Toll-like receptors 1 and 2 cooperatively mediate immune responses to curli, a common amyloid from enterobacterial biofilms

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Summary

Responses to host amyloids and curli amyloid fibrils of *Escherichia coli* and *Salmonella enterica* serotype Typhimurium are mediated through Toll-like receptor (TLR) 2. Here we show that TLR2 alone was not sufficient for mediating responses to curli. Instead, transfection experiments with human cervical cancer (HeLa) cells and antibody-mediated inhibition of TLR signalling in human macrophage-like (THP-1) cells suggested that TLR2 interacts with TLR1 to recognize curli amyloid fibrils. TLR1/TLR2 also serves as a receptor for tri-acylated lipoproteins, which are produced by *E. coli* and other Gram-negative bacteria. Despite the presence of multiple TLR1/TLR2 ligands on intact bacterial cells, an inability to produce curli amyloid fibrils markedly reduced the ability of *E. coli* to induce TLR2-dependent responses in vitro and in vivo. Collectively, our data suggest that curli amyloid fibrils from enterobacterial biofilms significantly contribute to TLR1/TLR2-mediated host responses against intact bacterial cells.

Introduction

Amyloids are protein deposits with a fibrillar cross β-sheet quaternary structure, which exhibit a starch (amylose)-like ability to stain with iodine. Host amyloid deposition in tissue is a result of protein misfolding and is associated with a number of illnesses, such as Alzheimer’s disease and prion diseases. In contrast, many bacteria produce functional amyloid deposits, which are an important component of their extracellular biofilm matrix (Larsen et al., 2007; Jordal et al., 2009). Perhaps the best-characterized bacterial amyloid is encoded by the csgDEFG csgBA gene cluster of *Escherichia coli* and *Salmonella enterica* serotype Typhimurium (S. Typhimurium) (Olsen et al., 1989; Hammar et al., 1995; Römling et al., 1998), which directs the biosynthesis of extracellular amyloid fibrils, termed curli, composed of the CsgA protein (Chapman et al., 2002). Curli fibrils are a major extracellular matrix component contributing to biofilm formation in *E. coli* (Vidal et al., 1998; Prigent-Combaret et al., 2000) and *S. Typhimurium* (Römling et al., 2003).

Amyloids of host and bacterial origin share a number of characteristics, including an ability to trigger innate immune responses. Host amyloid deposits induce chronic inflammation, which in turn results in the tissue injuries responsible for neurodegenerative diseases, such as Alzheimer’s disease (Akiyama et al., 2000). Similarly, expression of bacterial amyloid fibrils during infection is associated with the induction of inflammatory responses (Bian et al., 2000; 2001; Tükel et al., 2005; 2009). Recent research on the mechanisms by which amyloids initiate innate immune responses has revealed some diversity. For example, receptors implicated in inducing responses to β-amyloid from plaques of Alzheimer’s patients include Toll-like receptor (TLR)2 (Jana et al., 2008; Richard et al., 2008; Udan et al., 2008; Reed-Geagahan et al., 2009; Tükel et al., 2009), TLR4 (Tang et al., 2008; Udan et al., 2008; Reed-Geagahan et al., 2009) and a TLR4/TLR6 receptor complex (Stewart et al., 2010). Acute phase serum amyloid A, a host amyloid, stimulates innate responses through TLR2 (He et al., 2009; Chen et al., 2010), while pro-inflammatory cytokine expression is further enhanced when cells express a TLR2/TLR1 receptor complex (Cheng et al., 2008). The multitude of different receptors and receptor complexes implicated in mediating responses to host amyloids illustrates that it is not straightforward to predict by which mechanisms bacterial amyloids stimulate innate immunity.
Recent studies show that responses to amyloid fibrils formed by the S. Typhimurium CsgA protein are mediated through TLR2 (Tükel et al., 2005; 2009). Since both TLR1 and TLR6 have been implicated as co-receptors for different host amyloids (Cheng et al., 2008; Stewart et al., 2010), we explored the possibility that TLR2 forms functional hetero-dimers with TLR1 or TLR6 to mediate responses against bacterial amyloid fibrils formed by the CsgA protein. In addition to amyloid fibrils, TLR2 mediates responses against other pathogen-associated molecular patterns (PAMPs) present in bacterial cells. For example, lipoproteins of Gram-negative bacteria contain conserved tri-acylated cysteines at their N-termini (Hantke and Braun, 1973), which stimulate innate immune responses through a receptor complex containing TLR2 and TLR1 (Takeuchi et al., 2002). Lipoproteins of Mycoplasma spp. contain di-acylated cysteines (Shibata et al., 2000), a conserved structure recognized by TLR2 cooperatively with TLR6 (Takeuchi et al., 2001). To test the biological relevance of recognizing bacterial amyloids, we investigated whether formation of amyloid fibrils contributes significantly to TLR2-mediated responses against whole bacterial cells.

**Results**

**Cooperativity between TLR1 and TLR2 in initiating responses to CsgA**

Toll-like receptor 2 is necessary for initiating responses against amyloids in HEK293 cells (Tükel et al., 2005; 2009), a cell line intrinsically producing TLR1 and TLR6 (Kurt-Jones et al., 2004; Buwitt-Beckmann et al., 2006). To determine whether TLR2 cooperates with other receptors to initiate these responses, transfection experiments were performed with human cervical cancer (HeLa) cells, which lack TLR expression. HeLa cells carrying a NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells)-dependent luciferase reporter fusion were transfected transiently with the empty cloning vector (mock transfection) alone or in combination with constructs encoding human TLR1, human TLR6 or human TLR2 (Fig. 1). Stimulation with di-acylated synthetic lipopeptide (Pam3CSK4) resulted in marked induction of the NFkB luciferase reporter only when HeLa cells were transfected with both TLR2 and TLR6 (Fig. 1A). In contrast, stimulation with tri-acylated synthetic lipopeptide (Pam2CSK4) induced the NFkB luciferase reporter only when HeLa cells were transfected with both TLR2 and TLR1 (Fig. 1B). These data validated our transfection approach and confirmed that tri-acylated lipopeptides stimulate a receptor complex containing TLR2 and TLR1 (Takeuchi et al., 2002), while di-acylated lipopeptides are recognized by TLR2 cooperatively with TLR6 (Takeuchi et al., 2001).

To determine whether TLR2 cooperates with other receptors to mediate responses to amyloids present in bacterial biofilm, curli fibrils were purified from S. Typhimurium biofilm and used for stimulation of HeLa cells. Transfection with TLR2 alone did not confer responsiveness to curli, suggesting that TLR2 alone was not sufficient for mediating responses to amyloid fibrils purified from bacterial biofilm (Fig. 1C). A marked induction of the NFkB luciferase reporter was observed only with HeLa cells transfected with both TLR1 and TLR2. The main component of curli fibrils is the CsgA protein (Olsen et al., 1998). To further investigate the responsiveness of HeLa cells to curli, a fusion between glutathione S-transferase (GST) and the S. Typhimurium CsgA protein was affinity purified from the E. coli cytosol. Contamination with lipoprotein cannot account for the agonist activity of this fusion protein, because TLR2-mediated responses to GST–CsgA, but not to Pam3CSK4, can be abrogated by pretreatment with protease K (Tükel et al., 2005). Stimulation with GST–CsgA induced expression of the NFkB luciferase reporter only in HeLa cells transfected with both TLR1 and TLR2 (Fig. 1D). HeLa cells did not respond to control stimulation with GST protein purified from E. coli by the same method. Collectively, these data suggested that a TLR1/TLR2 receptor complex is sufficient for mediating responses to the CsgA protein from curli fibrils.

TLR1 and TLR2 initiate signalling cascades leading to NFkB translocation into the nucleus by engaging an intracellular adaptor protein, termed ‘myeloid differentia- tion primary response protein 88’ (MyD88) (Underhill et al., 1999; Wang et al., 2001). In HeLa cells transfected with both TLR1 and TLR2, expression of a dominant negative form of MyD88 resulted in a significantly (P < 0.05) blunted expression of the NFkB luciferase reporter in response to stimulation with Pam3CSK4, purified curli fibrils or GST– CsgA protein (Fig. 2). Our data were consistent with a role of MyD88 in initiating TLR1/TLR2-mediated responses to tri-acylated lipopeptide and to CsgA.

**TLR1 and TLR2 are necessary for responses to CsgA in human macrophage-like cells**

While transfection of HeLa cells represents a convenient model for investigating the contribution of individual TLRs to host responses (Fig. 1), drawbacks of this approach include artificial levels of receptor expression and the questionable relevance of using cervical cancer cells to study responses to E. coli or S. Typhimurium. We therefore investigated responses to curli fibrils using THP-1 cells, a human macrophage-like cell line naturally expressing TLR1 and TLR2.

Stimulation of THP-1 cells with purified curli fibrils resulted in increased mRNA levels of IL8, the gene
encoding CXCL8 (formerly known as interleukin-8) (Fig. 3A). IL8 mRNA levels were significantly \( (P < 0.05) \) reduced when the stimulation was performed in the presence of blocking anti-TLR2 and/or anti-TLR1 antibodies. These data were consistent with the idea that responses to curli fibrils are mediated through TLR2 and TLR1 in THP-1 cells. Similarly, THP-1 cells responded to stimulation with synthetic tri-acylated lipopeptide (Pam3CSK4) by producing increased mRNA levels of IL8. Induction of IL8 expression by Pam3CSK4 was significantly \( (P < 0.05) \) blunted, when stimulation was performed in the presence of blocking anti-TLR2 and/or anti-TLR1 antibodies. These data were consistent with previous reports implicating a TLR1/TLR2 receptor complex in responses to tri-acylated lipoproteins (Takeuchi et al., 2002). Next, THP-1 cells were stimulated with a synthetic peptide containing residues 111–151 of S. Typhimurium CsgA (CsgA111–151), a region of the protein involved in amyloid formation and sufficient for stimulating TLR2-mediated responses (Tükel et al., 2009). Compared with mock infected cells, expression of IL8 was markedly upregulated in THP-1 cells upon stimulation with CsgA111–151. Incubation with blocking anti-TLR1 or anti-TLR2 antibodies significantly \( (P < 0.05) \) blunted IL8 expression in response to CsgA111–151. In contrast, anti-TLR2 and anti-TLR1 antibodies did not block induction of IL8 mRNA in THP-1 cells treated with lipopolysaccharide (LPS) (Fig. 3B). The use of the synthetic Pam3CSK4 and CsgA111–151 peptides (Fig. 3A) excluded the possibility that responses of THP-1 cells were due to contamination with other PAMPs. We conclude that the TLR1/TLR2 receptor complex mediates responses against both tri-acylated lipoprotein and curli amyloid fibrils.
TLR2-mediated responses to whole bacterial cells are markedly influenced by expression of curli fibrils

In addition to curli fibrils, intact *E. coli* cells contain tri-acylated lipoproteins (Hantke and Braun, 1973), which stimulate responses through a TLR2/TLR1 receptor complex (Takeuchi et al., 2002). Due to the presence of multiple agonists for TLR2/TLR1, the biological relevance of curli in mediating responses to whole bacterial cells is not apparent from stimulating host cells with purified ligands (Fig. 1). To address this issue, we analysed responses to the *E. coli* wild-type (MC4100), an isogenic csgBA mutant (LSR13) and the csgBA mutant complemented with the cloned csgBA genes from *S. Typhimurium* (LSR13[pCsgBA]). Expression of CsgA was detected by Western blot in whole-cell extracts from the *E. coli* wild-type (MC4100) and the complemented csgBA mutant (LSR13[pCsgBA]), but not in the csgBA mutant (LSR13) (Fig. 4A). Biofilm formation of the *E. coli* wild-type (MC4100) on agar plates results in secretion of extracellular matrix, which contains curli amyloid fibrils (Hammar et al., 1995). Bacterial colonies formed under this growth condition bind Congo red, a dye that stains amyloid deposits (Hammar et al., 1995). Consistent with the elaboration of curli fibrils, colonies of the *E. coli* wild-type (MC4100) and the complemented csgBA mutant (LSR13[pCsgBA]) bound Congo red (Fig. 4B). In contrast, colonies of the csgBA mutant did not exhibit Congo red binding.

Stimulation with whole bacterial cells induced expression of the NFκB luciferase reporter only in HeLa cells transfected with TLR2/TLR1, but not cells transfected with either TLR1, TLR2, TLR6 or TLR2/TLR6 (Fig. 4C). These data suggested that *E. coli* cells contain agonists for TLR1/TLR2, but not for any of the other receptors tested. Expression of the NFκB luciferase reporter was significantly (*P < 0.05*) higher after stimulation with the *E. coli* wild-type (MC4100) than with the csgBA mutant (LSR13), which illustrated that curli production contributed markedly to TLR1/TLR2-mediated responses induced by whole bacterial cells. Complementation (LSR13[pCsgBA]) restored the level of NFκB luciferase reporter expression to levels...
observed after stimulating cells with *E. coli* wild-type (MC4100). A single mutation in csgA (*E. coli* strain MHR204) also reduced NFκB luciferase reporter expression in HeLa cells transfected with TLR1/TLR2 (Fig. 4D).

Expression of the NFκB luciferase reporter induced by stimulation with the csgBA mutant was significantly higher (*P* < 0.05) than expression in mock-stimulated HeLa cells (Fig. 4C). This stimulation of the NFκB luciferase reporter by the csgBA mutant was likely due to the induction of TLR1/TLR2-mediated responses by other ligands, such as tri-acylated lipoproteins (Hantke and Braun, 1973). We next investigated responses to LPS, a TLR4 agonist that was predicted to be expressed equally by all three bacterial strains. The *E. coli* wild-type (MC4100), the csgBA mutant (LSR13) and the complemented csgBA mutant (LSR13[pCsgBA]) induced similar expression levels of the NFκB luciferase reporter in HeLa cells transfected with TLR4, MD2 and CD14 (Fig. 4E). Thus, the reduced ability of the csgBA mutant to induce the NFκB luciferase reporter in HeLa cells transfected with TLR1/TLR2 (Fig. 4C) was not due to a general defect of the bacterial strain in inducing host responses in HeLa cells (Fig. 4E), but to its inability to produce curli (Fig. 4A and B).

The important role of amyloids in stimulating TLR1/TLR2-mediated responses against whole bacterial cells may relate to the accessibility of curli on the cell surface. In contrast, the incorporation of other TLR1/TLR2 ligands into the cell envelope may make these less accessible to pattern recognition receptors in intact bacterial cells. To test this idea, HeLa cells were stimulated with whole bacterial cell lysates to release internal PAMPs. Whole-cell lysates of the *E. coli* wild-type (MC4100) and the csgBA mutant (LSR13) elicited similar NFκB luciferase reporter expression levels (Fig. 3F), suggesting that after a substantial release of other PAMPs from bacterial cells, curli is no longer the major ligand responsible for stimulating the TLR1/TLR2 receptor complex. Collectively, our results provided evidence that curli fibrils contributed markedly to TLR1/TLR2-mediated responses to intact bacterial cells *in vitro*.

While HeLa cells are not able to kill bacterial cells, which would result in the release of PAMPs, lysates of *E. coli* cells may occur *in vivo* during an infection. To determine whether curli are an important contributor to TLR2-mediated responses generated by bacteria *in vivo*, mice were injected intraperitoneally with the *E. coli* wild-type (MC4100), the csgBA mutant (LSR13) or the complemented csgBA mutant (LSR13[pCsgBA]) and transcript levels of Nos2, encoding inducible nitric oxide synthase (iNOS), were quantified 8 h after infection using quantitative real-time PCR (Fig. 5). Expression of Nos2 in the liver was significantly (*P* < 0.05) higher after stimulation with the *E. coli* wild-type (MC4100) than with the csgBA mutant (LSR13) (Fig. 5A), although both bacterial strains were recovered in similar numbers from this organ (Fig. 5B). Reduced Nos2 production elicited by the csgBA
mutant could be complemented in vivo by introducing the cloned csgBA genes from S. Typhimurium (LSR13[pCsgBA]). Differences between the E. coli wild-type (MC4100) and the csgBA mutant (LSR13) in inducing Nos2 expression were TLR2-dependent, because both strains elicited similar Nos2 mRNA levels when the experiment was repeated with TLR2-deficient mice (Fig. 5A). These data show that curli fibrils contribute significantly to TLR2-mediated responses against bacterial cells in vivo.

Fig. 4. Curli contribute to recognition of whole E. coli cells through TLR1/TLR2.

A. Detection of CsgA expression in the indicated E. coli strains using Western blot with rabbit anti-CsgA serum.
B. Congo red binding activity of bacterial colonies formed by the indicated E. coli strains.
C–F. HeLa cells carrying a NFκB luciferase reporter were mock-transfected (vector) or transfected with the indicated pathogen recognition receptors. Cells were stimulated with the indicated E. coli strains (C, D and E) or with whole-cell lysates of the indicated E. coli strains (F). Non-stimulated cells served as negative controls. Activity of the NFκB luciferase reporter was monitored by measuring RLU. Bars represent averages from at least three independent experiments ± standard error.
Discussion

Biofilms of *E. coli* and *S. Typhimurium* contain extracellular amyloid deposits, termed curli (Vidal et al., 1998; Prigent-Combaret et al., 2000; Römling et al., 2003), which can elicit inflammatory responses in the host (Bian et al., 2000, 2001; Tükel et al., 2005, 2009). While TLR2 is necessary for initiating these responses (Tükel et al., 2005, 2009), here we show that this receptor was not sufficient for initiating inflammatory gene expression after stimulation with curli. Instead, a receptor complex containing TLR1 and TLR2 was sufficient for mediating responses to curli amyloid fibrils in HeLa cells. In addition to curli, TLR2 has been implicated in recognizing other PAMPs present in *E. coli*, including tri-acylated lipoproteins (Takeuchi et al., 2002), peptidoglycan (Schwandner et al., 1999) and B subunits of type II heat-labile enterotoxins (Hajishengallis et al., 2005; Liang et al., 2007). The *E. coli* isolate used in this study does not express a heat-labile enterotoxin. Furthermore, ultra pure peptidoglycan preparations do not activate TLR2, thus calling into question whether peptidoglycan is indeed a genuine TLR2-ligand (Travassos et al., 2004). Thus, bacteria used in this study expressed at least two classes of TLR1/TLR2 ligands, curli amyloid fibrils and tri-acylated lipoproteins. The latter group consists of 96 distinct lipoproteins, some of which, such as the Braun lipoprotein (Hantke and Braun, 1973), are highly expressed in *E. coli* (Brokx et al., 2004).

Despite the presence of multiple TLR2 ligands, an inability to produce curli fibrils markedly reduced the ability of HeLa cells transfected with TLR1/TLR2 to respond to stimulation with intact bacterial cells. Elaboration of curli fibrils on the surface of *E. coli* induces Nos2 expression in a mouse sepsis model (Bian et al., 2001) and here we show that this effect was TLR2-dependent. Curli fibrils might serve as an important PAMP because these structures are secreted, thereby being more readily accessible to detection by pattern recognition receptors than lipoproteins, which are buried in the cell envelope of intact bacterial cells. The importance of bacterial amyloids as PAMPs is further supported by work in animal models, where secretion of curli fibrils contributes significantly to host responses against *E. coli* or *S. Typhimurium* (Bian et al., 2001; Tükel et al., 2005, 2009). Although expression of curli fibrils is induced in *E. coli* at ambient temperature (26°C) in rich medium, expression at body temperature (37°C) can be observed after culturing bacteria under conditions of iron starvation, which is encountered in the mammalian host (Römling et al., 1998). Serum from mice infected with *S. Typhimurium* or patients recovering from *E. coli* sepsis contain anti-CsgA antibodies, which provides indirect evidence for in vivo expression of curli fibrils (Bian et al., 2000; Humphries et al., 2005). Collectively, these studies identify bacterial amyloid from biofilm material as a significant PAMP recognized by the innate immune system. Amyloid fibrils are not only produced by members of the *Enterobacteriaceae*, but are also commonly present in biofilm material from bacteria belonging to the *Firmicutes*, *Bacteroides* and *Actinobacteria* phyla (Larsen et al., 2007; Jordal et al., 2009), whose representatives are dominant constituents of the intestinal microbiota. Thus recognition of amyloids is of broad significance for detection of bacterial cells by the innate immune system.

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Experimental procedures

Bacterial strains and plasmids

Curli producing wild-type *E. coli* strain MC4100, an isogenic curli mutant LSR13 (csgBA mutant) and a csgA::TN105 mutant (MHR204) were kindly provided by Dr Scott Hultgren and Dr Matthew Chapman (Wang and Chapman, 2008) (Hammars et al. 1995). To complement curli production in strain LSR13, the csgBA genes were amplified from S. Typhimurium by using primers 5'-GGATCCGGGTTGACAGGTTGAAAACAAAATTGTTA-3' and 5'-GGAATTCCTTTATGCGACAGGCTAAATTAATACTGGTTA GCCGTGGGC-3'. The resulting PCR product was digested with EcoRI and BamHI and ligated into low-copy plasmid pWSK29 giving rise to plasmid pCsgBA.

Growth conditions for biofilm formation and purification of curli

Extracellular matrix formation was induced on T-medium plates at 28°C for 48 h (Collinson et al., 1991). Curli production was monitored by the addition of Congo red to this medium to a final concentration of 20 mg l⁻¹ (Hammars et al., 1995). *E. coli* cells were recovered from the plates in phosphate buffered-saline and OD₆₀₀ was adjusted to 0.5. Bacteria were killed by the addition of 1% sodium azide for 5 min or lysed by heat treatment at 100°C for 5 min. Na-Azide was removed by washing in PBS three times.

Curli was purified from the surface of the S. Typhimurium msbB mutant (RPW3) according to an established protocol (Collinson et al., 1991). Briefly, cells were removed from T-medium plates and lysed by sonication followed by enzymatic digestion and preparative sodium dodecyl-sulfate gel electrophoresis (SDS-PAGE). Insoluble material (curli fibrils) retained in the well of the SDS-PAGE gel was collected after the electrophoresis.

Western blot

Bacteria were recovered from the T-medium plates and curli was depolymerized by 90% formic acid treatment as described previously (Collinson et al., 1991). The sample was resuspended in SDS-PAGE sample buffer, boiled for 10 min and analysed by SDS-PAGE on a 15% gel. Following electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore) using a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. CsgA antisemur (Humphries et al., 2003) and a horse radish peroxidase conjugated goat anti-rabbit secondary antibody (Bio-Rad) were used to detect curli expression.

Purification of GST fusion proteins

Plasmid pSW5-50, containing the csgA gene cloned in the GST fusion protein vector pGEX-4T-2, was described previously (Humphries et al., 2003). Fusion proteins GST and GST–CsgA were purified from *E. coli* strains DH5α (pGEX-4T-2) and DH5α (pSW5-50), respectively, using glutathione sepharose (Amer sham Pharmacia) columns as described previously (Humphries et al., 2003). The protein concentration in each sample was determined by Bradford assay (Ausubel et al., 1994).

Tissue culture cells and reagents

The HeLa 57A cell line stably transfected with an NF-kB luciferase reporter was kindly provided by Dr R.T. Hay (the Wellcome Trust Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, UK). HeLa 57A cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS at 37°C in 5% CO₂ atmosphere. Human monocytic cell line THP-1 was kindly provided by Dr Vernon Tesh. THP-1 cells were maintained in RPMI containing 10% FBS and glutamine. The synthetic diacylated lipopeptide (Pam₃CSK₄) and synthetic triacylated lipopeptide (Pam₃CSK₄) were purchased from InvivoGen.

Transfection of HeLa cells

HeLa 57A cells were seeded at a density of 1 × 10⁶ cells per well in 96-well tissue culture plates containing DMEM + 10% FBS at 24 h prior to transfection. Then cells were transiently transfected using ExGen 500 reagent (Fermentas) according to the manufacturer’s instructions. Vectors carrying mTLR4, mMD2, mCD14, hTLR2, hTLR1, hTLR6 and LacZ (Keestra et al., 2007; Keestra and van Putten, 2008) were added in various combinations to a total amount of 150 ng transfected plasmid DNA. Human dominant negative MyD88 (DMyD88) vector was purchased from InvivoGen and added to the transfection reaction. In all transfections, the LacZ vector was used to normalize the transfection efficiency. Luciferase assays were performed at 48 h post transfection.

Luciferase assay

The Luciferase assay System (containing luciferase reagent and reporter lysis buffer) and β-galactosidase Enzyme Assay System were purchased from Promega. TLR signalling was assessed using the NF-kB luciferase reporter system. For luciferase assays, cells were stimulated with purified curli (2.5 µg well⁻¹), GST (2.5 µg well⁻¹), GST–CsgA (2.5 µg well⁻¹), Pam₃CSK₄ (0.05 µg well⁻¹), or Pam₃CSK₄ (0.05 µg well⁻¹) for 6 h prior to determining luciferase activity. For luciferase assays with whole bacterial cells, HeLa cells were stimulated with 4 × 10⁵ colony-forming units (cfu) well⁻¹ of the indicated bacterial strains for 4 h prior to determining luciferase activity. To determine luciferase activity, cells were washed three times with PBS, and lysed with 60 µl of reporter lysis buffer. Luciferase activity was measured using a multimode plate reader (Analyzer GT, Molecular Devices). Luciferase values were adjusted to β-galactosidase values to normalize the efficiency of transfection. Results were expressed in relative luciferase units.

Stimulation of THP-1 cells

To differentiate monocytes into macrophages, THP-1 cells were stimulated with 50 ng ml⁻¹ phorbol myristic acid (PMA) (Sigma) and seeded at a density of 5 × 10⁵ cells per well in 24-well plates. Following a 48 h incubation at 37°C in 5% CO₂ atmosphere, PMA containing media was removed. Macrophages were washed twice with PBS, and replaced with PMA-free medium. Fresh media was added daily (500 µl), and cell assays were performed after 7 days of incubation.
Blocking anti-human TLR2 (T2.5) and TLR1 (GD2,F4), anti-bodies were purchased from eBioscience. Macrophages were treated with 10 μg per well of antibody or medium (control) 1 h prior to stimulation. Purified curli fibrils, synthetic CsgA \(_{111-151}\) (5 μM) or Pam3CSK4 (0.1 μg ml\(^{-1}\)) were added to the wells for 4 h. After stimulation, RNA was extracted to determine \(IL8\) mRNA levels by real-time PCR.

**Real-time PCR**

RNA samples were prepared using TRizol reagent (Molecular Research Center). Real-time PCR was performed using the SYBR Green method (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Reverse transcription of total RNA (1 μg) was performed at 48°C for 30 min. Real-time PCR was performed for each cDNA sample (5 μl per reaction) in duplicate using gene specific primers (human \(GAPDH\) and human \(IL8\)) (Stylianou et al., 2002) and an ABI Prism 7700 thermocycler (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Real-time PCR amplification of \(GAPDH\) transcripts was used to normalize the cDNA concentrations of the other gene transcripts. Results were given as 2\(^{-\Delta CT}\) ± S.E, where S is the standard deviation.

**Animal experiments**

For mouse experiments, 4- to 6-week-old female C57BL/6 mice or congenic TLR2-deficient mice (Takeuchi et al., 1999) were purchased from Jackson Laboratories. Groups of 4 mice were intraperitoneally infected with \(1 \times 10^6\) cfu in PBS or sterile PBS (mock infection). At 8 h after infection, mice where sacrificed a prior to stimulation. Purified curli fibrils, synthetic CsgA \(_{111-151}\) (mock infection). At 8 h after infection, mice were sacrificed and

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