Nephrol Dial Transplant (2011) 26: 4079–4090
doi: 10.1093/ndt/gfr217
Advance Access publication 1 June 2011

Human peritoneal mesothelial cells respond to bacterial ligands through a specific subset of Toll-like receptors

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Abstract
Background. Bacterial infection remains a major cause of morbidity and mortality in peritoneal dialysis (PD) patients worldwide. Previous studies have identified a key role for mesothelial cells, lining the peritoneal cavity, in coordinating inflammation and host defense. Toll-like receptor (TLR) involvement in early activation events within the mesothelium, however, remains poorly defined. To investigate the initiation of bacterial peritonitis, we characterized TLR activation by bacterial ligands in human peritoneal mesothelial cells (HPMC).
Methods. Primary HPMC were isolated from omental biopsies and TLR expression detected by real-time polymerase chain reaction (PCR), reverse transcription (RT)–PCR and flow cytometry. The responsiveness of HPMC to specific bacterial TLR agonists was determined using chemokine production as a biological readout. The requirement for CD14 in HPMC responses to a clinically relevant *Staphylococcus epidermidis* cell-free supernatant (SES) was investigated using soluble CD14 or anti-CD14-blocking antibodies.
Results. Real-time PCR detected TLR1-6 messenger RNA expression in HPMC and responses to TLR2/1 and TLR2/6 ligands and SES. No cell surface TLR4 expression or responses to lipopolysaccharide were detectable in HPMC, but they did respond to flagellin, a TLR5 ligand. SES-mediated responses were dependent on TLR2 but did not require CD14 in HPMC for optimal efficiency, unlike peripheral blood mononuclear cells. HPMC expression of TLR2 was also modulated by TLR2 ligands and inflammatory cytokines.
Conclusions. These data suggest that mesothelial cell activation by TLR2/1, TLR2/6 and TLR5 contributes to bacterial recognition influencing the course of the infective process and has implications for improving treatment of infection in PD patients.

Keywords: bacterial infection; human peritoneal mesothelial cells; peritonitis; peritoneal dialysis; Toll-like receptors

Introduction

Bacterial peritonitis is a feature of long-term peritoneal dialysis (PD) and a major cause of impaired peritoneal function and treatment failure. It is generally accepted that diminished peritoneal function is in part related to infection/inflammation-driven structural changes within the membrane including mesothelial cell loss, sub-mesothelial compact zone thickening and degenerative vascular changes [1]. Traditionally, PD-associated peritonitis is related to Gram-positive *Staphylococcal* species with *Staphylococcus epidermidis* and *Staphylococcus aureus* found in ~30 to 50% of cases [2]. Clinically severe infections with Gram-negative bacteria have become more frequent over the past decade, resulting in increased treatment failure and worse patient outcomes [3]. Previous studies by our group and others have identified a central role for the mesothelial cell in orchestrating peritoneal responses during inflammation and infection [4–7]. Human peritoneal mesothelial cells (HPMC) are activated by various stimuli, including bacteria, and regulate leucocyte recruitment through cytokines and chemokine secretion and adhesion molecule expression [8–11]. The ability of human mesothelial cells to respond directly to bacterial challenge has been previously suggested, but their responses to bacterial ligands mediated by the Toll-like receptor family have not been fully characterized [12].

Toll-like receptors (TLR) play a critical role in innate immune responses by specifically recognizing molecular patterns from a range of microorganisms, including bacteria, fungi and viruses [13]. TLR4 was initially identified as the TLR responsible for Gram-negative bacteria-induced responses through its recognition of lipopolysaccharide (LPS). Recognition of Gram-positive bacteria is primarily mediated by TLR2, which recognizes an array of microbial molecules in part by hetero-dimerization with other TLRs (e.g. TLR1 and TLR6) or unrelated receptors (e.g. Dectin-1) [13]. TLR activation triggers nuclear factor-kappa

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B (NF-κB), interferon (IFN) regulatory factor and mitogen-activated protein kinase signalling leading to altered gene expression, including pro-inflammatory cytokine and IFN-inducible genes [13]. TLRs are highly expressed on professional phagocytes but also to some degree in other cell types [14–16]. A full characterization of TLR expression and responsiveness to bacterial ligands in primary HPMC has not been carried out previously to our knowledge. In the present study, we have investigated the recognition of bacterial ligands by TLR family members in HPMC. Our data demonstrate the expression of a specific subset of TLRs by HPMC which enables the detection of both Gram-positive and Gram-negative bacteria. These findings emphasize the potentially important role the mesothelium plays in regulating local peritoneal host defense.

Materials and methods

Reagents

Pam_3Cys and Pam_2Cys were purchased from EMC Microcollections, (Tübingen, Germany) and ultra-pure LPS (*Escherichia coli* O111:B4), peptidoglycan and flagellin (*Salmonella typhimurium*) from Invivogen (San Diego, CA). Interleukin-1 beta (IL-1β) and *tumour necrosis factor-*α were purchased from R&D Systems (Minneapolis, MN). A cell-free supernatant from a clinical isolate of *S. epidermidis* (SES) was prepared as described previously [17]. sCD14 was purified from human milk, as described previously [18].

Isolation, culture and cell activation of HPMC

HPMC were isolated by tryptic digest of omental tissue from consenting patients undergoing abdominal surgery and characterized as previously described [8]. Prior to experimentation, HPMC monolayers were growth arrested for 48 h in serum-free culture medium and stimulated for 24 h, as indicated. Culture supernatants were harvested, rendered cell free by centrifugation (300 *g*, 5 min) and stored at −80°C until use in commercially available ELISA kits (BD Biosciences, Oxford, UK and R&D Systems).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation from whole blood of healthy volunteers and adherence (2 h, 37°C). Cells were stimulated as indicated for 20 h in phenol red-free RPMI 1640 medium (Invitrogen, Paisley, UK) with 0.5% human AB serum (TCS Biosciences, Buckingham, UK). For blocking experiments, the cells were preincubated with the anti-CD14-blocking antibody (MY4; Beckman Coulter, High Wycombe, UK) for 1 h and the stimulus was then added for the indicated time.

Culture and stimulation of transfected Human Embryonic Kidney Cells

Human embryonic kidney (HEK) 293 plasmid control-transfected cells were cultured in complete medium: Dulbecco's-modified Eagle's medium containing 4500 mg/mL glucose (Invitrogen) supplemented by 2 mM L-glutamine and 10% fetal calf serum (FCS) and specific additives as follows: 400 μg/mL hygromycin B (Calbiochem, San Diego, CA) for HEK 293-TLR2 [19] or 200 μg/mL Geneticin (G418) (Sigma) for HEK 293-TLR4/MD2 (kindly provided by Prof. Douglas T. Golenbock, University of Massachusetts Medical School, North Worcester, MA, USA). Cells were trypsinized and washed five times with phosphate-buffered saline (PBS) to eliminate sCD14 before being stimulated as indicated.

Detection of TLR expression by immunocytochemistry

HPMC were fixed on slides for 5 min in acetone at −20°C and left in PBS overnight at 4°C. They were then blocked in PBS containing 1% (w/v) bovine serum albumin (BSA) and 10% (v/v) normal rabbit serum for 1 h before incubation with either an anti-TLR2-specific monoclonal antibody (TLR2.5; eBioscience, San Diego, CA) or an isotype-matched mouse monoclonal antibody (eBioscience) and then Alexa 568-conjugated Goat anti-mouse IgG antibody (kindly provided by Prof. Valerie O'Donnell, Cardiff University, Cardiff, UK).

Detection of TLR cell surface expression by flow cytometry

HPMC were incubated with blocking buffer (50% flow cytometry buffer: PBS pH 7.3, 10 mM EDTA, 1% BSA, 15 mM sodium azide, 25% normal rabbit serum (Dako, Glostrup, Denmark), 25% human AB serum) before immunolabelling at 4°C with primary unconjugated antibodies directed against human TLR1 (GD2.F4), TLR2 (2.1), TLR4 (HTA125), TLR6 (hPer6) and isotype-matched control antibodies (all eBioscience) and anti-CA125 (Fujirebio Diagnostic, Malvern, PA) and appropriate goat anti-mouse/rat phycoerythrin (PE)-conjugated secondary antibodies (Dako) or PE-conjugated anti-CD14 (BD Biosciences) and anti-gp130 (R&D Systems). Up to 20 000 events were acquired using a FACSCalibur flow cytometer (BD Biosciences) using previously defined settings for HPMC [20] and specific primary antibody staining compared against the isotype control.

RNA isolation, reverse transcription–polymerase chain reaction and real-time polymerase chain reaction

Total RNA was isolated from HPMC or murine peritoneal membrane using Tri-Reagent (Sigma, Poole, UK) and following a chloroform–isopropanol isolation protocol according to the manufacturer's instructions. Before performing reverse transcription–polymerase chain reaction (RT-PCRs), messenger RNAs (mRNAs) were treated with RNAse-free DNAse I (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed using 1 μg of RNA with the SuperScript II reverse transcriptase (Invitrogen). PCR amplification was performed with Ampli*Taq* Gold polymerase (Applied Biosystems, Warrington, UK; Table 1). For real-time PCR, RNA was analysed with the NanoDrop-1000 spectrophotometer (Thermo Fisher, Pittsburgh, PA). Reverse transcription was performed with 1 μg RNA with the High Capacity complementary DNA (cDNA) Reverse Transcription kit (for HPMC; Applied Biosystems) or SuperScript II reverse transcriptase (for peritoneal membrane; Invitrogen) using the manufacturer's protocol. cDNA (diluted 1:5) was used to perform real-time PCR using Power SYBR Green PCR master mix (Applied Biosystems) and standard primers (Table 2; from Invitrogen) (for HPMC) or Taqman Universal PCR master mix and primer probe sets (for murine peritoneal membrane, Eukaryotic 18s RNA VIC-labelled, 4310893E and murine *Tlr2*, FAM-labelled probe, Mm00442346_m1; all Applied Biosystems), using the manufacturer's protocol and the following cycling conditions: (i) 1 cycle, 95°C 10 min, (ii) 40 cycles, 95°C 15 s, 60°C 1 min, (iii) 1 cycle, 95°C 15 s, 60°C 15 s, 95°C 15 s, using an Applied Biosystems ABI 7900 HT machine. Baseline and threshold for C_t values were determined using the Sequence Detection System 2.3 software (Applied Biosystems). Results are expressed as relative induction from the housekeeping gene *Gapdh* (HPMC) or 18s RNA (peritoneal membrane) ($2^{-\Delta Ct}$) or using the $2^{-\Delta\Delta Ct}$ method [22].

NF-κB reporter assay

HEK-TLR2 or HEK-TLR4/MD2 cells (1×10^5 cells per well) were transiently transfected (Lipofectamine transfection reagent; Invitrogen) with 0.25 μg of NF-κB-responsive firefly luciferase reporter (pNF-κB Luc; Stratagene, La Jolla, CA) and 0.05 μg Renilla luciferase under control of a constitutive SV40 promoter (pRL-SV40; Promega, Southampton, UK). After 48 h, cells were stimulated for 16 h with SES concentrations as indicated. Luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega).

Induction of acute peritoneal inflammation

Experiments were performed using 8- to 12-week-old 129/C57BL6/J mice, according to Home Office regulations (PPL:30/2269). Peritoneal inflammation was induced via the intraperitoneal route with 500 μL SES and at designated time points the peritoneal membrane was snap-frozen [6].

Statistical analysis

Data presented are expressed as means (± SEMs) and statistical analysis was performed using a two-tailed Student's *t*-test. A P-value of <0.05 was considered significantly different.

Results

HPMC express a distinct subset of TLRs

The major resident cell populations within the peritoneal cavity, i.e. mesothelial cells, macrophages and lymphocytes,

Table 1. Primer sequence and protocol used for RT-PCR

Primer	Sequence (5′–3′) Forward	Reverse
Tlr2	TGGCCAGCAGGTTCAGGATG	TCGCAGCTCTCAGATTTACCC
Tlr4	CCTCCAGGTTCTTGATTACAG	GTGCCGCCCCAGGACACT
Tlr6	AGAACTCACCAGAGGTCCAACC	GAAGGCATATCCTTCGTCATGAG
Actin	CGGCCAGCCAGGTCCAGA	GTGGGCATGGGTCAGAAGGATT
PCR amplification	32 cycles (TLR2 and TLR4) or 22 cycles (actin) at 94°C for 40 s, 56°C for 40 s and 72°C for 90 s	

Table 2. Primer sequence used for real-time PCR primer sequences were designed using Primer3 [21]

Human *Tlr*	Sequence (5′–3′) Forward	Reverse	Accession number
Tlr1	CAATGCTGCTGTTCAGCTCTTC	GCCCAATATGCCTTTGTTATCC	NM_003263.3
Tlr2	AATCCTCCAATCAGGCTTCTCT	TGTAGGTCACTGTTGCTAATGTAGGT	NM_003264.3
Tlr3	GAAAGGCTAGCAGTCATCCAAC	GTCAGCAACTTCATGGCTAACA	NM_003265.2
Tlr4	AGAACCTGGACCTGAGCTTTAATC	GAGGTGGCTTAGGCTCTGATATG	NM_138554.2
Tlr5	ACAAGATTCATACTCCTGATGCTACTG	CCAGGAAAGCTGGGCAACTA	NM_003268.3
Tlr6	ATTGTTAAAAGCTTCCATTTTGTTTG	CTAAGACTTTGGTTTTCAGCGGTAG	NM_006068.2
Tlr7	TCTCATGCTCTGCTCTCTTCAAC	TTGTCTCTTCAGTGTCCACATTGGAAA	NM_016562.3
Tlr8	GCTTGACTTACGTGGAAACAAACTAC	AACTTAAATCGAGGTGCTTCAGACTAC	NM_016610.2
Tlr9	TATTCATGGACGGCAACTGTTATT	TACTTGAGTGACAGGTGGGTGAG	NM_017442.2
Tlr10	CGATTCCACGCATTTATTTCATAC	TAGCTTTCATAAAGGCAAATCAAGATAG	NM_001017388.1
Gapdh	CCTCTGACTTCAACAGCGACAC	TGTCATACCAGGAAATGAGCTTGA	NM_002046.3

are believed to modulate responses to infection by orchestrating leucocytes trafficking [5 6 8 17 23]. While monocyte/macrophage TLR expression has been well described [24], human mesothelial TLR expression and responsiveness to bacterial ligands has not been characterized. To investigate TLR expression (TLR1–TLR10) by HPMC, we performed real-time PCR analysis and found a range of relative expression ranging high expression (TLRs 1–3), low/moderate levels (TLRs 4–6) to very low/undetectable expression (TLRs 7–10) (Figure 1A). A relative comparison with TLR mRNA expression in PBMC revealed lower levels of all the TLRs in HPMC, except TLR3 (Table 3).

To evaluate responses to Gram-positive bacteria and Gram-negative bacteria, we further examined TLR2 and TLR4 expression by reverse transcription–polymerase chain reaction (RT-PCR) and flow cytometry (Figure 1B and C, respectively). We also measured TLR1 and TLR6 expression, which form heterodimers with TLR2 [13]. TLR2 and TLR6 mRNA expression were detected by RT–PCR but only an indistinct product observed for TLR4 (Figure 1B). TLR1 and TLR2 expression were detected on the cell surface by flow cytometry, but TLR4 and TLR6 were undetectable on either growth-arrested or stimulated HPMC (Figure 1C and data not shown). TLR2 expression was also consistently detected in HPMC by immunohistochemistry (Figure 1D), despite variable expression observed between different HPMC lines by flow cytometry (Figure 1E).

To eliminate the possibility that TLR4 downregulation (also called 'LPS tolerance' [25]) might account for the lack of TLR4 detected in cultured HPMC, we analyzed cells by flow cytometry, directly following their isolation (i.e. without passage). As reported previously under these conditions, HPMC displayed high-cell surface expression of CA125 [26] and gp130 [20]. TLR2 expression was also detectable, but we were unable to detect TLR4 (Figure 1F).

HPMC respond to ligands for TLR2 and TLR5 but not TLR4

To characterize mesothelial cell responses to Gram-positive and Gram-negative bacteria, we treated HPMC with ligands to TLR2/1 (Pam_3Cys), which does not mediate local or systemic effects in TLR2-deficient mice [27], and TLR4 (LPS), plus a well-characterized cell-free supernatant derived from a clinical *S. epidermidis* isolate (SES), previously used to investigate acute inflammatory regulation *in vivo* [6, 17]. Chemokine (CXCL8, CCL2 and CCL5) and cytokine (IL-6) production were assessed. SES and Pam_3Cys induced a dose-dependent increase in CXCL8, IL-6 and CCL2 (Figure 2A), whereas LPS had no effect, even at extremely high doses (up to 5 μg/mL) (Figure 2A and B). As previously mentioned, cells may lose responsiveness to LPS, but certain genes are resistant to this process, e.g. the chemokine CCL5/RANTES [28]. However, CCL5 levels were not increased by LPS stimulation of HPMC at high or low concentrations (Figure 2B and Supplementary Figure 1).

Stimulation with Pam_2Cys (MALP-2 analogue, a TLR2/6 ligand) also triggered a dose-dependent increase in CXCL8 secretion by HPMC (Figure 2C), demonstrating that the low TLR6 expression observed (Figures

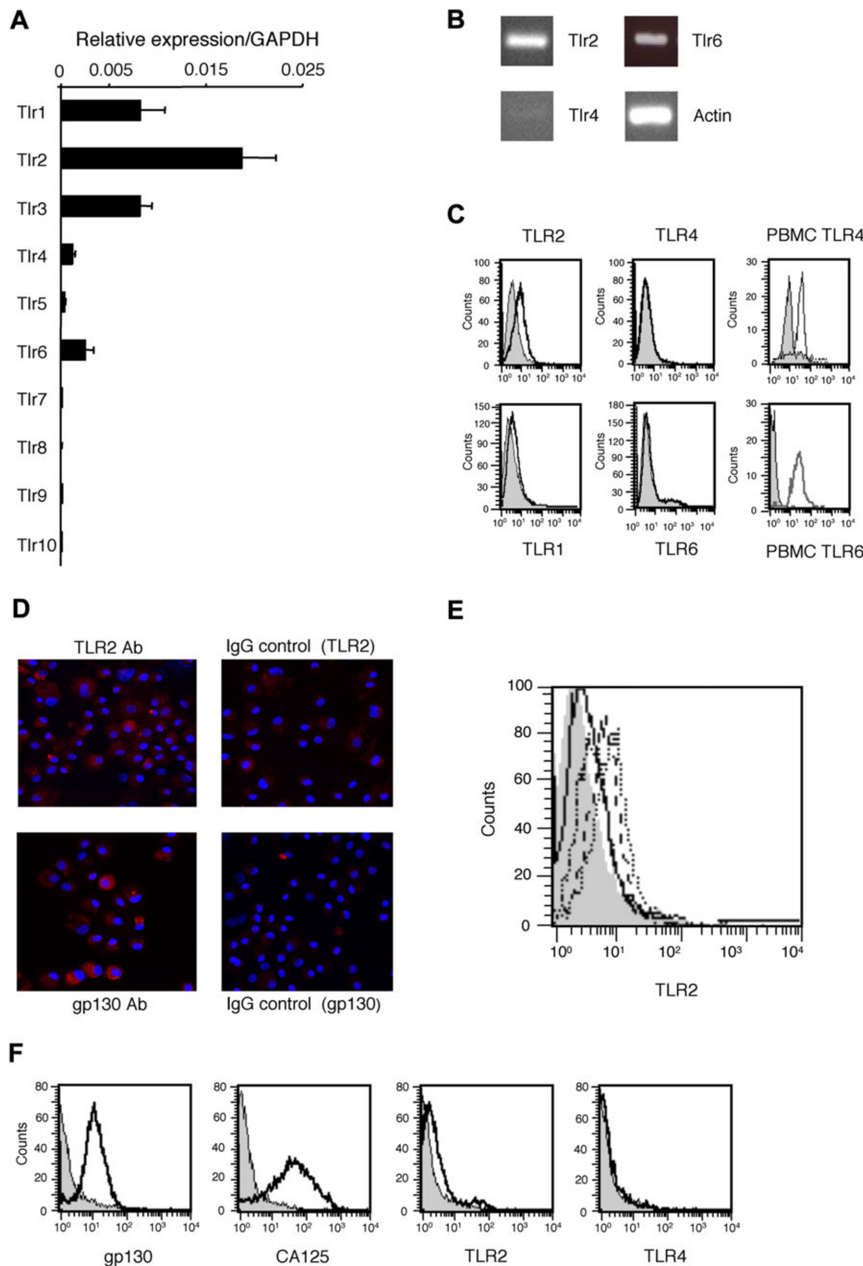


Fig. 1. Analysis of TLR expression by primary HPMC. (**A**) Analysis of TLR1-TLR10 mRNA expression in HPMC was performed by real-time PCR. Specific primers were used to amplify human TLR1-TLR10 cDNAs prepared from total RNA from unstimulated HPMC. Expression of each mRNA was determined by the calculation of $2^{-\Delta Ct}$ as described in the Methods section and was expressed relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**B**) Expression of mRNA for TLR2, TLR4, TLR6 and β-actin was assessed by RT-PCR using specific primer pairs in unstimulated HPMC and analyzed by agarose gel electrophoresis. (**C**) Cell surface expression of TLR2, TLR4 and TLR6 was assessed by flow cytometry using specific antibodies (open plots). Control profiles corresponding to the immunostaining with isotype control antibodies are also shown (shaded plots). These results are representative of seven different cell lines from individual donors. Human PBMC were used as a positive control for the TLR4 and TLR6 antibodies. (**D**) Unstimulated HPMC were assessed for TLR2 expression by immunocytochemistry using the TLR2.5 monoclonal antibody. A gp130 antibody was used as a positive control and control antibodies were used as negative controls for both TLR2 and gp130 staining. Results are representative of immunostaining of two different primary cell lines. (**E**) Primary HPMC originating from different donors were stained with TLR2.1 monoclonal antibody and analyzed by flow cytometry. The negative control staining from the isotype control antibody is shaded in gray and is identical for all donors. The results shown were obtained from four primary cell lines. (**F**) Mesothelial cells were analyzed for cell surface marker expression directly following tryptic digests of omental tissue. Cells were stained with antibodies specific for gp130, CA125, TLR2 and TLR4 and cell surface expression detected by flow cytometry.

1A–C) is sufficient to allow responses to Pam_2Cys. Indeed, the relatively low TLR1 and TLR2 expression detected by flow cytometry nevertheless allowed responses to TLR2/1 ligands, e.g. Pam_3Cys or peptidoglycan (data not shown). HPMC did not respond to zymosan, another TLR2/6 ligand (data not shown), potentially due to a lack of Dectin-1 expression, the coreceptor for zymozan [29].

Table 3. Relative TLR expression in HPMC[a]

Tlr gene	*Tlr* ($2^{-\Delta Ct}$) HPMC	PBMC	Relative expression to PBMC
Tlr1	0.0041	0.0547	0.0749
Tlr2	0.0134	0.2943	0.0455
Tlr3	0.0089	0.0010	8.9
Tlr4	0.0018	0.0330	0.0545
Tlr5	0.0003	0.0033	0.0909
Tlr6	0.0012	0.0351	0.0342
Tlr7	2.13×10^{-5}	0.0022	0.0097
Tlr8	2.37×10^{-6}	0.0540	4.39×10^{-5}
Tlr9	4.55×10^{-5}	0.0029	0.0157
Tlr10	7.96×10^{-6}	0.0037	0.0022

[a]We evaluated by quantitative PCR the expression of TLR1 to TLR10 in HPMC (as shown in Figure 1A) as well as in PBMC, known to express most TLRs. Results are expressed as the ratio of the Tlr gene ($2^{-\Delta Ct}$) to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase ($2^{-}\Delta Ct$) for each cell type as described in the Methods section and then as the relative difference in expression between HPMC and PBMC (HPMC/PBMC).

As shown previously, although HPMC responded to Gram-positive bacterial ligands, they were unable to respond to the TLR4 agonist, LPS (Figure 2A and B). Real-time PCR data, however, suggested a low TLR5 expression (Figure 1A), which detects a flagellum component (flagellin), commonly found on many Gram-negative bacteria [30] We stimulated HPMC with increasing doses of flagellin and observed a dose-dependent increase in CXCL8 (Figure 2D), implying that direct responses to Gram-negative bacteria in HPMC may occur through TLR5 not TLR4. The results obtained in Figure 2A–C regarding HPMC response to LPS, Pam3Cys, Pam_2Cys, and SES were confirmed by measurement of NF-κB activation by electrophoretic mobility shift assay (Supplementary Figure 2).

CD14 is not required for a coreceptor for TLR2-mediated responses in HPMC

We determined that the clinically relevant stimulus, SES, specifically activated responses through TLR2, which would be present on peritoneal mesothelial cells and resident peritoneal leucocytes and not via TLR4 (present on resident leucocytes only) (Figure 3). These experiments were carried out using stably transfected HEK293 cells expressing high levels of TLR2 or TLR4 and its co-factor MD2 (HEK-TLR2 and HEK-TLR4/MD2, respectively, Figure 3A) and plasmid control-transfected cells (HEK-pDR2). TLR2, but not TLR4 or control cells, responded to SES stimulation in a dose-dependent manner either through increased CXCL8 production or through NF-κB reporter activity (Figure 3B–D), demonstrating that TLR2, but not TLR4, is required for SES-mediated responses. SES may activate other pattern recognition pathways, notably the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), NOD1 and NOD2. NOD1 and NOD2 recognize dipeptides derived from bacterial peptidoglycan (NOD1: Gram-negative and specific Gram-positive bacteria, not including *Staphylococcus* species; NOD2: all Gram-negative and Gram-positive bacteria) [31, 32]. An analysis of HPMC responses to NOD1- and NOD2-specific agonists [gamma-D-glutamyl-meso-diaminopimelic acid/ (eE-DAP) and muramyl dipeptide/MDP, respectively] revealed a modest induction of CXCL8 in response to iE-DAP and no induction in response to MDP (data not shown). These results are similar to the findings of Park *et al.* [7] in murine mesothelial cells, which identified responses to NOD1, but not NOD2.

To test whether CD14 was required for TLR2-mediated responses triggered by SES in HPMC, we analysed the cell surface expression of CD14 by flow cytometry. No cell surface expression of CD14 was detected in HPMC regardless of serum, unlike PBMC, which expressed high levels of cell surface CD14 (Figure 4A). To investigate whether low levels of CD14, potentially below the limit of detection by flow cytometry, were capable of enhancing TLR2-mediated responses, we performed experiments using an anti-CD14-blocking antibody. The anti-CD14-blocking antibody had no effect on SES-induced CXCL8 in HPMC (Figure 4B), while it significantly reduced CXCL8 production induced by both SES and LPS in PBMC (Figure 4C). This finding implies that if TLR2 requires CD14 for efficient signalling, it has to be provided in its soluble form. We therefore measured CXCL8 production in HPMC treated with Pam_3Cys and SES with or without serum, as a source of sCD14. Pam_3Cys and SES triggered a significantly higher response in the presence of serum (Figure 4D and E). To investigate the potential of sCD14 specifically to enhance responses to SES stimulation, we stimulated HPMC in the presence or absence of sCD14. As shown in Figure 4F, SES-induced CXCL8 secretion was not altered by sCD14. Therefore, our results suggest that CD14 is required for SES-mediated responses in PBMC, but not in HPMC.

Modulation of TLR2 expression by inflammatory cytokines and TLR ligands

TLR2 expression can be modulated following activation with cytokines and microbial ligands [33, 34]. In order to study the regulation of TLR2 expression, we examined TLR2 mRNA expression in HPMC by real-time PCR following treatment with cytokines and TLR ligands. We observed that TLR2 mRNA expression was up-regulated upon 3 h poststimulation

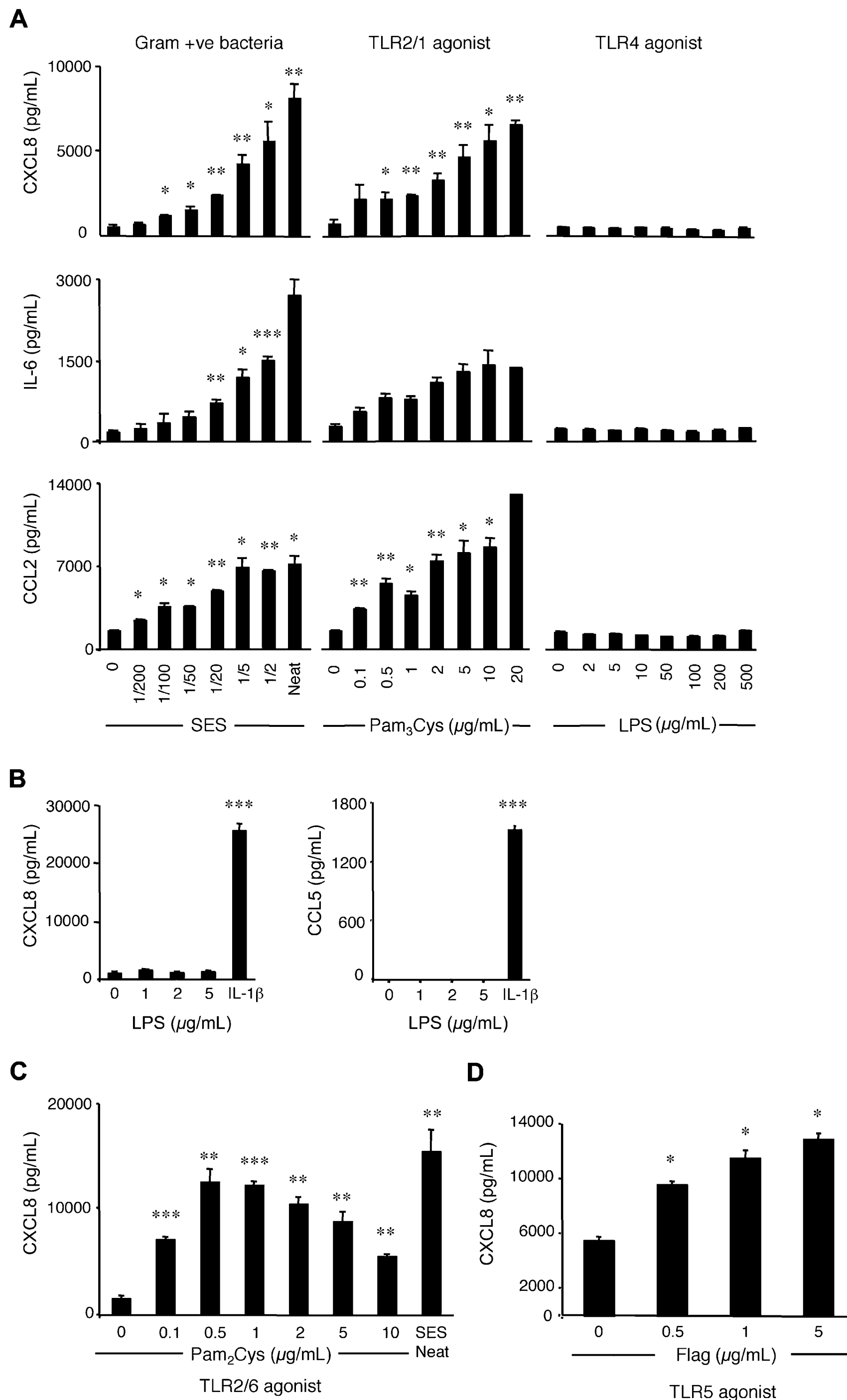
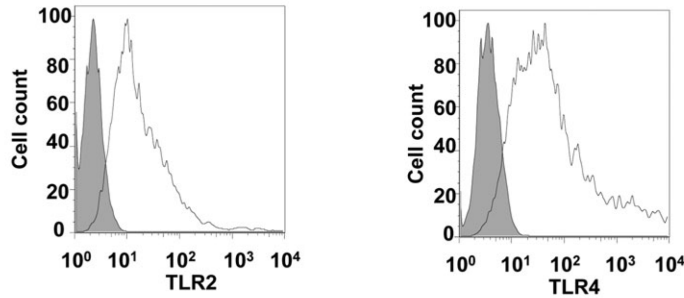
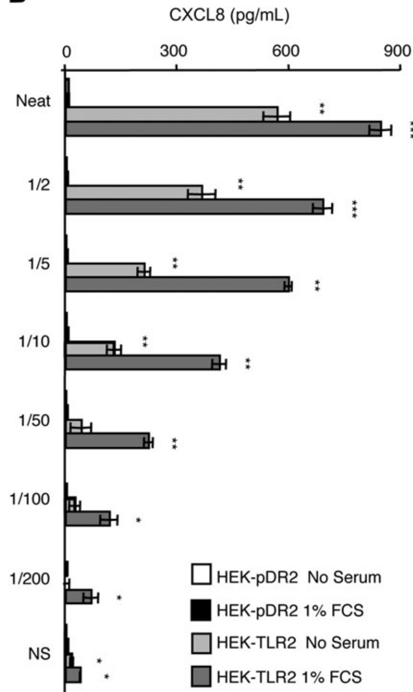
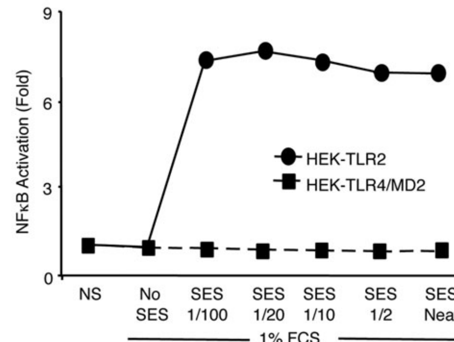


Fig. 2. Response of HPMC to bacterial TLR agonists. (**A**) HPMC were stimulated with serial dilutions of SES (1/200-Neat) Pam_3Cys (0.1–20 μg/mL) or LPS (2–500 ng/mL). After 24-h incubation CXCL8, IL-6 or CCL2 levels were quantified in cell-free supernatants using ELISA. Data shown are a result representative of at least three experiments performed using primary cell lines from different donors (*$P < 0.05$; **$P < 0.01$ between unstimulated and stimulated cells with the indicated ligands). (**B**) HPMC were stimulated with high doses of LPS (1–5 μg/mL). After 24-h incubation, CXCL8 and CCL5 levels were quantified in cell-free supernatants using ELISA (***$P < 0.001$). (**C**) HPMC were stimulated for 24 h with increasing doses of Pam_2Cys (MALP-2 analogue) or neat SES (with 0.1% FCS) and CXCL8 levels were quantified by ELISA in cell-free supernatants. Data presented are results representative of four independent experiments (**$P < 0.01$; $P < 0.001$ between unstimulated and stimulated cells with Pam_2Cys. (**D**) HPMC were stimulated for 24 h with increasing doses of flagellin and CXCL8 levels were quantified by ELISA in cell-free supernatants. Data presented are results representative of two independent experiments (*$P < 0.05$ between unstimulated and stimulated cells with flagellin).

with IL-1β or Pam_3Cys (Figure 5A), as previously described in epithelial cell lines [35] and murine pleural mesothelial cells [36]. This TLR2 up-regulation by Pam_3Cys was confirmed by immunocytochemistry (Figure 5B), using a protocol validated using HEK-TLR2 transfectants (Supplementary Figure 3). TLR1 and TLR6 mRNA expression did not change from







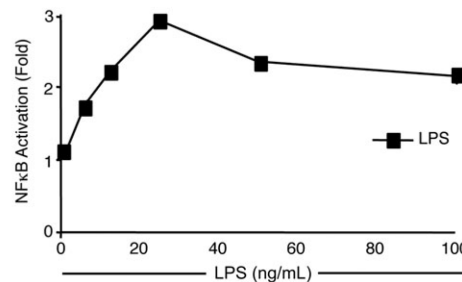


Fig. 3. Responses to SES are mediated by TLR2 but not TLR4. (**A**) Profile of TLR2 expression in HEK-TLR2 cell transfectants (left) was obtained by staining with PE-conjugated anti-TLR2, TL2.1 or an isotype-matched control antibodies. Profile of TLR4 expression in HEK-TLR4 cell transfectants (right) was obtained by staining with the unconjugated anti-TLR4 monoclonal antibody, HTA125 or an isotype-matched control IgG, followed by incubation with a secondary PE-conjugated anti-mouse antibody. Immunostained cells were analysed by flow cytometry. (**B**) HEK293 cells stably transfected with an empty plasmid vector (HEK-pDR2) or TLR2 expression vector (HEK-TLR2) were stimulated for 15 h with increasing concentration of SES in the presence or absence of 1% FCS. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are a representative result of three experiments performed in triplicate (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ between HEK-pDR2 and HEK-TLR2 stimulated in the absence of serum and between HEK-TLR2 stimulated in the presence or absence of 1% FCS). (**C**) HEK-TLR2 or HEK-TLR4/MD2 cells were transiently transfected with an NF-κB luciferase Firefly reporter and constitutive Renilla luciferase vectors and following 48 h in culture stimulated with SES (1/100 dilution to Neat) for 16 h. Dual luciferase activity was then assayed and the Firefly reporter results normalized against the Renilla signal obtained as the fold change above the control. (**D**) HEK-TLR4/MD2 cells were transiently transfected with an NF-κB-responsive Firefly luciferase reporter and constitutive Renilla luciferase vectors and following 48 h in culture stimulated with LPS (0–100 ng/mL), sCD14 and LBP (0.5 μg/mL and 0.1 μg/mL, respectively) for 16 h. Dual luciferase activity was then assayed and the Firefly reporter results normalized against the Renilla signal obtained as the fold change above the control.

basal levels following stimulation with the same stimuli (data not shown).

The IFN-γ and TLR signalling pathways are important regulators of macrophage function [37] and IFN-γ up-regulates several TLR family mRNAs, in particular TLR2 [33, 34]. Given the crucial role played by IFN-γ in regulating peritoneal immune responses [6, 38], TLR2 expression was analysed by real-time PCR following stimulation of HPMC with IFN-γ. Over a 24-h period, we showed a 7-fold increase of TLR2 expression peaking at 6 h and returning to baseline levels by 12 h (Figure 5C). We also observed a similar pattern of TLR2 expression following stimulation with SES with an earlier peak at 3 h (Figure 5C). To complement these *in vitro* observations, we used our well-established *in vivo* model of peritoneal inflammation to examine TLR mRNA expression during SES-induced activation [6, 17]. Real-time PCR was performed using RNA extracted from the

parietal peritoneal membrane at various time points following SES injection. This analysis showed a rapid and transient 3-fold increase of TLR2 mRNA expression (Figure 5D). This peak in TLR2 expression coincides with the time point of peak chemokine expression (CXCL1/KC and CXCL2/MIP-2) [6, 17].

Discussion

Repeated peritonitis is a feature of PD treatment and predominantly caused by Gram-positive bacteria, especially *Staphylococcus* species [2]. However, Gram-negative bacterial infections also represent a significant problem and overall have a worse prognosis in terms of patient outcomes [3]. It is a generally thought that host defenses are compromised in PD patients as a result of dialysis solution exposure, dilution of humoral factors and alteration in resident and recruited leucocytes function [4, 39]. The initial peritoneal immune response involves contributions not only from resident peritoneal leucocytes [23, 40] but also from the peritoneal mesothelial cells, lining the parietal and visceral peritoneum [41]. Mesothelial cells express adhesion molecules [42], synthesize cytokines and chemokines [6, 8, 17] and present antigen via major histocompatibility complex Class II, promoting $CD4^+$ T-cell responses [11, 43, 44]. Therefore, we examined TLR expression by HPMC and characterized their responses to bacterial agonists. To our knowledge, this study represents the most comprehensive characterization of TLR expression and responsiveness to bacterial ligands in primary HPMC to date.

An analysis of TLR expression by HPMC identified expression of a restricted subset of TLR family members (TLR1-6), which was in agreement with findings from murine peritoneal mesothelial cells [7, 45]. However, experiments examining the responsiveness of HPMC to bacterial TLR agonists revealed important differences between human and murine systems. Although HPMC responded to TLR1/2, TLR2/6 and TLR5 ligands in a similar manner to murine cells, human cells, unlike reports from murine cells, were unresponsive to the TLR4 agonist LPS [7, 45]. As in our previous studies, we went to great lengths (using differential subculture) to ensure that HPMC cultures were not contaminated with peritoneal fibroblasts or residual resident macrophages that might contribute to apparent TLR4 expression [8, 46–48].

Lack of TLR4 responsiveness may also be due to TLR4 downregulation at the cell surface, known as 'LPS tolerance' [25]. Analysis of freshly isolated mesothelial cells and quantification of the tolerization-resistant chemokine CCL5 appeared to exclude tolerance for the lack of TLR4 responsiveness [28]. Poor responses to TLR4 ligands in airway smooth muscle cells were overcome by co-culture with PBMC in an IL-1β-dependent manner [49]. Therefore, peritoneal macrophages may act as important sensors of Gram-negative bacterial infections, indirectly activating mesothelial cells [4, 39, 50]. Alternatively, mesothelial LPS hypo-responsiveness may be beneficial in avoiding excessive inflammation. In this respect, intestinal epithelial cells lose their ability to respond to LPS after birth [51]. Overall, our data suggest that HPMC respond to bacterial infections through TLR2 (Gram-positive bacteria) and TLR5 (flagellated bacteria, predominantly Gram negative) and that of others to viral pathogens via TLR3 recognition of double-stranded RNA [52]. These data do not exclude the possibility that other pattern recognition receptor families, e.g. NOD-like receptors NLRs and retinoic acid-inducible gene-I-like receptors (RLRs), may also play a role in pathogen recognition in HPMC [32].

TLR5 mediates recognition of flagellin, a flagellum component, present on many motile bacteria [28]. Interestingly, TLR5 expression in the intestinal epithelium is limited to the basolateral surface and is activated upon bacterial invasion of the gut wall [53, 54]. TLR5-deficient mice develop a TLR4-dependent spontaneous colitis, suggesting a critical role for TLR5 in maintaining the host-microflora balance within the gastro-intestinal tract [55]. TLR5 expression on mesothelial cells may therefore be critical in sensing invasion of flagellated bacteria into the peritoneal cavity. Translocation of intestinal bacteria is a potential cause of infection in PD patients, along with access through the intraperitoneal catheter[3], and many flagellated bacteria are Gram-negative species, with a poor outcome in PD-associated peritonitis [2].

TLR2, but not TLR4, expression was critical for the recognition of a *S. epidermidis*-derived cell free supernatant (SES), which has been extensively utilized to model acute peritoneal inflammation [16, 17]. Similarly, others have demonstrated that TLR2 is required for cellular responses to and clearance of *Staphylococcus* species [56, 58]. TLR2 may also act in concert with co-receptors e.g. other TLRs (TLR1/6), Dectin-1, CD36 or CD14, a co-factor for both TLR2- and TLR4-mediated responses [13, 59]. CD14 blockade significantly reduced SES-induced responses in PBMC indicating that, in leucocytes, TLR-mediated responses are highly CD14 dependent. HPMC lacked cell surface expression of CD14, and although serum, a source of soluble CD14, had a significant effect on SES-mediated response in HPMC, purified sCD14 did not. These results indicate that (i) the requirement for CD14 in TLR2 activation is cell specific and (ii) there may be other soluble co-factors in serum that co-operate with TLR2 signalling.

Finally, we identified the up-regulation of TLR2 expression in response to both TLR2-specific agonists and pro-inflammatory cytokines in HPMC and in response to SES *in vivo*. It is well established in other cell types that both TLR and cytokine signalling may influence TLR expression and responsiveness [33–36, 60]. In particular, van Aubel *et al.* [60] described a differential cross-talk between TLRs 2, 4 and 5, and TLR4-induced IFN-γ production led to a sensitization of TLR2-dependent activation in a model of Gram-negative sepsis [61]. Interestingly, the induction of TLR2 expression *in vivo* coincides with the peak of neutrophil-attracting chemokine expression [6, 17, 62], suggesting the involvement of TLR2 in these critical early immune activation events. Mesothelial cells are a major resident cell type within the peritoneal cavity and are therefore likely to encounter infecting bacteria, triggering local inflammation and host defence [50]. This study is the first to fully

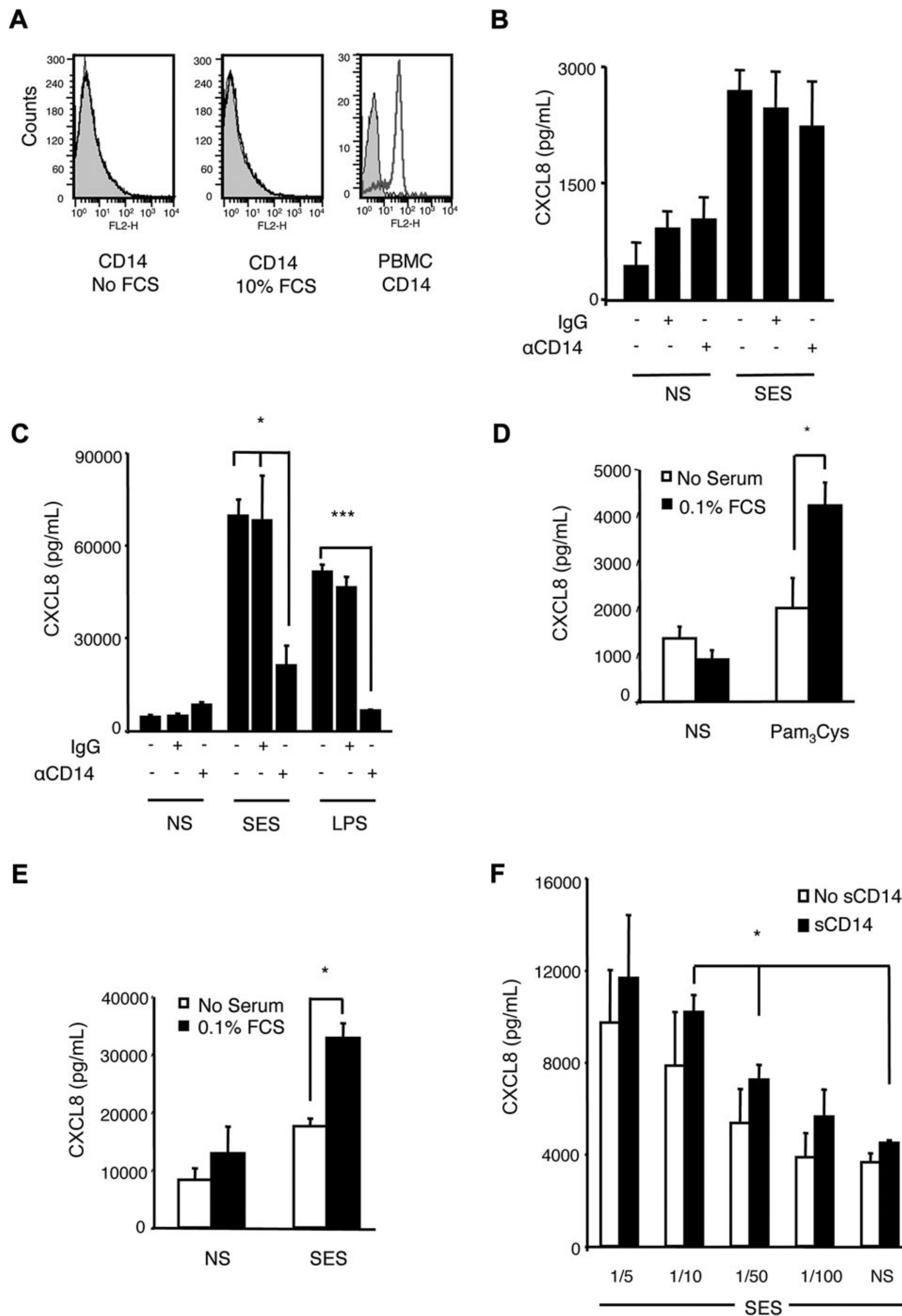


Fig. 4. CD14 can act as a co-receptor for SES. (**A**) HPMC were immunostained with a PE-conjugated CD14 monoclonal antibody in the presence or absence of 10% serum and human PBMC were used as a positive control for CD14 expression (isotype control antibody shaded in gray and CD14 expression outlined in black). Data presented are a representative result of six experiments performed with primary cell lines derived from separate donors. (**B**) HPMC were stimulated in medium with SES (1/10 dilution) in the presence or absence of anti-CD14-blocking antibody (10 μg/mL) for 1 h 30 min. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are representative of three experiments performed in triplicate with HPMC derived from three independent donors. (**C**) PBMC were isolated from healthy human volunteers and were stimulated in medium containing 0.5% human AB serum with LPS (50 ng/mL) or SES (1/80 dilution) in the presence or absence of anti-CD14-blocking antibody (10 μg/mL) for 1 h. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are representative of three experiments performed in triplicate with PBMC derived from three independent donors (**$P < 0.01$). (**D**) HPMC were stimulated with Pam_3Cys (2 μg/mL) in the presence or absence of 0.1% FCS (as a source of sCD14) for 24 h. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are representative of three experiments performed in triplicate with primary cell lines derived from three independent donors (*$P < 0.05$). (**E**) HPMC were stimulated with SES (Neat) in the presence or absence of 0.1% FCS (as a source of sCD14) for 24 h. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are representative of three experiments performed in triplicate with primary cell lines derived from three independent donors (*$P < 0.05$). (**F**) HPMC were stimulated with increasing doses of SES in medium without serum in the absence or presence of sCD14 (500 ng/mL) for 24 h. CXCL8 levels were quantified in cell-free culture supernatants by ELISA. Data presented are representative of three experiments performed in triplicate with HPMC derived from three independent donors. (*$P < 0.05$ between unstimulated cells and the indicated doses of SES).

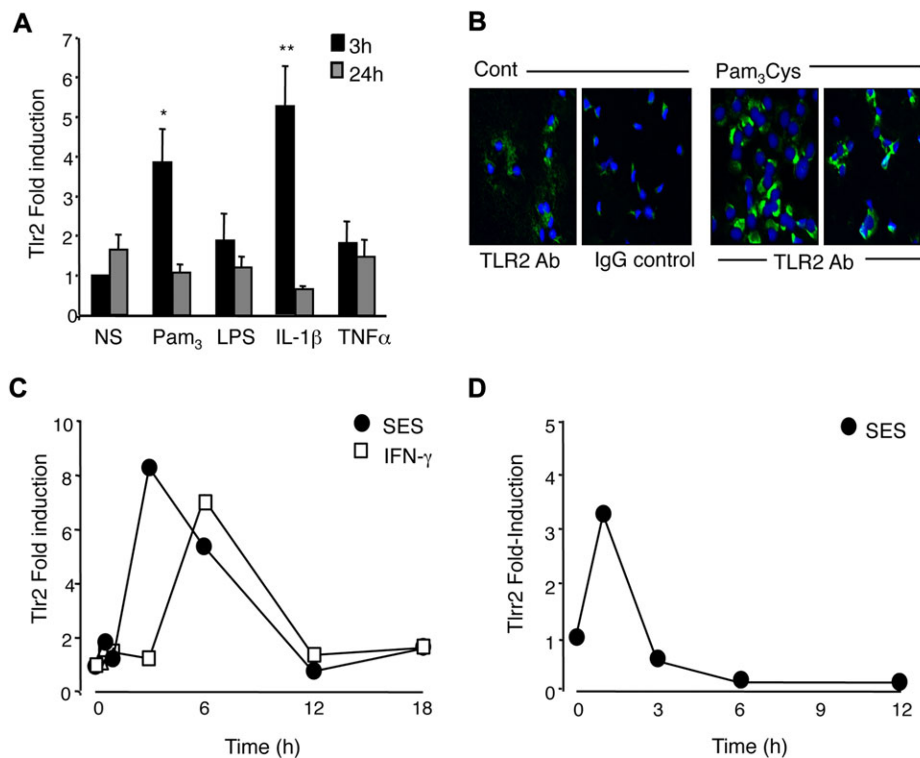


Fig. 5. Modulation of TLR2 expression by inflammatory cytokines and TLR ligands. (A) TLR2 mRNA expression was assessed by real-time RT–PCR following stimulation of HPMC for 3 or 24 h with Pam_3Cys (2 μg/mL), LPS (250 ng/mL), IL-1β (100 pg/mL) or TNFα (100 pg/mL). NS represent unstimulated cells. Data presented are representative of three experiments performed in triplicate with primary cell lines derived from three independent donors (*$P < 0.05$; **$P < 0.01$). (**B**) TLR2 expression in HPMC was detected by immunocytochemistry using the TLR2.5 monoclonal antibody. TLR2 expression was tested on cells stimulated with Pam3Cys. Control staining was carried out using an isotype-matched control antibody. Data presented are representative of two experiments performed with primary cell lines derived from two independent donors. (**C**) TLR2 mRNA expression was assessed following stimulation of HPMC for 24 h with SES (Neat) or IFN-γ (10 ng/mL) by real-time RT-PCR. Data presented are representative of three experiments performed in triplicate with primary cell lines derived from three independent donors (*$P < 0.05$; **$P < 0.01$). (**D**) Wild-type mice were administered via the *intraperitoneal (i.p.)* route with SES. At defined intervals, TLR2 mRNA expression levels were quantified by real-time RT–PCR following total RNA extraction from samples of parietal peritoneal membrane. This data represents the results from one experiment.

characterize HPMC expression of TLRs and their responses to bacterial ligands. An understanding of TLR-mediated responses during peritonitis will be important in improving compromised host defense in PD patients, reducing infection and improving clinical outcome.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

Acknowledgements. Real-Time PCR analysis and flow cytometry were performed in the Central Biotechnology Services (CBS) Core Facility in the School of Medicine, Cardiff University (www.cardiff.ac.uk/medic/cbs).

N.T., C.S.C. and S.A.J. were supported by a Wellcome Trust University Translational Award. C.A.F. was supported by a Kidney Research UK Career Development Fellowship (CDF02/2006) and a Wellcome Trust Value in People Award from Cardiff University.

Conflict of interest statement. We have had no involvements that might raise the question of bias in the work reported or in the conclusions, implications or opinions stated. The results presented in this paper have not been published previously, in whole or in part, except in abstract form.

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Received for publication: 10.1.11; Accepted in revised form: 29.3.11