

# Non-enterobacterial endotoxins stimulate human coronary artery but not venous endothelial cell activation via Toll-like receptor 2

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## Abstract

**Objective:** To determine whether non-enterobacterial endotoxins, which are likely to constitute the majority of the circulating endotoxin pool, may stimulate coronary artery endothelial cell activation.

**Methods and results:** Interleukin-8 secretion, monocyte adhesion, and E-selectin expression were measured in human umbilical vein endothelial cells (HUVECs) and coronary artery endothelial cells (HCAECs) challenged *in vitro* with highly purified endotoxins of common host colonisers *Escherichia coli*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and *Bacteroides fragilis*. HCAECs but not HUVECs expressed Toll-like receptor (TLR)-2 and were responsive to non-enterobacterial endotoxins. Transfection of TLR-deficient HEK-293 cells with TLR2 or TLR4/MD2 revealed that while *E. coli* endotoxin utilised solely TLR4 to signal, the endotoxins, deglycosylated endotoxins (lipid-A), and whole heat-killed bacteria of the other species stimulated TLR2-but not TLR4-dependent cell-signalling. Blockade of TLR2 with neutralizing antibody prevented HCAEC activation by non-enterobacterial endotoxins. Comparison of each endotoxin with *E. coli* endotoxin in limulus amoebocyte lysate assay revealed that the non-enterobacterial endotoxins are greatly underestimated by this assay, which has been used in all previous studies to estimate plasma endotoxin concentrations.

**Conclusion:** Circulating non-enterobacterial endotoxins may be an underestimated contributor to endothelial activation and atherosclerosis in individuals at risk of increased plasma endotoxin burden.

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**Keywords:** Endotoxins; Endothelial receptors; Infection/inflammation; Signal transduction; Atherosclerosis

## 1. Introduction

Inflammatory activation of the endothelial cell (EC) is a critical step in the development of atherosclerosis [1], and the agents responsible are being sought. Bacterial endotoxin (lipopolysaccharide, LPS), the major lipid component of the Gram-negative cell wall, is increasingly being considered to be a potentially significant contributor to EC activation and atherosclerosis [2–4] and reviewed in Ref. [5]. Recent studies have revealed that endotoxin exists in the plasma of all healthy individuals at concentrations between 3 and 200 pg/ml [3,6–9]. While such low concentrations of

endotoxin were previously considered to be insufficient to affect the vasculature, recent evidence has shown that some markers of endothelial activation are increased following exposure to as little as 50 pg/ml endotoxin [2] and that baseline endotoxin levels greater than 50 pg/ml correlate with a three-fold increase in risk of incident atherosclerosis [8]. Moreover, the pro-atherogenic potential of endotoxin has been confirmed in both mouse [10] and rabbit [11] models of endotoxin-accelerated atherosclerosis.

The signalling receptor for enterobacterial endotoxin is Toll-like receptor (TLR)-4, a member of a family of ten human innate immune sensors that serve to detect microbial infection and initiate inflammation [12]. Recent genetic studies in mice have revealed that TLR-signalling plays a major role in atherosclerosis, as deletion of MyD88, a shared

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signalling adaptor of the TLRs, results in an approximately 60% reduced plaque burden in apolipoprotein-E<sup>-/-</sup> mice [13,14]. Together with the discovery that deficiency of either TLR2 or TLR4 alone also reduces atherosclerosis in this mouse background [14,15], these findings have led to considerable efforts to identify candidate ligands of TLR-signalling in the vascular wall [11,12,16,17].

The majority of previous studies of the effects of endotoxin on vascular tissues have employed enterobacterial (e.g. *Escherichia coli* or *Salmonella*) endotoxins, largely because the endotoxins of these two organisms are well characterised and readily available commercially. However, much less is known about the effects on the vasculature of non-enterobacterial endotoxins (NE-LPS), which are likely to contribute a much larger fraction to the circulating endotoxin pool. Non-enterobacterial colonisers greatly outnumber enterobacteria as colonists of the host mucosal surfaces, perfusion of which is considered to be the leading source of the plasma LPS pool [3,5,18–20]. For example, *Bacteroides fragilis* alone outnumbers the Enterobacteriaceae at least 1000:1 in the gut [18], and in the oral cavity non-enterobacterial organisms are also predominant.

Previous studies of NE-LPS induced endothelial cell activation have concluded that endothelial cells are almost entirely resistant to activation by NE-LPSs and that NE-LPSs act as antagonists of enterobacterial LPS mediated TLR4-signalling [21–24]. However, as these studies investigated the responses of umbilical vein endothelial cells, which are less relevant to atherogenesis, we sought to determine whether arterial endothelial cells are similarly unresponsive to NE-LPSs, and to elucidate further the receptors involved in NE-LPS induced endothelial cell-signalling. As the circulating endotoxin pool is likely to derive mainly from colonists of the mucosal surfaces [3,5,18–20], we investigated arterial endothelial cell responses to endotoxins extracted from organisms representative of those that may colonise or cause infection at each of the three major mucosal surfaces: the gut (*B. fragilis*), the oral cavity (*Porphyromonas gingivalis*) and the lung (*Pseudomonas aeruginosa*).

## 2. Materials and methods

### 2.1. Bacterial strains and endotoxin extractions

Endotoxins of *Escherichia coli* R1 (NCTC-13114), *B. fragilis* (NCTC-9343), *P. aeruginosa* (PAC-611) and *P. gingivalis* (NCTC-11834), prepared as described previously [25], were kind gifts of Professor Ian Poxton (University of Edinburgh, UK). All LPS samples were repurified to remove protein and lipoprotein contamination as described by Hirschfeld et al. [26]. Briefly, LPS samples were adjusted to 0.2% triethylamine and 0.5% deoxycholate and subjected to two rounds of phenol/water re-extraction. Aqueous phases were pooled and adjusted to 75% ethanol and 30 mM sodium acetate. LPS was then precipitated at –20 °C for 1 h, harvested by centrifugation (10 min at 10000 ×g),

washed in 1 ml cold 100% ethanol, air dried and resuspended in the original volume of pyrogen-free water. Heat-killed bacteria of the same strains were prepared by growing cultures in depyrogenated glassware (180 °C dry heat for 4 h) before resuspending in sterile saline and boiling at 100 °C for 10 min.

### 2.2. Lipid-A preparation

Lipid-A was prepared from each LPS by hydrolysis in 1% acetic acid at 100 °C for 1 h, washed three times in pyrogen-free water and redissolved in 1:1 chloroform:methanol. Samples were stored under nitrogen at –20 °C until use. Aliquots were dried down under nitrogen flow in sterile Eppendorfs before resuspending in warm medium by vortexing for 1 min.

### 2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) were purchased from Cambrex or Cascade Biologics and cultured in Medium 200 supplemented with low-serum growth supplement (Cascade Biologics, Mansfield, UK) or EGM-2MV (Cambrex, Wokingham, UK) between passages 3 and 7. U937 and THP-1 cells (ECACC, UK) were maintained in RPMI-1640 supplemented with 10% fetal-calf serum (FCS) and HEK-293 cells were maintained in DMEM/10% FCS.

### 2.4. Determination of endothelial cell activation

ECs were challenged with indicated concentrations of LPS and supernatant IL-8 was measured at 18 h by ELISA (R&D, Abingdon, UK). Cell-surface E-selectin ELISA was performed at 4 h on fixed EC monolayers as described previously [27]. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min at 4 °C, blocked with 1% BSA in PBS for 30 min, then labelled with HRP-conjugated anti-human E-selectin (HBT, Netherlands) for 2 h prior to development of signal with Sureblue peroxidase substrate (KPL, Maryland, US). In some experiments, cells were cultured with 10 µg/ml of a TLR2-neutralizing monoclonal antibody (clone TL2.5, HBT), TLR6-neutralizing antibody (pAb-hTLR6, Invivogen, Toulouse, France) or isotype matched antibody control (TLR3.7, HBT) for 30 min before challenge with LPS or the synthetic lipopeptides Pam<sub>3</sub>CSK<sub>4</sub> or FSL-1 (Invivogen). Monocyte adhesion was determined by challenging ECs in 12-well plates with 1 µg/ml LPS for 4 h, washing twice in RPMI and adding 10<sup>5</sup> U937 cells per well for 30 min. Monolayers were gently washed three times, then fixed in 4% paraformaldehyde before counting adherent cells per field under light microscopy.

### 2.5. Transfection assays

TLR-deficient HEK-293 cells were plated in 96-well plates at 8 × 10<sup>3</sup> cells per well and transfected after 24 h using Genejuice (Novagen, USA) according to manufacturer's instructions. Amounts of construct per well were 30 ng of

human TLRs 1, 2, 4 (co-expressing MD-2) or 6 (Invivogen), 30 ng of pCD14 (kind gift of Professor Christopher Gregory, University of Edinburgh), 20 ng of renilla reporter construct and 10 ng of luciferase-reporter construct driven by the NF- $\kappa$ B regulated E-selectin promoter (pELAM), with the balance made up with empty pCDNA3. 72 h after transfection cells were challenged for a further 18 h with medium alone (DMEM/1% FCS), 0.1–1000 ng/ml of each LPS, 1  $\mu$ g/ml of each lipid-A or  $10^7$  heat-killed bacteria per ml. Reporter levels were normalised to co-transfected renilla expression as an internal transfection efficiency control. Promoter expression is represented as fold induction of normalised pELAM signal relative to cells cultured in medium alone  $\pm$  S.D. from triplicate wells. Endogenous expression of TLRs in HEK-293 was ruled out by RT-PCR (data not shown).

## 2.6. Determination of TLR expression profiles

PCR (30 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 30 s) was carried out on cDNA prepared from HUVECs and HCAECs using the following primers: TLR1 — GCTGCTGTTCAGCTCTTGTG, GAACGTGGATGAGACCGTTT; TLR2 — CAAGCAGGATCCAAAGGAGA, TCCTGTTGTTGGACAGGTCA; TLR4 — GGCTCGAGGAAGAGAAGACA, GGAACCACCTCCGTGATAAA; TLR6 — CCCTTAGGATAGCCACTGC, CTCACAA-TAGGATGGCAGGA; CD14 — GAGCTCAGAGGTTTCGGAAGA, CCAGTAGCTGAGCAGGAACC;  $\beta$ -actin — TACCCATTGAACACGGCA, TGGGCACAGTGTGGGTGAC. To confirm TLR expression profiles, real-time PCR was performed using the same primer set with SYBR-Green master mix in a Roche light-cycler for 40 cycles. Abundance of each transcript was normalised to percentage of  $\beta$ -actin expression. Western blot was performed on 10  $\mu$ g of whole cell lysates obtained from unchallenged HEK-293, HEK293 cells transfected with TLR2 or TLR4/MD2, HUVEC, HCAEC and THP-1 cells using antibodies to TLR2 (sc10739, Santa-Cruz, US), TLR4 (sc10741, Santa-Cruz), TLR6 (3653, Prosci, CA, US) or loading control GAPDH (sc25778, Santa-Cruz).

## 2.7. Plasma collection and LAL assay

The limulus amoebocyte lysate (LAL) assay is an *in vitro* assay for the detection of endotoxin based on an invertebrate-derived enzyme system. Endotoxin present in test samples added to LAL reagent stimulates an enzyme cascade that results in a colour change that can be compared against a standard curve made from reference endotoxin. To investigate the sensitivity of the LAL assay for NE-LPSs, dilutions of each endotoxin from 100 ng/ml to 1 pg/ml were prepared in pyrogen-free water. 50  $\mu$ l of each dilution was added to 50  $\mu$ l of LAL reagent (Pyrochrome, Quadratch, Epsom, UK) in duplicate in pyrogen-free 96-well plates as described previously [6,8], incubated at 37 °C for 30 min prior to halting reaction with 10  $\mu$ l 50% acetic acid and measuring absorbance at 405 nm in an automated plate reader. To

measure circulating endotoxin, heparinised plasma was obtained by venipuncture from seven healthy subjects (four male, three female, aged 20–44) according to local ethical procedures and the principles outlined in the Declaration of Helsinki. Plasma was diluted 1:10 in pyrogen-free water, heated at 70 °C for 10 min to inactivate plasma proteins that otherwise inhibit the LAL assay and endotoxin was quantified in triplicate as described above using standard curves prepared with *E. coli* LPS or each of the NE-LPSs.

## 2.8. Statistical analysis

Results are presented as mean  $\pm$  S.D. Differences were analysed using one way ANOVA and considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Non-enterobacterial LPSs stimulate coronary artery but not venous EC activation

Human arterial or venous ECs were challenged *in vitro* with 1–1000 ng/ml of enterobacterial (*E. coli*) or non-enterobacterial (*P. gingivalis*, *B. fragilis*, *P. aeruginosa*) endotoxins. HUVECs released the pro-inflammatory chemokine IL-8 in response to low concentrations of *E. coli* LPS but not to any of the non-enterobacterial LPSs tested (Fig. 1A). By contrast, HCAECs released IL-8 in response to

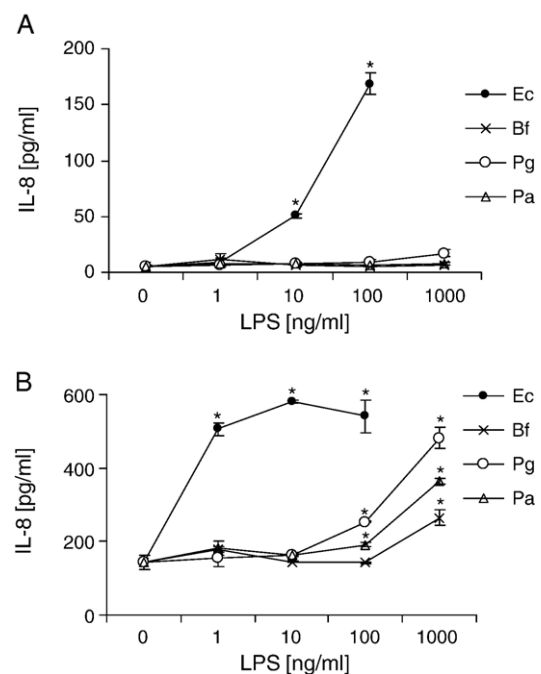


Fig. 1. HCAECs but not HUVECs release interleukin-8 in response to NE-LPSs. HUVECs (A) or HCAECs (B) were challenged with medium alone or 1 to 1000 ng/ml of each LPS. Supernatant IL-8 protein levels were measured by ELISA after 18 h. \* $P < 0.05$  vs cells cultured in medium alone. Ec = *E. coli*, Bf = *B. fragilis*, Pg = *P. gingivalis*, Pa = *P. aeruginosa*. Data shown are representative of three experiments.

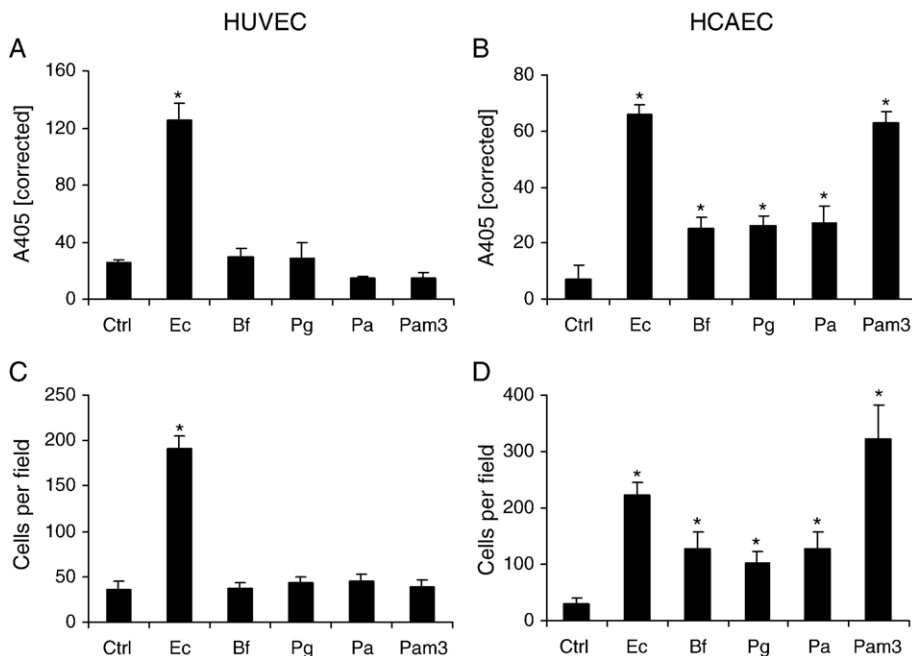


Fig. 2. NE-LPSs increase E-selectin expression and monocyte adhesion to HCAECs but not HUVECs. HUVECs or HCAECs were challenged with medium alone or 1  $\mu\text{g}/\text{ml}$  of each LPS or 100  $\text{ng}/\text{ml}$  Pam<sub>3</sub>CSK<sub>4</sub> for 4 h, then E-selectin expression was measured by cell based ELISA (A,B) or adhesion of human monocytic U937 cells was measured by light-microscopy (C,D). Results are means of triplicate wells  $\pm$ S.D. and are representative of at least 3 independent experiments. Ec = *E. coli*, Bf = *B. fragilis*, Pg = *P. gingivalis*, Pa = *P. aeruginosa*. \* $P < 0.05$  vs cells cultured in medium alone.

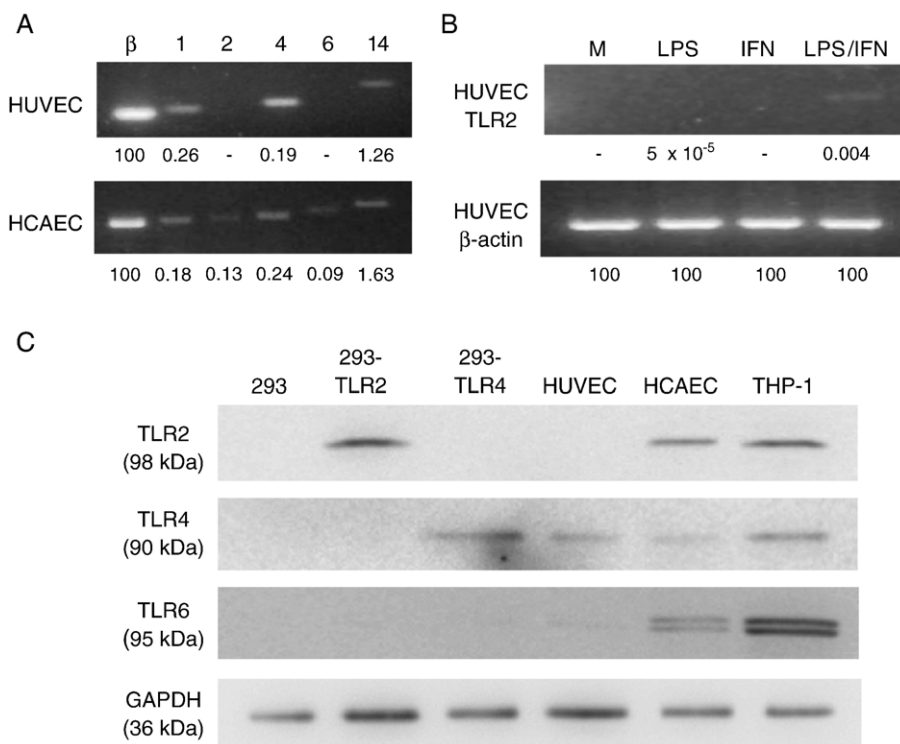


Fig. 3. Resting HCAECs but not HUVECs express TLR2. RT-PCR was performed on RNA from unstimulated HUVECs and HCAECs using primers specific for  $\beta$ -actin, TLR1, 2, 4, or 6 or CD14. Numbers beneath images show abundance of each transcript relative to  $\beta$ -actin as determined by real-time PCR. Dash indicates not detected within 40 cycles. (A). TLR2 mRNA expression was also measured in HUVECs cultured in medium alone, 1  $\mu\text{g}/\text{ml}$  *E. coli* LPS, 10  $\text{ng}/\text{ml}$  IFN- $\gamma$  or LPS and IFN- $\gamma$  combined for 18 h (B). Western blot for TLR2, TLR4, TLR6 and loading control GAPDH was performed on 10  $\mu\text{g}$  of whole cell lysate from the TLR-deficient cell line HEK-293, HEK-293 cells transfected with TLR2 or TLR4/MD2, HUVECs, HCAECs or the human monocytic cell-line THP-1 (C).

all of the LPSs, though responses to *E. coli* LPS occurred at lower concentrations than the NE-LPSs (Fig. 1B). NE-LPSs also induced adhesion of monocytic cells and expression of the adhesion molecule E-selectin in HCAECs, but not HUVECs (Fig. 2). Similarly, HCAECs, but not HUVECs, were responsive to the synthetic bacterial lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> (Fig. 2).

### 3.2. Venous but not coronary artery ECs are highly resistant to TLR2 expression

As responses to Pam<sub>3</sub>CSK<sub>4</sub> are dependent on the pattern-recognition receptor TLR2, we investigated whether differential expression of TLR2 may explain the different sensitivities of HUVECs and HCAECs. RT-PCR and real-time PCR revealed that while both HUVECs and HCAECs expressed TLR4, the receptor for enterobacterial LPS, HCAECs, but not HUVECs, expressed TLR2 (Fig. 3A). HCAECs expressed mRNAs for the TLR2 co-receptors TLR1 and TLR6, while HUVECs expressed only TLR1. *E. coli* LPS and IFN- $\gamma$  alone had very little effect on TLR2 mRNA expression in HUVECs, and only combined treatment with LPS and IFN- $\gamma$  led to appreciable TLR2 expression (Fig. 3B). Western blots confirmed that TLR4

protein was expressed in both cell types, but TLR2 and TLR6 protein was expressed only in HCAECs (Fig. 3C).

### 3.3. Non-enterobacterial LPSs stimulate TLR2 but not TLR4-signalling

Next, as there is some debate as to whether TLR2 or TLR4 is employed in the recognition of certain types of LPS [28–31], we examined signalling in a highly sensitive TLR-transfection reporter assay. In the absence of TLR-transfection, HEK-293 cells were insensitive to even high concentrations of all LPSs tested (Fig. 4A). Transfection of TLR4 conferred sensitivity to picomolar quantities of *E. coli* LPS, but concentrations of non-enterobacterial LPSs as high as 1  $\mu$ g/ml showed no TLR4-dependent cell-signalling (Fig. 4B). By contrast, TLR2 transfection enabled recognition of the non-enterobacterial LPSs, but not *E. coli* LPS (Fig. 4C). Because crude LPS preparations are understood to contain contaminating bacterial proteins and lipopeptides, which led to confusion in earlier studies of endotoxin receptor utilisation [26], we investigated the TLR2 signalling potential of *E. coli* LPS before and after purification to remove these agents. Consistent with earlier studies [26,32], the phenol re-extraction process (which was applied to all of

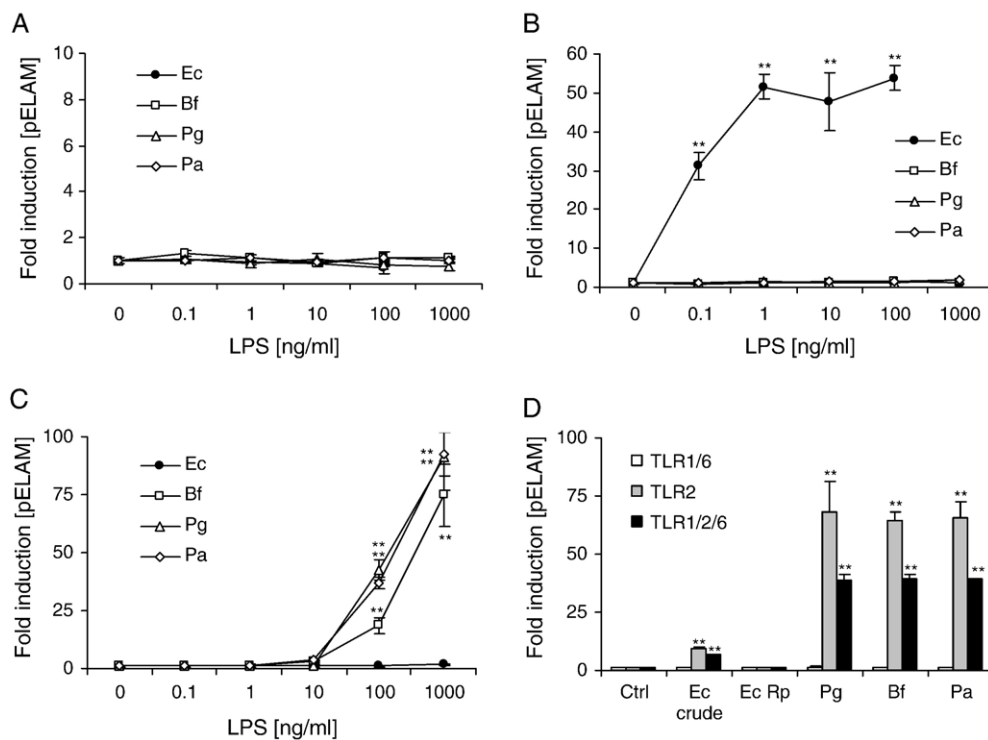


Fig. 4. NE-LPSs but not *E. coli* LPS stimulate TLR2-dependent signalling. TLR-deficient HEK-293 cells were transfected with CD14 and reporter construct alone (A), or with additional TLR4/MD2 (B) or TLR2 (C), then challenged with 0.1 to 1000 ng/ml of each type of LPS for 18 h. Data are expressed as fold induction of NF- $\kappa$ B dependent reporter expression normalised to internal transfection efficiency control vs cells cultured in medium alone, and are representative of at least four experiments. Ec = *E. coli*, Bf = *B. fragilis*, Pg = *P. gingivalis*, Pa = *P. aeruginosa*. (D) Phenol re-extraction removes all TLR2-stimulatory activity from a crude *E. coli* LPS preparation and neither TLR1 nor TLR6 is sufficient or required for responsiveness to NE-LPSs. HEK-293 cells were transfected with TLR1 and TLR6, TLR2 only, or the combination of TLRs 1, 2 and 6 together. Cells were then challenged with 1  $\mu$ g/ml of crude *E. coli* LPS, phenol re-extracted *E. coli* LPS, or each of the NE-LPSs for 18 h. \*\* $P$  < 0.01 vs cells cultured in medium alone.

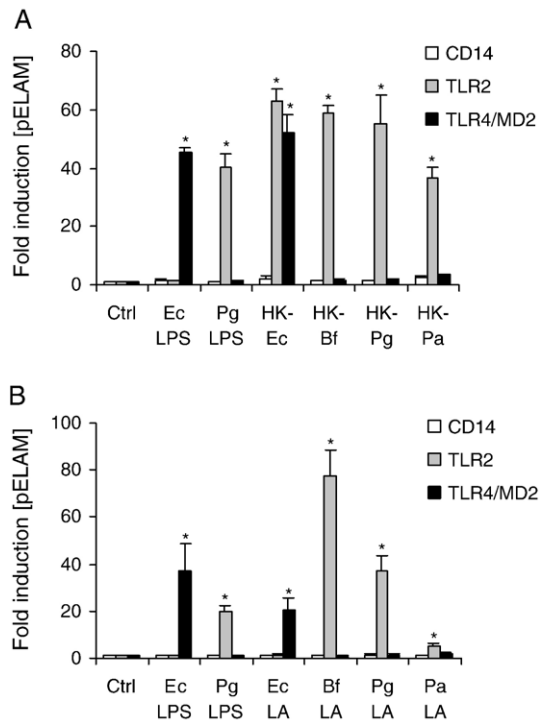


Fig. 5. Whole non-enterobacterial organisms and lipid-As can stimulate TLR2-dependent signalling. TLR-deficient HEK-293 cells were transfected with CD14, or with additional TLR2 or TLR4/MD2 co-expression, as described in the legend of Fig. 4. Cells were then challenged with  $10^7$  whole heat-killed bacteria per ml (A), or  $1 \mu\text{g/ml}$  of lipid-A (LA) of each organism, for 18 h (B). Responses to  $10 \text{ ng/ml}$  *E. coli* or *P. gingivalis* LPS are included as positive controls for TLR4 and TLR2 signalling respectively. Data are expressed as mean fold induction of NF- $\kappa$ B dependent reporter expression normalised to internal transfection efficiency control vs cells cultured in medium alone, and are representative of three experiments. Ec = *E. coli*, Bf = *B. fragilis*, Pg = *P. gingivalis*, Pa = *P. aeruginosa*. \* $P < 0.05$  vs medium alone.

the endotoxins examined in this study) removed all TLR2-signalling contaminants from a crude *E. coli* LPS preparation (Fig. 4D). Accordingly, the protein content of the re-extracted endotoxins was below the limit of detection of the Bradford assay.

#### 3.4. Neither TLR1 nor TLR6 is required for responsiveness to NE-LPSs

We next investigated whether other lipid-sensing TLRs may contribute to the recognition of NE-LPSs. Transfection of HEK-293 cells with TLR1 and TLR6 together did not confer sensitivity to any of the LPSs tested while transfection of TLR2 alone did (Fig. 4D). Fold induction of reporter in response to NE-LPSs was slightly reduced in cells co-transfected with TLR1/2/6, although it should be noted that this occurred only because TLR1/2/6 co-expression increased background promoter activation in unchallenged cells. Outright pELAM expression (i.e. not normalised to unchallenged cells) in response to NE-LPSs was approximately 30% higher than in cells transfected with TLR2 alone, which is consistent with the observation that genetic

deletion of TLR1 or TLR6 blunts but does not abolish responsiveness to bacterial lipopeptides in mice [33].

#### 3.5. Non-enterobacterial heat-killed bacteria and lipid-A stimulate TLR2 but not TLR4-signalling

It has been suggested that the lack of TLR4-stimulating capacity of NE-LPSs observed in previous studies may be an artefact of LPS purification [34]. To address this possibility, we assessed the TLR2 and TLR4 stimulating potential of whole, unextracted heat-killed bacteria. Whole bacteria did not stimulate NF- $\kappa$ B signalling in HEK-293 cells in the absence of TLRs, while transfection with TLR2 enabled responsiveness to all types of bacteria. Transfection with TLR4/MD2 enabled responsiveness to *E. coli*, but not the other types of bacteria (Fig. 5A). Next, as the lipid-A region of the LPS molecule is the part recognised by the TLR4/MD2 complex [35], we investigated whether lipid-A extracted from each endotoxin could stimulate TLR2 or TLR4 signalling. The lipid-A of *E. coli* was found to stimulate only TLR4-dependent signalling, while the non-enterobacterial lipid-As stimulated TLR2-dependent signalling but not TLR4-dependent signalling (Fig. 5B). Lipid-A of *P. aeruginosa* consistently demonstrated

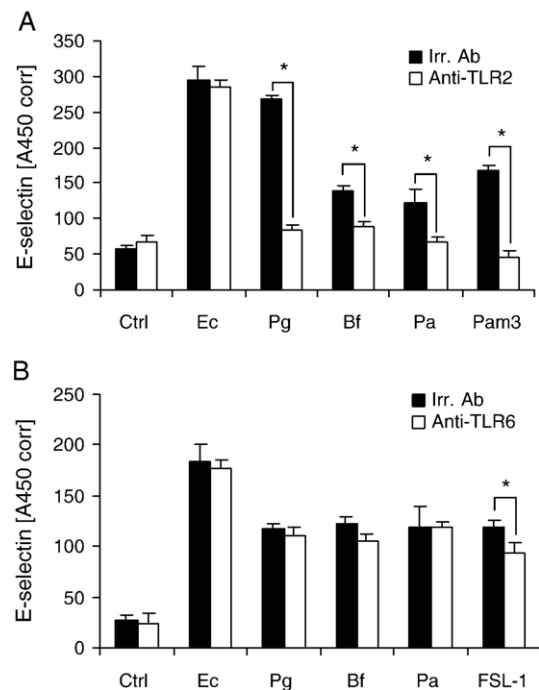


Fig. 6. TLR2-neutralizing antibody blunts HCAEC responses to NE — but not *E. coli* LPS. HCAECs were incubated with  $10 \mu\text{g/ml}$  of a TLR2-neutralizing monoclonal antibody, TL2.5 (Anti-TLR2) or isotype matched antibody control, TLR3.7 (Irr. Ab) for 30 min prior to challenge with  $1 \mu\text{g/ml}$  of each LPS or  $100 \text{ ng/ml}$  Pam<sub>3</sub>CSK<sub>4</sub> for 4 h (A). Alternatively, HCAECs were preincubated with  $10 \mu\text{g/ml}$  of TLR6-neutralizing polyclonal antibody (Anti-TLR6) or irrelevant antibody before challenge with  $1 \mu\text{g/ml}$  of each LPS or  $10 \text{ ng/ml}$  of the synthetic diacyl lipopeptide FSL-1 for 4 h (B). Cell-surface ELISA was then performed to measure relative expression of E-selectin. Results are representative of three experiments and are presented as mean  $\pm$  S.D. \* $P < 0.05$  by Student's *t*-test.

a comparatively weak TLR2 signal at 1  $\mu\text{g/ml}$ , although higher concentrations induced TLR2 signalling comparable with the other NE-lipid-As (not shown).

### 3.6. Blockade of TLR2 in HCAECs blunts responsiveness to NE-LPSs

To determine whether TLR2 played a functional role in the detection of NE-LPSs by HCAEC, we measured cellular E-selectin expression in response to LPSs following pre-incubation with the TLR2-neutralizing mAb TL2.5. Responses to all NE-LPSs and to the established TLR2 ligand Pam<sub>3</sub>CSK<sub>4</sub> were blunted significantly following exposure to TL2.5 but not to an isotype matched control antibody (Fig. 6A). However, the response to the diacyl synthetic lipopeptide FSL-1, which is thought to depend on both TLR2 and TLR6 signalling, was blunted by a TLR6 blocking antibody, while responses to the endotoxin preparations were unchanged (Fig. 6B). Consistent with earlier reports [36], treatment of cells with TLR4-specific antibodies failed to inhibit LPS signalling (data not shown).

### 3.7. Non-enterobacterial endotoxin may be underestimated in human plasma

All previous studies that have estimated the range of concentrations of endotoxin in human plasma have employed

the limulus amoebocyte lysate (LAL) assay [3,6–8]. By convention, this assay is calibrated using enterobacterial *E. coli* LPS. To determine whether NE-LPSs are efficiently recognised by the LAL assay enzymes, we assessed the potential of each chosen NE-LPS to stimulate the LAL reaction. The LAL assay was found to be dramatically less sensitive to NE-LPS than it is to *E. coli* LPS (Fig. 7). Purified endotoxin of *P. gingivalis* was underestimated by a factor of  $\sim 16$ , that of *P. aeruginosa* by  $\sim 200$  fold and that of *B. fragilis* by  $\sim 2200$  fold. In order to estimate the potential circulating concentrations of NE-LPS in human plasma, plasma from seven healthy subjects was measured by LAL assay using the NE-LPSs as the basis for four separate standard curves. Circulating endotoxin was low in all volunteers, with a maximum of 25 pg/ml *E. coli* LPS equivalent, 0.7 ng/ml *P. gingivalis* equivalent, 2.5 ng/ml *P. aeruginosa* LPS equivalent and 45 ng/ml *B. fragilis* LPS equivalent.

## 4. Discussion

Recent studies indicate that bacterial endotoxin is present in the plasma of all healthy individuals [3,6–9], and that elevated levels correlate with an increased risk of atherosclerosis [8,9]. However, while the majority of previous cardiovascular studies of endotoxin have employed enterobacterial (e.g. *E. coli* or *S. minnesota*) LPS, the circulating plasma endotoxin pool is almost certainly composed mainly of endotoxins of non-enterobacterial origin [18,19]. Those studies that have investigated the effects of NE-LPSs on EC function have concluded that ECs are almost entirely unresponsive to endotoxins of these organisms [21–23]. Our results confirm that HUVECs are indeed unresponsive to these LPSs, but show also that HCAECs respond readily to these endotoxins (Figs. 1–2), and that this responsiveness is due to TLR2-dependent recognition of a substance present in these endotoxin preparations (Figs. 3–6).

The nature of this substance remains to be fully characterised, though our results suggest that it is likely to be non-enterobacterial lipid-A (Fig. 5). While this confirms previous findings [30], we cannot exclude the possibility that the observed TLR2-dependent signalling may be due to non-LPS components that remain associated with LPS even after the many purification and hydrolysis steps employed. As pointed out by ourselves and others [30,32,37], this component is unlikely to be lipoprotein as phenol re-extraction removed all TLR2 signalling contaminants from a crude *E. coli* LPS preparation (Fig. 3D). Further studies, including chemical synthesis of the lipid-As of these organisms, will be required to dissect whether it is the endotoxin or an associated molecule that is responsible for the TLR2 stimulation seen by ourselves and other groups [28–31]. Nevertheless, studies employing bacteria-derived endotoxin such as ours may be more relevant than studies of synthetic lipid-A derivatives as circulating endotoxin almost certainly associates with the same TLR2-stimulating molecules present in our preparations and whole bacteria.

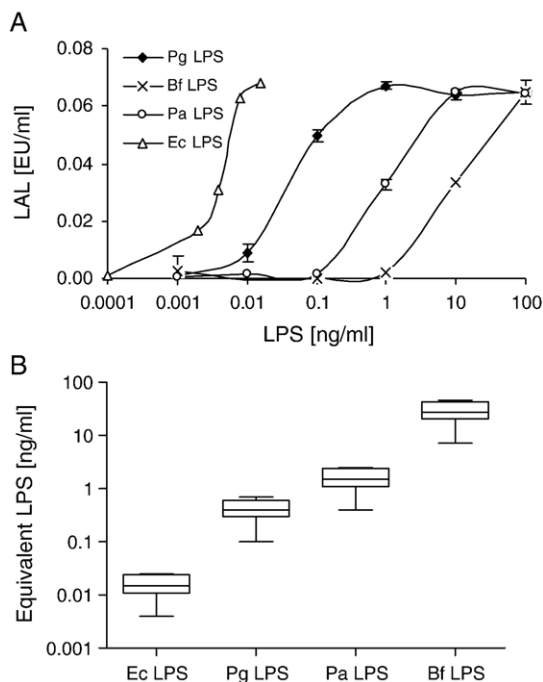


Fig. 7. NE-LPSs are greatly underestimated by LAL assay. Serial dilutions of each LPS were made from 1 mg/ml stocks in pyrogen-free water and assessed for ability to promote the limulus reaction by chromogenic endpoint analysis (A). Endotoxin was quantified in plasma from seven healthy subjects by LAL assay calibrated with standard curves prepared using each of the four LPS types. Median, interquartile range and total range are shown and are representative of three similar experiments (B).

While it has been established beyond any doubt that enterobacterial LPS stimulates TLR4- and not TLR2-dependent signalling [26,35,38], several groups have continued to report that highly purified forms of LPS from certain non-enterobacterial strains stimulate TLR2 signalling even in the absence of lipoprotein contamination [28–31]. This issue remains controversial, however, as other groups have reported that NE-LPSs from bacteria adapted to particular conditions can also stimulate TLR4-dependent signalling [37,39,40]. Species-specific TLR-signalling disparities may account for some of these divergent findings, as certain lipid-A structures that are agonists of murine TLR4 are antagonists of human TLR4 [38,40]. Additional confusion may have arisen from the fact that non-enterobacterial lipid-A structures vary considerably not only between different species, but also between different strains of the same species [30,35,40] and can even vary considerably depending on the growth conditions and temperature of the bacteria prior to harvest [30,40]. For example, human TLR4/MD2 recognises hexa-acyl endotoxin of *P. aeruginosa* adapted to growth in the cystic fibrosis lung, but not penta-acyl lipid-A of alternatively adapted *P. aeruginosa* strains [40]. Thus, it should be stressed that while our results show that endotoxins of certain non-enterobacterial strains can stimulate TLR2-signalling independently of TLR4, they do not exclude the possibility that endotoxins of some other non-enterobacterial strains may also stimulate TLR4-dependent signalling [37,39,40].

These findings may be of relevance to coronary artery disease, as it has been shown that TLR2 deficiency reduces atherosclerosis in ApoE<sup>-/-</sup> mice [15], while TLR2 stimulation accelerates atherosclerosis [41]. Arterial regions at higher risk of atherosclerosis, such as outer curvatures and bifurcations, express increased levels of TLR2 and this has been proposed as a potential contributor to the regio-specificity of atherosclerosis [42].

However the question remains of whether NE-LPSs may reach concentrations sufficient to stimulate arterial ECs, and under which conditions these may be reached. We found that the endotoxins used in our study are greatly underestimated by the LAL assay, though even when correcting for the low activity of NE-LPSs in this assay, we found in a preliminary survey of healthy subjects that circulating NE-LPSs are unlikely to reach concentrations sufficient to stimulate arterial cell activation in most healthy individuals (Fig. 7). Wider studies of plasma endotoxin, such as the Bruneck study which measured circulating LPS in 516 individuals, found that about 10% of individuals showed resting endotoxin concentrations up to 15 times higher than the median [8]. It therefore remains possible that in a small proportion of the population, perhaps transiently during episodic bacteraemia missed by intermittent sampling or as a result of ongoing disease processes, levels of NE-LPSs may approach those required to stimulate the artery.

Interestingly, recent evidence suggests that individuals at risk of higher circulating NE-LPS levels have a higher risk of

atherosclerosis. Transient bacteraemia is common in individuals with periodontitis and connections have long been made between oral infection and cardiovascular risk with systemic endotoxin release recently being implicated in this process [20]. Increased plasma endotoxin has been shown to correlate with chronic bacterial infections, which in turn have been found to correlate well with incident atherosclerosis [9]. Consistent with this proposed mechanism, a rat model of *P. aeruginosa* infection revealed a prominent atherogenic effect even in the absence of elevated cholesterol [43]. Finally, a role for gut commensal-derived endotoxin cannot be excluded [3]. Elevated plasma endotoxin has been observed in many studies of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis [44] and it has been discovered recently that inflammatory bowel disease correlates with increased risk of atherosclerosis, independently of traditional risk factors [45]. The mechanism of increased atherosclerosis risk in these groups is not well understood, it is possible that systemic endotoxin release plays a contributing role.

To conclude, we have shown that in contrast to previous thinking, endotoxins of *P. gingivalis*, *P. aeruginosa* and *B. fragilis* are capable of stimulating coronary artery EC activation. The presence of these and similar endotoxins in human plasma may have been greatly underestimated by previous studies and it is possible that among individuals at risk of increased plasma endotoxin load, endothelial TLR2-signalling initiated by these endotoxins may contribute to the development of atherosclerosis.

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