCs) distinguishable by light microscopy when cultures are grown in a glucose-defined medium. In addition, bacterial cells which appear to be non-capsulate by light microscopy may produce a marginal micro-capsule (MC) of approximately 35 nm in size visible as an electron-dense layer adjacent to the outer membrane, visible by electron microscopy. The electron-dense MC is also visible beneath the LC by electron microscopy, but is not associated with the SC (Patrick *et al.*, 1986). On primary isolation, individual cultures usually contain a mixture of LC, SC and MC cells. Populations of *B. fragilis* enriched for each of the different capsules can, however, be obtained by using Percoll density-gradient centrifugation (Reid *et al.*, 1987).

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Abbreviations: AP, aqueous phenol; BWP, boiling water/proteinase K; LAL, *Limulus* amoebocyte lysate; LC, large capsule; MC, micro-capsule; PCP, phenol/chloroform/petroleum spirit; PS, polysaccharide; SC, small capsule; TLR, Toll-like receptor; TM/TMP, Triton/magnesium chloride without and with proteinase K treatment.

Examination of the MC- and LC-enriched population indicates that there are multiple antigenically different polysaccharides variably expressed within single populations (Patrick *et al.*, 1999). The determination of the complete genome sequence of *B. fragilis* NCTC 9343 indicated that it lacks a single operon with similar genetic structure to the loci involved in O-antigen or lower-molecular-mass lipid A linked K antigen synthesis in *E. coli*. Unusually, however, there are eight PS loci (A–H) that are related to variable MC expression and each contains genes predicted to encode O-antigen polymerase (Wzy) and flippase (Wzx)-like proteins. The variable expression at seven of these loci is controlled by site-specific serine recombinase directed inversion of the promoter region (Cerdeno-Tarraga *et al.*, 2005; Patrick *et al.*, 2003). Investigation of NCTC 9343 by targeted gene deletion suggests that the antigenically variable MC equates to enteric LPS, but instead of the classical low-molecular-mass O-antigen, it contains a high-molecular-mass polysaccharide that forms the antigenically variable electron-dense MC visible by electron microscopy outside and adjacent to the outer membrane (Patrick *et al.*, 2009). Thus the LPS of *B. fragilis* differs from classical enteric LPS not only with respect to phosphorylation of the lipid A diglucosamine and 3-deoxy-D-manno-octulosonate (reviewed by Patrick, 2002), but also with respect to the O-antigen.

It is recognized widely today that innate immunity against LPS is considered to be one of the principal defences against Gram-negative bacterial infection. Of particular interest in this regard was the discovery of Toll-like receptors (TLRs), suggested to be a cornerstone in the LPS immuno-sensing machinery since they guided the elucidation of the cascade of molecules which collectively recognize LPS and eventually lead to the production of the innate inflammatory response (Alexander & Rietschel, 2001). Although TLR4, in particular, is considered to be the main signal transducer of classic LPS (Hirschfeld *et al.*, 2000; Lien *et al.*, 2000; Poltorak *et al.*, 1998), there are contradictory reports as to whether TLR2 (Erridge *et al.*, 2004, 2007) or TLR4 (Mancuso *et al.*, 2005) plays a principal role in signalling *B. fragilis* LPS as a non-classical LPS.

The main aim of this study was to investigate whether different *B. fragilis* LPS preparations from different extraction methods and different phenotypic subpopulations of *B. fragilis* signalled through the same Toll-like receptor.

METHODS

Bacterial strains and culture conditions. Strains of *B. fragilis* (NCTC 9343: MPRL 1669), *E. coli* O18K[−] (MPRL 1275) and *Porphyromonas gingivalis* (NCTC 11834) were used. MPRL numbers indicate strains from our laboratory culture collection. *B. fragilis* was grown in proteose-peptone yeast extract medium (Holbrook *et al.*, 1977) or defined medium (Van Tassell & Wilkins, 1978) under anaerobic conditions at 37 °C. *E. coli* O18K[−] was grown in nutrient

broth under aerobic conditions at 37 °C. *P. gingivalis* was grown in brain heart infusion (BHI) broth (Oxoid) supplemented with haemin (7.7 mM) under anaerobic conditions at 37 °C. All cultures were checked for purity by Gram stain and subculture under aerobic and anaerobic conditions.

Extraction of LPSs. Five different *B. fragilis* LPS preparations were obtained by extraction of lyophilized bacterial cells by four different extraction methods, namely: (i) the aqueous phenol (AP) method as described originally by Westphal & Luderitz (1953); (ii) the phenol/chloroform/petroleum spirit (PCP) method of Galanos *et al.* (1969) with the modification of Qureshi *et al.* (1982); (iii) the Triton/magnesium chloride method, applied to extract two kinds of LPS with (TMP) and without (TM) proteinase K treatment according to the work of Uchida & Mizushima (1987); and finally (iv) the boiling water/proteinase K treatment (BWP) method, which was applied to extract LPSs as proposed by Eidhin & Mouton (1993). *E. coli* and *P. gingivalis* LPSs were extracted using the AP method (i) only. Full details of extraction methods (i) and (ii) are described in Hancock & Poxton (1988).

Repurification of LPS preparations. All LPS samples were subjected to a repurification step to eliminate the protein contaminants which might be responsible for TLR2 signalling according to the procedure described by Manthey & Vogel (1994) and further detailed by Hirschfeld *et al.* (2000).

Preparation of heat-killed bacteria. For TLR specificity assays, three different subpopulations of *B. fragilis* were prepared by discontinuous Percoll density-gradient centrifugation of broth cultures grown in a defined medium (Van Tassell & Wilkins, 1978) which contained a mixed population of LC, SC and electron-dense MC layer cells. Populations enriched for expression of each of the LC, SC or MC were prepared by subculture from the 0–20% Percoll, 20–40% Percoll and 60–80% Percoll interface layers, respectively. Enrichment for capsule expression was monitored by negative capsule staining (Patrick *et al.*, 1986). The bacteria were heat-killed in a 95 °C water bath for 30 min.

Analysis of LPS preparations. After LPSs were extracted, the preparations were examined by three procedures: firstly, PAGE (12% without SDS in the gel buffers, but included in the sample buffer) was prepared with the buffer system of Laemmli (1970) according to the method of Poxton & Brown (1979). To visualize LPSs, gels were processed according to the method of Tsai & Frasch (1982) as described by Hancock & Poxton (1988). Secondly, protein bound to LPS samples was detected by colloidal gold total protein stain (Bio-Rad) according to the manufacturer's instructions. Finally, the endotoxic activity of LPS samples was determined by *Limulus* amoebocyte lysate assay (LAL assay) using the Pyrochrome LAL kit (Associates of Cape Cod) according to the manufacturer's instructions. The A₄₀₅ was read on an Anthos 2001 automated plate reader.

Cells and transfection assays. LPS and heat-killed bacteria signalling via TLR2 or TLR4/MD2 was assessed using a TLR-deficient human embryonic kidney (HEK) 293 cell line transfection assay as previously described (Erridge *et al.*, 2007). Briefly, HEK-293 cells were plated in 96-well plates at 8×10^3 cells per well and transfected after 24 h using GeneJuice (Novagen) according to the manufacturer's instructions. Amounts of construct per well were 10 ng human pTLR2, 30 ng human pTLR4/MD2 (Invivogen), 30 ng pCD14 (kind gift of Professor Christopher Gregory, University of Edinburgh, UK), 20 ng renilla reporter construct and 10 ng luciferase-reporter construct driven by the NF- κ B-regulated E-selectin promoter (pELAM), with the balance made up with empty pCMV. A plasmid encoding human mCD14 as a co-transfectant was always included in every transfection assay for both TLR2 and TLR4 transfections. The

control transfection lacking TLR plasmids had CD14 plasmid and reporters alone. Seventy-two hours after transfection, cells were challenged for a further 18 h with medium alone (DMEM/1% FCS), 0.1–1000 ng each LPS ml⁻¹ and 10⁷ heat-killed bacteria ml⁻¹. Reporter levels were normalized to co-transfected renilla expression as an internal transfection efficiency control. Promoter expression is represented as fold induction of normalized pELAM signal relative to cells cultured in medium alone $\pm$ SD from triplicate wells.

Statistical analysis. Statistical analysis was done by ANOVA.

RESULTS AND DISCUSSION

In this study, *B. fragilis* LPS preparations were extracted by five different methods and then all were further repurified according to the method of Hirschfeld *et al.* (2000). These methods were chosen to produce different forms of LPS: predominantly smooth, high-molecular-mass material is extracted by the AP method and rough, low-molecular-mass material is extracted by the PCP method. The other three extraction methods, TM, TMP and BWP treatment, are much milder than the first two methods as proposed by Poxton & Edmond (1995). LPS yields ranged from 0.23% of the dry weight of the bacterial cell for the PCP-extracted material to 5.12% of the dry weight of the bacteria for the BWP method, with the others between 1 and 2%. The PCP-extracted material was also the most difficult to solubilize. This is in agreement with previous studies that reported that applying the PCP method to extract LPS from *B. fragilis* dry bacteria results in only a small amount of the LPS (Hofstad *et al.*, 1977). In fact, it is found that

high-molecular-mass capsular polysaccharide makes the cell envelope more hydrophilic, to the extent it becomes insoluble in the hydrophobic PCP mixture (Weintraub *et al.*, 1985). It is also generally recognized that the solubility of rough LPS in water is poor (Hellman *et al.*, 2003). The other LPS preparations were readily soluble.

Analysis by PAGE (results not shown) revealed that all non-purified extracts contained protein material as revealed by colloidal gold staining, with the PCP-extracted material (Bf3) being the most contaminated and the AP-extracted material (Bf5) the least. All non-purified extracts also contained some high-molecular-mass polysaccharide materials with the exception of the Bf3 preparation. Only after repurification was the lipid A and core oligosaccharide revealed, appearing as rough-form LPS as a single band at the gel front. At this stage, all protein bands were absent while the high-molecular-mass polysaccharide material was apparent in all but Bf3. The *E. coli* extracts all contained lipid A and core oligosaccharide at the gel front with the typical higher-molecular-mass ladder pattern of smooth LPS. In addition to LPS PAGE analysis, the endotoxic activity of *B. fragilis* non-purified and purified LPSs was measured by the end-point method of the LAL assay and the results are shown in Fig. 1. This demonstrates that the LAL activity of most *B. fragilis* LPSs is much lower than the activity of classical LPSs like those of *E. coli*. However, Bf5n, which represents LPS extracted by the AP method, is approximately fivefold more active than other *B. fragilis* LPSs at a concentration of 0.1 ng LPS ml⁻¹ (Fig. 1). It also showed a statistically significant difference among all other

Fig. 1. Comparison of the ability of non-purified and purified *B. fragilis* and *E. coli* endotoxins to stimulate the LAL reaction at 0.1 ng (black bars) and 0.01 ng (grey bars) LPS ml⁻¹. Bf1n, non-purified *B. fragilis* LPS extracted by TM; Bf1p, purified Bf1n LPS; Bf2n, non-purified *B. fragilis* LPS extracted by TMP; Bf2p, purified Bf2n LPS; Bf3n, non-purified *B. fragilis* LPS extracted by PCP; Bf3p, purified Bf3n LPS; Bf4n, non-purified *B. fragilis* LPS extracted by BWP; Bf4p, purified Bf4n LPS; Bf5n, non-purified *B. fragilis* LPS extracted by AP; Bf5p, purified Bf5n LPS; Ec, purified *E. coli* LPS extracted by AP; HPW, highly purified water; Blank, empty wells. Results represent the means ($\pm$ SD) of two similar experiments. *Bf5n LAL activity significantly higher than for other samples at 0.1 ng ml⁻¹ except for Ec ($P < 0.01$). †Ec LAL activity significantly higher than for other samples at 0.1 ng ml⁻¹ ($P < 0.01$).

B. fragilis non-purified and purified LPSs ($P > 0.01$) at 0.1 ng ml^{-1} but not at 0.01 ng ml^{-1} . This enhanced activity of the AP-extracted material may have been an artefact of the purification process as weight for weight it contains more LPS than the more heavily protein-contaminated material of the other extracts. It is reported that the endotoxic activity of *B. fragilis* LPS when extracted by the PCP method is considered to be lower than that of the classical LPSs such as those of *E. coli* (Erridge *et al.*, 2007; Lindberg *et al.*, 1990; Weintraub *et al.*, 1989). However, *B. fragilis* LPSs extracted by the AP method showed high endotoxic activity which may be comparable to that of classical LPS (Delahooke *et al.*, 1995; Poxton & Edmond, 1995). In this regard, the LAL assay is one of the most sensitive and highly specific methods for detecting and quantifying the existence of endotoxin and comparing the endotoxin intensity between different LPS preparations (Nakagawa *et al.*, 2002; Seydel *et al.*, 2003). However, it is unknown whether LPS activity in this assay is linked to the ability of LPS to induce inflammatory immune responses for reasons like the obvious phylogenetic distance between the horseshoe crab and human cells (Nakagawa *et al.*, 2002). Therefore, the assay may not always be a reliable endotoxicity indicator at the cellular and *in vivo* levels (Seydel *et al.*, 2003).

It has been observed that LPS activity can differ significantly according to the type of organism, method of cultivation and the technique by which the LPS is extracted (Fukushi *et al.*, 1964; Morrison & Leive, 1975). Moreover, although LPS activity is particularly associated with the lipid A moiety, it can also be affected by other materials which can be present in the final preparation, such as outer-membrane proteins (Hellman *et al.*, 2003; Morrison *et al.*, 1976), the specific O-antigen subunit (Vukajlovich & Morrison, 1985) and the O-antigen carbohydrates (Morrison *et al.*, 1987). In the case of *B. fragilis*, LPS-associated contamination is not always assumed to be of protein origin (Mancuso *et al.*, 2005). Additionally, previous studies have revealed that non-protein (Muroi *et al.*, 2003) and protein (Lee *et al.*, 2002) components which are presented in association with LPS preparations have LPS-like activities. One early study using purified high-molecular-mass capsular polysaccharide of *B. fragilis* showed that these molecules have the capacity to induce interleukin-8 production from human monocytes or polymorphonuclear leukocytes (Gibson *et al.*, 1996). Although highly purified LPS is essential for studying its biological activity *in vitro* (Kutuzova *et al.*, 2001), LPS in itself is a particularly difficult ligand to work with because of its amphipathicity, which neither makes it easily soluble nor allows the formation of LPS monomers in aqueous suspension (Lien *et al.*, 2000). Furthermore, during extraction, when LPS is subjected to denaturing steps, many of the contaminating proteins and other substances become tightly bound to the LPS molecule and cannot be detached completely (Rudbach & Proctor, 2001). Therefore, use of a repurification procedure to prepare

LPS in a highly purified form has been one of the important goals in the LPS research field, especially when it is intended to investigate the specific signalling activity of the LPS (i.e. the TLR specificity).

The repurification method of Manthey & Vogel (1994) was used by many investigators until Hirschfeld *et al.* (2000) gave this method another dimension when it was used to verify the actual TLR specificity of LPS through eliminating the protein contaminants which are usually associated with LPS preparations. This repurification method had a noticeable effect on both the gel appearance of *B. fragilis* LPS preparations tested in this study (removing the protein contamination) and the intensity of the signalling response. However, it is totally unreasonable to consider that the non-purified LPS is without its uses since it represents a powerful tool for studying an immune inflammatory response against LPS. After all, these non-purified LPS preparations more closely mimic the LPS that would be encountered by host cells subjected to either whole bacteria or released LPS-containing membranous vesicles. For the same reason, different heat-killed bacterial populations were involved in the TLR specificity assay as discussed later. In fact, one study supports this notion since it reported that fluorescein-labelled *E. coli* was able to pass across the rat intestinal epithelium but fluorescein-conjugated *E. coli* LPS was unable to do so, even after disruption of the intestinal epithelium with a potent mucolytic agent (Benoit *et al.*, 1998). The ability to penetrate the intestinal barrier exposes LPS directly to immune cells such as monocytes. Therefore, it is plausible to suggest that using both LPS extraction methods and an extra technique to repurify LPS contributed much to this study in terms of comparing different LPS preparations of different levels of purity.

Using a transient transfection with the efficiency control of TLR4/MD2, TLR2 and CD14 receptors into HEK-293 cells, comparison experiments were done to examine the TLR specificity. The five non-purified and five purified *B. fragilis* LPS preparations extracted by different methods together with the heat-killed *B. fragilis* from the three different subpopulations were tested in this assay. The results presented in Fig. 2 clearly demonstrate the TLR2 highly significant specificity of all five non-purified *B. fragilis* LPS preparations. Most of them showed a stronger TLR2 specificity than the TLR2-positive control LPS (i.e. *P. gingivalis* LPS), except Bf3n, which showed an almost equal TLR2 specificity to that of the TLR2 positive control LPS. Bf1n LPS which was extracted by the TM method showed the strongest TLR2 specificity. However, none of the five non-purified *B. fragilis* LPS preparations demonstrated a TLR4 specificity that was comparable to that of the TLR4 positive control LPS (i.e. *E. coli* LPS). It should be noted that when non-purified LPS extracted by the PCP method from *E. coli* is used at 100 ng ml^{-1} in such an assay there was clearly TLR2 specificity as well as TLR4/MD2, at 9.1 and 13.7-fold induction (pELAM), respectively (data not shown).

Fig. 2. Determination of TLR-signalling pathways initiated by five non-purified *B. fragilis* endotoxin preparations. TLR-deficient HEK-293 cells were transfected with CD14 alone (unfilled bars), with TLR4/MD2 (grey bars) or with CD14 plus TLR2 (black bars). Cells were then challenged with 1 μg non-purified *B. fragilis* endotoxin ml^{-1} for 18 h. The degree of induction of a co-transfected NF- κ B-sensitive reporter construct (pELAM) is shown, relative to cells cultured in medium alone (Ctrl). Results are presented as means of triplicate wells and are representative of three independent experiments. Ec, Repurified *E. coli* LPS at 10 ng ml^{-1} ; Pg, repurified *P. gingivalis* LPS at 100 ng ml^{-1} . *TLR2 signal significantly higher than for TLR4/MD2, CD14 and cells cultured in medium alone ($P < 0.05$). *B. fragilis* preparations as in Fig. 1.

For the purified LPS preparations of *B. fragilis* (Fig. 3), the results again reveal the high TLR2 specificity of Bf1p, Bf4p and Bf5p LPSs, which were extracted by the TM, BWP and AP methods, respectively, and good TLR2 specificity but

with a lower response with Bf2p and Bf3p, which were extracted by the TMP and PCP method, respectively. The TLR2 positive control LPS response was stronger than that for all five purified *B. fragilis* LPS preparations. Bf5p LPS

Fig. 3. Determination of TLR-signalling pathways initiated by five purified *B. fragilis* endotoxin preparations. TLR-deficient HEK-293 cells were transfected with CD14 alone (unfilled bars), with TLR4/MD2 (grey bars) or with CD14 plus TLR2 (black bars). Cells were then challenged with 1 μg purified *B. fragilis* endotoxin ml^{-1} for 18 h. The degree of induction of a co-transfected NF- κ B-sensitive reporter construct (pELAM) is shown, relative to cells cultured in medium alone (Ctrl). Results are presented as means of triplicate wells and are representative of three independent experiments. Ec, Repurified *E. coli* LPS at 10 ng ml^{-1} ; Pg, repurified *P. gingivalis* LPS at 100 ng ml^{-1} . *TLR2 signal significantly higher than for TLR4/MD2, CD14 and cells cultured in medium alone ($P < 0.05$). *B. fragilis* preparations as in Fig. 1.

shows the strongest TLR2 response and none of the five purified *B. fragilis* LPS preparations demonstrated a TLR4 specificity that was comparable with that of the TLR4 positive control LPS. In fact, the TLR2 specificity for all of the five purified *B. fragilis* LPS preparations was significantly higher than their TLR4 and CD14 specificities ($P > 0.05$). To examine the possibility of the presence of TLR4 agonist attached to *B. fragilis*, heat-killed populations enriched for expression of either the LC, SC or MC were examined for their TLR specificity. The results are shown in Fig. 4, where the TLR specificity of MC, SC and LC heat-killed *B. fragilis* populations shows the same pattern as the TLR2 positive control LPS. Again, none of the preparations demonstrated any TLR4 specificity that was comparable with that of the TLR4/MD2 positive control LPS (i.e. *E. coli*).

The main aim of this study was to elucidate the TLR specificity of different *B. fragilis* LPS preparations extracted by different methods and different heat-killed *B. fragilis* populations. Comparison between Figs 2 and 3 clearly demonstrates that the repurification method made a noticeable difference in the intensity of the TLR2 signalling but contributed nothing to the TLR specificity. The contribution of the repurification method was clearly profound with all extraction methods and especially with the TMP and PCP methods. It is also possible to assume that although different extraction methods may affect the conformational properties of lipid A moieties of different *B. fragilis* LPSs, this effect did not change the TLR specificity. This is clearly illustrated through all preparations of non-purified and purified *B. fragilis* LPSs, together

with different heat-killed *B. fragilis* populations which had an obvious TLR2 specificity through the transfection experiment. None of them produced an LPS structure that signalled through TLR4 even after applying the repurification method (Figs 2, 3 and 4).

Using heat-killed *B. fragilis* subpopulations that express different surface polysaccharide structures was further confirmation that *B. fragilis* does not have a ligand that can signal through TLR4. However, our findings support the notion that biologically active LPS-associated components are also present in *B. fragilis* since the TLR2 signalling response from heat-killed bacteria or non-purified *B. fragilis* LPSs was higher than that from purified LPSs. This may be in part due to other pro-inflammatory LPS-associated molecules without excluding the role of ligands such as peptidoglycan fragments which are known to signal via TLR2. Many experimental differences between *in vitro* assays and *in vivo* models to test LPS-signalling machinery should be taken into consideration when trying to understand what really happens inside the host. For example, the *in vitro* assay, whatever its type, a fresh blood sample from volunteers or cell line culture, is not subjected to the remarkable decrease in numbers, as occurs in *in vivo* model, of circulatory monocytes in particular and white blood cells counts in general after LPS administration as shown by Richardson *et al.* (1989). One study suggested that TLR2 and TLR4 are initially downregulated on monocytes 2 h after the *in vivo* LPS administration, and they were then upregulated reaching a significant level for TLR2 by 8 h (Marsik *et al.*, 2003).

Fig. 4. Determination of TLR-signalling pathways initiated by three populations of heat-killed (Hk) *B. fragilis* expressing MCs, SCs and LCs. TLR-deficient HEK-293 cells were transfected with CD14 alone (unfilled bars), with TLR4/MD2 (grey bars) or with CD14 plus TLR2 (black bars). Cells were then challenged with 10^7 heat-killed *B. fragilis* for 18 h. The degree of induction of a co-transfected NF- κ B-sensitive reporter construct (pELAM) is shown, relative to cells cultured in medium alone (Ctrl). Results are presented as means of triplicate wells and are representative of three independent experiments. Ec, Repurified *E. coli* LPS at 10 ng ml^{-1} ; Pg, repurified *P. gingivalis* LPS at 100 ng ml^{-1} . *TLR2 signal significantly higher than for TLR4/MD2, CD14 and cells cultured in medium alone ($P < 0.05$).

The reason for the inconsistency between this study's findings together with those of Erridge *et al.* (2004, 2007), which all showed TLR2 specificity of *B. fragilis* LPS and heat-killed *B. fragilis*, and those of Mancuso *et al.* (2005), which showed TLR4 specificity of *B. fragilis* LPS, is unclear. However, this discrepancy may be due to culture conditions or even mixed bacterial cultures. In this regard, the findings of the current study are consistent for a diverse range of *B. fragilis* LPS preparations together with different heat-killed whole *B. fragilis* cells.

Although the TLR specificity of the classical LPSs, such as those of *E. coli*, went through a period of some controversy, it is now generally accepted that TLR4 is an essential receptor for such an LPS despite the fact that as yet there is no clear evidence for the direct physical contact between TLR4 and LPS. This is not to say, however, that TLR4 is the universal and only receptor from the TLR receptor family that detects different LPS structures. In fact, many important studies demonstrating TLR4 as an essential receptor for LPS signalling have concluded this by using enterobacterial LPS of a classical kind, especially that extracted from *E. coli* or *Salmonella* (Faure *et al.*, 2000; Hirschfeld *et al.*, 2000; Hoshino *et al.*, 1999; Lien *et al.*, 2000; Poltorak *et al.*, 1998, 2000; Tapping *et al.*, 2000). This may have occurred because endotoxin preparations from these bacteria are commercially available and the structures of both are well characterized as mentioned by Erridge *et al.* (2007).

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