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Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition

Leonardo H. Travassos[1,5], Stephen E. Girardin[2], Dana J. Philpott[1], Didier Blanot[6], Marie-Anne Nahori[3], Catherine Werts[3] & Ivo G. Boneca[4+]

[1]Groupe d'Immunité Innée et Signalisation, [2]Unité de Pathogénie Microbienne Moléculaire, [3]Unité Postulante Cytokines et Inflammation, and [4]Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris, France, [5]Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil, and [6]Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, Université Paris-Sud, Orsay, France

Toll-like receptor 2 (TLR2) has been shown to recognize several classes of pathogen-associated molecular patterns including peptidoglycan (PG). However, studies linking PG with TLR2 recognition have relied mainly on the use of commercial *Staphylococcus aureus* PG and have not addressed TLR2 recognition of other PG types. Using highly purified PGs from eight bacteria (*Escherichia coli, Pseudomonas aeruginosa, Yersinia pseudotuberculosis, Helicobacter pylori, Bacillus subtilis, Listeria monocytogenes, Streptococcus pneumoniae* and *S. aureus*), we show that these PGs are not sensed through TLR2, TLR2/1 or TLR2/6. PG sensing is lost after removal of lipoproteins or lipoteichoic acids (LTAs) from Gram-negative and Gram-positive cell walls, respectively. Accordingly, purified LTAs are sensed synergistically through TLR2/1. Finally, we show that elicited peritoneal murine macrophages do not produce tumour necrosis factor-α or interleukin-6 in response to purified PGs, suggesting that PG detection is more likely to occur intracellularly (through Nod1/Nod2) rather than from the extracellular compartment.

Keywords: peptidoglycan; LTA; Nod2; cytokine; macrophage; TLR

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[1]Groupe d'Immunité Innée et Signalisation, [2]Unité de Pathogénie Microbienne Moléculaire, [3]Unité Postulante Cytokines et Inflammation, [4]Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, 28 Rue du Dr Roux, 75724 Paris cedex 15, France
[5]Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Bloco I-Cidade Universitária-ilha do Fundão, CEP 21941-590 Rio de Janeiro-RJ, Brasil
[6]Enveloppes Bactériennes et Antibiotiques, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, Unité Mixte de Recherche 8619 CNRS, Université Paris-Sud, Bât. 430, 91405 Orsay, France
[+]Corresponding author. Tel: +33 1 4438 9516; Fax: +33 1 4061 3640;
E-mail: bonecai@pasteur.fr

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INTRODUCTION

The discovery of Toll-like receptors (TLRs) markedly increased our understanding of how the innate immune system recognizes and triggers a response towards microbes (Takeda & Akira, 2003). TLRs detect pathogen-associated molecular patterns (PAMPs) and mediate the induction of pro-inflammatory cytokines and co-stimulatory cell-surface molecules through the activation of transcription factors such as nuclear factor-κB (NF-κB). These responses then contribute to the clearance of the infectious agent from the host organism.

The best-characterized TLRs are TLR4 and TLR2. Whereas TLR4 recognizes lipopolysaccharide (LPS), TLR2 recognizes several molecules including lipoteichoic acid (LTA), lipoarabinomannan, lipoproteins and peptidoglycan (PG), which is a polymer composed of repeating *N*-acetylglucosamine-β-1,4-*N*-acetylmuramic acid (GlcNAc-MurNAc) disaccharide units linked by short peptides. Although the role of TLR2 as a PG receptor has been extensively examined, these studies have been mainly conducted with commercial *Staphylococcus aureus* PG preparations (Takeda & Akira, 2003). During our investigations describing the muramyltripeptide recognized by the cytosolic PG sensor Nod1 (Girardin *et al*, 2003a), we observed that highly purified PGs did not elicit TLR2-dependent activation in transiently transfected HEK293T cells. Consequently, we hypothesized that TLR2–PG stimulatory activity could be attributed to other cell wall components present in commercial PG preparations or partially purified PG.

Here, we used highly purified PGs from eight different Gram-positive and Gram-negative bacteria to clearly show that purified PG is not detected by TLR2. The observed PG stimulatory activity towards TLR2 is due to the presence of LTA or lipoproteins in the cell walls from Gram-positive or Gram-negative bacteria, respectively.

RESULTS AND DISCUSSION

Does TLR2 recognize different PGs?

Different PG chemotypes differ mainly according to variations in the third amino acid of the peptidic chain and the nature of the

crossbridge. We prepared highly purified PGs from Gram-positive and Gram-negative bacteria and tested their recognition by TLR2 at different steps of the purification procedure. Due to their distinct cell wall architecture, the purification procedure, outlined in supplementary Fig 1 online, is radically different between Gram-negative and Gram-positive bacteria.

As Nod2 detects muramyldipeptide (MDP; Girardin *et al*, 2003b; Inohara *et al*, 2003), and thus can detect PG purified from either Gram-negative or Gram-positive bacteria, we co-transfected Nod2 in HEK293T cells with the same amount of partially or highly purified PG as a positive control for our purification procedures. Accordingly, with increasing purity of PG, we observed higher levels of Nod2-dependent NF-κB activation (Fig 1A,C). However, all PG preparations from *S. aureus* and *Streptococcus pneumoniae* elicited a poor activation through Nod2. Muramidase digestion of *S. aureus* PG did not enhance Nod2-dependent activity due to the fact that it produces trace amounts of MDP (de Jonge *et al*, 1992). In contrast, muramidase digestion of *S. pneumoniae* PG produced a tenfold increase in Nod2-dependent NF-κB activation (H.L. Travassos and I.G. Boneca, unpublished observations) consistent with higher amounts of MDP. Finally, analyses of the amino-sugar and amino-acid composition for the eight highly purified PGs (Table 1) were consistent with previous reports indicating that at the end of the purification procedure, no other contaminants were present (Schleifer & Kandler, 1972; Quintela *et al*, 1995; Costa *et al*, 1999). The presence of LTA/wall teichoic acid (WTA) in these highly purified Gram-positive PG preparations would have given higher glucosamine/muramic acid (⩾2) and D-alanine/diamino acid (⩾3) ratios for *S. aureus*, *Listeria monocytogenes* and *Bacillus subtilis*, whereas for *S. pneumoniae* we would have also detected galactosamine.

Samples of each PG purification step were then tested for their ability to induce TLR2-dependent activity in transiently transfected HEK293T cells (Fig 1B,C). A general feature was that whereas TLR2 could detect some crude PG preparations from the initial purification steps, TLR2-dependent sensing was systematically lost after the last step of PG purification. Cell wall preparations from *Helicobacter pylori* and *S. aureus* lost their TLR2-activating ability immediately at the first purification step. Loss of TLR2-dependent activity was observed despite the fact that approximately 1 μg of PG was added to the cells (equal to 10^7–10^9 colony-forming units (CFUs); see Table 1). Note that we consider using higher amounts as physiologically artificial.

Interestingly, *Escherichia coli* and *Pseudomonas aeruginosa* cell walls lost their TLR2 stimulatory activity only after trypsin treatment (step 2a), arguing that Braun lipoprotein or analogous lipoproteins covalently anchored to these two PGs were responsible for TLR2 activation (Glauner, 1988; Quintela *et al*, 1995). Accordingly, *H. pylori* and *Yersinia pseudotuberculosis* PGs,

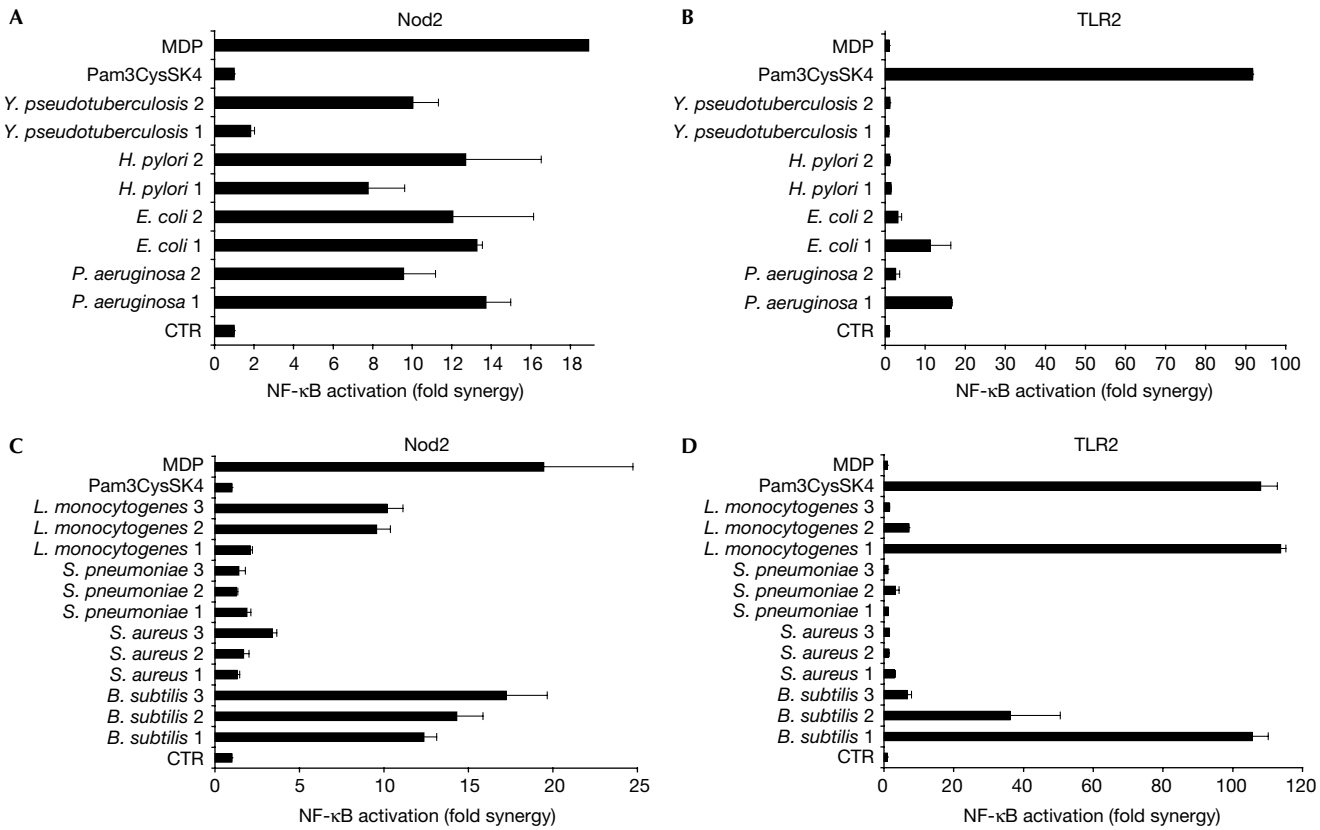

Fig 1 | Gram-negative (**A**,**B**) and Gram-positive PG (**C**,**D**) was purified and samples from each purification step were used to stimulate Nod2- (**A**,**C**) or TLR2- (**B**,**D**) transfected HEK293T cells. The purified PG preparations numbered 1–3 correspond to the different purification steps (supplementary Fig 1 online).

Table 1 | Amino-sugar and amino-acid composition of the different PGs

PGN	GlcNAc	MurNAc	Ala	Glx	Diamino acid[a]	Gly	Ser	Others	CFU equivalent to 1 μg PGN
E. coli	1.03	0.88	2.04	1.17	1	0.17	0.10	0.08	$3.3–5 \times 10^8$
P. aeruginosa	0.98	0.94	1.88	1.25	1	0.17	0.13	0.08	$\sim 5 \times 10^8$
Y. pseudotuberculosis	0.96	0.92	2.08	1.22	1	0.13	0.07	0.05	$\sim 5 \times 10^8$
H. pylori	0.99	0.91	2.00	1.21	1	1.16	0.10	0.05	$1.6–3.2 \times 10^9$
B. subtilis	0.97	0.86	1.71	1.22	1	0.13	0.09	0.07	$\sim 5 \times 10^7$
L. monocytogenes	0.95	0.76	1.67	1.20	1	0.05	0.04	0.02	$3.3–6.25 \times 10^7$
S. aureus	0.69	0.67	2.23	1.55	1	4.13	0.49	0.21	$1.25–6.6 \times 10^6$
S. pneumoniae	0.85	0.62	2.22	1.27	1	0.21	0.36	0.16	$\sim 5 \times 10^7$

[a]The diamino acid corresponds to L-lysine for *S. aureus* and *S. pneumoniae*, and *meso*-diaminopimelic acid for the remaining species. Values correspond to molar ratios taking diamino acid as the reference.

which do not have an equivalent covalently PG-bound lipoprotein (Costa *et al*, 1999; I.G. Boneca, unpublished observations), showed no TLR2-stimulating activity even at the first purification step.

Does TLR2 sense commercial and soluble PG?

The results presented above suggested that the activation of TLR2 by commercial *S. aureus* PG preparations was due to the presence of contaminating molecules. Hence, we compared the ability of 'raw' and partially re-purified commercial *S. aureus* PG to stimulate cells via TLR2. Interestingly, when commercial *S. aureus* PG was submitted to our first purification step (supplementary Fig 1 online), which removes most LTA and noncovalently bound lipoproteins, the TLR2-dependent stimulatory activity was lost (Fig 2A).

Soluble PG (sPG), as prepared by Schwandner *et al* (1999), is released by growing staphylococci at subinhibitory concentrations of penicillin, and sPG purified by vancomycin-affinity chromatography is reported to be a potent TLR2 agonist. Thus, we extended our studies to examine the effect of sPG on TLR2-dependent NF-κB activation. As we used an alternative approach to purify PG, we rendered *S. aureus* PG soluble by cleaving the pentaglycine bridges with lysostaphin, mimicking the effect of penicillin on sPG. However, lysostaphin treatment did not result in a TLR2 recognition of sPG (Fig 2B). To ascertain that the lysostaphin treatment was effective, we verified that sPG was able to activate Nod2 (Fig 2C). Furthermore, high-performance liquid chromatography (HPLC) analysis of the sPG showed a profile consistent with previous reports (Fig 2D; Sieradzki *et al*, 1999).

However, this contradiction can be explained by the fact that the procedure used by Schwandner and colleagues does not remove WTAs, which remain attached to PG. Furthermore, penicillin also induces a massive release of LTA (Tomasz & Waks, 1975; van Langevelde *et al*, 1998). Therefore, the sPG isolated using this protocol is potentially enriched in LTA. In fact, some studies use a tenfold higher concentration of sPG, therefore increasing the amount of 'contaminants'.

TLR2 confers responsiveness to heat-killed bacteria

As some crude PGs/cell walls did not induce TLR2, we wanted to ascertain that heat-killed (HK) bacteria *per se* were able to induce TLR2-dependent activation. To investigate this, we used HK bacterial suspensions standardized to obtain the same PG amount by gross (approximately 0.5–1 μg; see Table 1). Even though the bacterial suspensions presented similar PG amounts, TLR2 expression did not confer responsiveness to HK *S. aureus* and *S. pneumoniae* (Fig 3A).

As *S. aureus* and *S. pneumoniae* are able to induce strong TLR2-dependent NF-κB activation, we repeated these experiments with increasing amounts of HK bacteria. Interestingly, TLR2-dependent NF-κB activation was dose dependent and maximal only when all HK bacteria were present at roughly the same CFU per millilitre (Fig 3B). These results clearly indicate that TLR2 activation is bacterial concentration dependent, rather than PG content dependent, thereby arguing that additional cell wall components mediate TLR2-dependent activation.

TLR1 and TLR6 do not confer TLR2 responsiveness to PG

Recognition of triacyl and diacyl lipopeptides may require the formation of TLR2/1 and TLR2/6 heterodimers, respectively (Takeda & Akira, 2003). We decided to test whether TLR1 or TLR6 enhance PG sensing. The co-transfection of TLR1 and TLR6 with TLR2 did not result in PG-stimulated NF-κB activation (Fig 3C). LTA, however, was sensed efficiently via TLR2 (Fig 3D). Furthermore, TLR1 co-transfection resulted in synergistic effects with highly purified *L. monocytogenes* or *S. aureus* LTAs, commercial *B. subtilis* LTA or synthetic lipopeptide (Fig 3D,E). Commercial *S. aureus* LTA did not induce NF-κB activation corroborating previous results (Morath *et al*, 2001), whereas *S. pneumoniae* LTA activated mildly (Fig 3D). Interestingly, *S. pneumoniae* LTA has been shown to be less pro-inflammatory in comparison with *S. aureus* LTA (Han *et al*, 2003), consistent with the fact that the same amount of HK *S. pneumonia* induced less NF-κB activation via TLR2 (Fig 3B).

PG does not stimulate IL-6 and TNF-α production

Next, we stimulated peritoneal macrophages from C57BL6/J and TLR2-deficient mice with different PG preparations. Whereas cell walls and highly purified LTA (Fig 4A,B) were able to induce tumour necrosis factor-α (TNF-α) in a TLR2-dependent fashion, highly purified PGs were not (Fig 4C). Moreover, highly purified PGs did not induce interleukin-6 (IL-6) production even at 10 μg/

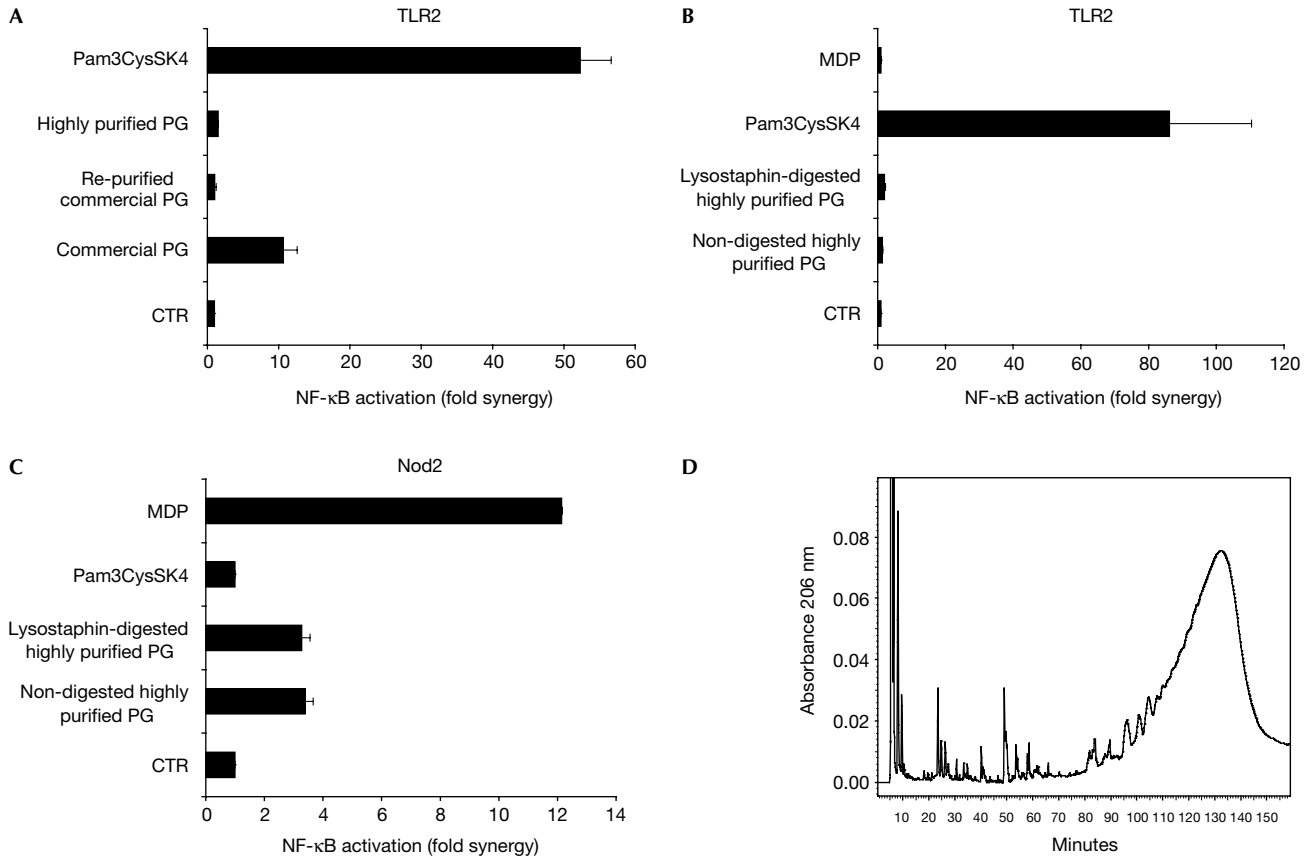

Fig 2 | No TLR2-dependent sensing of commercial and soluble peptidoglycan (PG). TLR2-transfected HEK293T cells were stimulated with 'raw' or partially re-purified commercial and highly purified *S. aureus* PG (**A**). Lysostaphin-digested highly purified *S. aureus* PG was used to stimulate TLR2- (**B**) or Nod2- (**C**) transfected HEK293T cells. (**D**) HPLC profile of the same *S. aureus* digested PG.

ml (Fig 4D), consistent with the results obtained with transfected HEK293T cells. A lack of cell responses to highly purified PG was observed despite Nod1 and Nod2 expression in these cells (Gutierrez *et al*, 2002; Chamaillard *et al*, 2003) consistent with the idea that PG must gain entry into the cells for the activation of Nod proteins.

A principal difficulty concerning the identification of which PAMP is detected by a specific TLR resides in the fact that most of these products need to be purified from bacterial cell walls, and therefore contamination with other cell wall components can often occur leading to erroneous conclusions. Indeed, our results suggest that the previous attribution of TLR2 as the receptor of PG is likely to be incorrect as many of these studies relied on impure PG as the stimulus. Our results strongly suggest that cell wall contaminants present in PG preparations are responsible for TLR2-dependent cell activation. For Gram-negative bacterial cell wall preparations, we have shown that TLR2 stimulatory activity is dependent on the presence of covalently bound lipoproteins. TLR2 stimulatory activity of Gram-positive cell walls is likely to be mediated by contaminating LTA.

Interestingly, although Gram-positive cell walls stimulated TLR2 either in transfected HEK293T cells (*L. monocytogenes* and *B. subtilis*) or macrophages (*S. pneumoniae*, *L. monocytogenes* and *B. subtilis*), after hydrofluoric acid treatment this stimulatory activity was completely lost. Hydrofluoric acid treatment hydrolyses LTA and WTA into their building block subunits (phosphate, D-alanine, choline, sugars, glycerol and/or lipid anchor). A principal argument in favour of LTA instead of WTA as a TLR2 agonist is on the basis of the fact that *S. aureus* and *S. pneumoniae* cell wall preparations, which still have WTA, were not able to induce TLR2 in HEK293T cells. As WTA corresponds grossly to half of the Gram-positive cell wall, the TLR2-stimulating activity present in *S. pneumoniae* cell walls observed with macrophages must be only in trace amounts, excluding WTA as a TLR2 agonist. Accordingly, highly purified LTAs induced a TLR2-dependent NF-κB response. Furthermore, we show for the first time that TLR2 seems to synergize at least with TLR1 to sense LTA. Moreover, chemically synthesized LTA has been reported as a potent inducer of cytokines in monocytes (Morath *et al*, 2002).

Consequently, it is conceivable that the TLR and Nod pathways cooperate to enhance the immunological response. Accordingly, crosstalk between TLR2 and Nod2 has been described recently (Chen *et al*, 2004; Netea *et al*, 2004; Watanabe *et al*, 2004). Cooperation between different sensing pathways is intuitively an advantage for the host, as the response can be more robust, avoiding marked responses to the occasional presence of individual PAMPs. Finally, our observations have the crucial consequence that Nod1 and Nod2 are more than just cytosolic

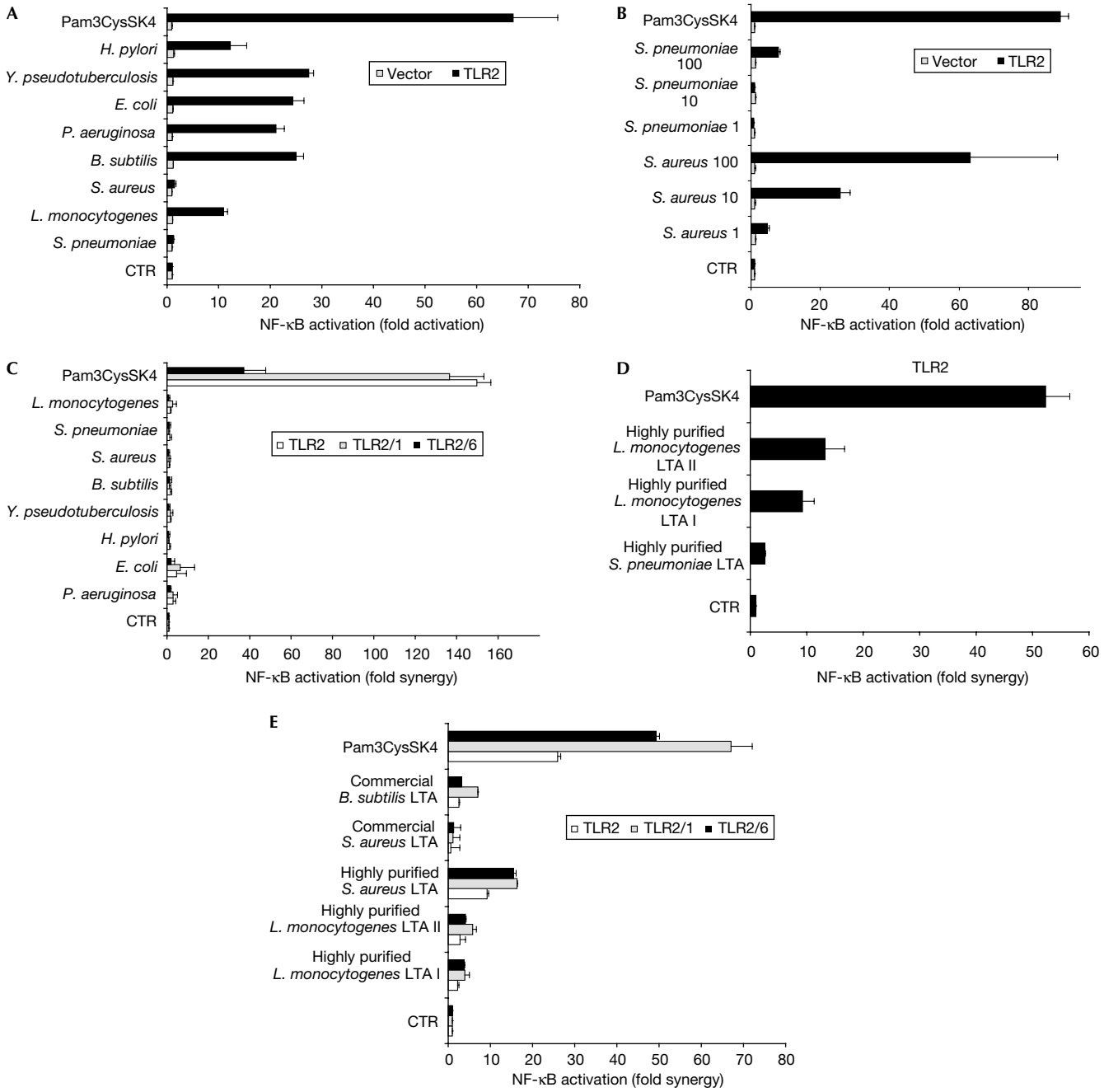

Fig 3 | TLR2-, TLR2/1- and TLR2/6-dependent sensing of heat-killed (HK) bacteria, peptidoglycan (PG) and lipoteichoic acid. The different heat-killed bacterial concentrations were adjusted to obtain equal PG amounts (approximately 1 μg) (**A**). Dose-dependent HEK293T cells stimulation with tenfold increasing amounts of HK *S. aureus* and *S. pneumoniae* (**B**). TLR2-, TLR2/1- or TLR2/6-transfected HEK293T cells stimulation with highly purified peptidoglycans (**C**). *S. pneumoniae* and *L. monocytogenes* LTAs were used to stimulate TLR2 (**D**). TLR2-, TLR2/1- or TLR2/6- transfected HEK293T cells stimulation with *L. monocytogenes*, *S. aureus* and commercial *B. subtilis* LTAs (**E**) *L. monocytogenes* LTAs type I and II differ by the addition of a phosphate group to the glycolipid anchor diglucosyldiacylglycerol.

'second fiddle', showing overlapping functions with TLR2 in PG sensing. In fact, the Nods show unique sensing specificities that are not shared by members of the TLR family (Girardin *et al*, 2003c), resolving the controversial findings that Nods and TLR2 seem to recognize the same ligands.

METHODS

Bacterial strains. Bacterial strains used to prepare PG and HK cells were *S. aureus* COL, *L. monocytogenes* EGD, *B. subtilis* 168, *S. pneumoniae* R800, *H. pylori* 26695, *E. coli* MC1061, *Y. pseudotuberculosis* IP32953 and *P. aeruginosa* O1.

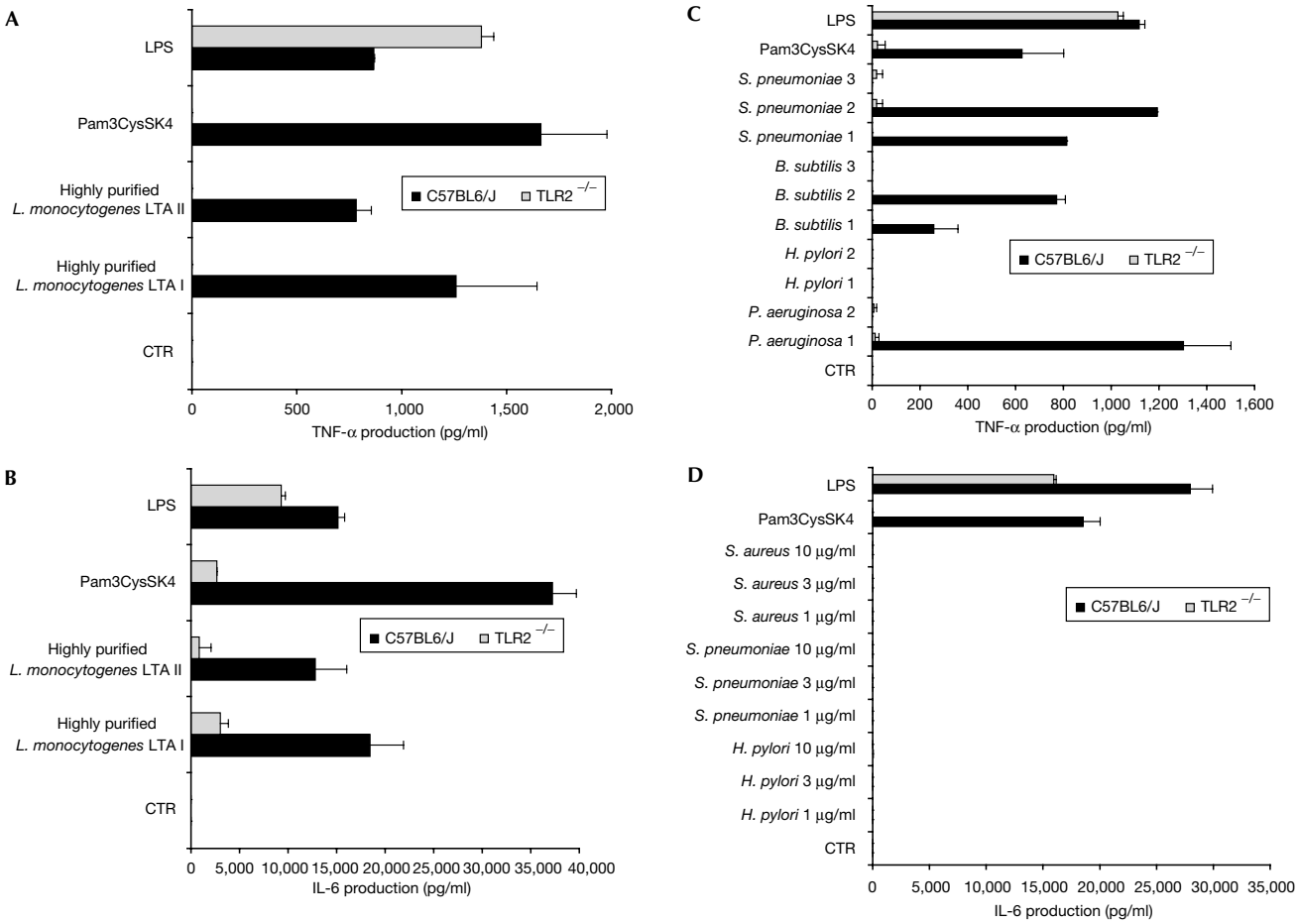

Fig 4 | Macrophage sensing of peptidoglycan (PG) and lipoteichoic acid. Peritoneal macrophages from C57BL6/J and TLR2$^{-/-}$ mice were stimulated with highly purified *L. monocytogenes* lipoteichoic acid (**A,B**), PG preparations from each purification step (**C**) and different concentrations of highly purified *H. pylori*, *S. pneumoniae* and *S. aureus* PGs (**D**). TNF-α and IL-6 productions were determined by enzyme-linked immunosorbent assay. The purified PG preparations numbered 1–3 correspond to the different purification steps (supplementary Fig 1 online).

Reagents. Pure LPS from *E. coli* F515 was obtained as described (Sanchez Carballo *et al*, 1999). Pam_3CysSK_4, MDP, commercial *S. aureus* PG and LTA, and *B. subtilis* LTA were from EMC microcollections (Tubingen, Germany), Calbiochem (San Diego, CA, USA), Fluka (Buchs, Switzerland), Sigma-Aldrich (St Louis, MO, USA) and Invivogen (San Diego, CA, USA), respectively. Highly purified *S. aureus* LTA was kindly donated by Thomas Hartung. *L. monocytogenes* ATCC 19115 serotype 4a LTAs type I and II, differing by the addition of a phosphate group to the glycolipid anchor diglucosyldiacylglycerol, and *S. pneumoniae* R6 LTA were kindly provided by Pascale Cossart. Endotoxin-free fetal calf serum (FCS) was from Hyclone (Logan, UT, USA). All cell culture reagents and antibiotics were from Life Technology (Cergy, France).

PG purification. PGs from Gram-negative and Gram-positive bacteria were purified as described (Girardin *et al*, 2003b). PG samples were lyophilized in a speed-vac to estimate the amount of PG and determine the yield per CFU. PG samples were resuspended in pyrogenic-free ultrapure water (Biochrom AG, Berlin, Germany). Amino-acid and amino-sugar compositions were determined with a Hitachi L8800 analyser (ScienceTec, Les Ulis, France) after hydrolysis of samples in 6 M HCl at 95 °C for 16 h.

HPLC analysis. *S. aureus* PG was digested with recombinant lysostaphin (50 μg/ml; Sigma) in 50 mM Tris–HCl (pH 8) at 37 °C with stirring for 18 h and was analysed by HPLC as described (Sieradzki *et al*, 1999), except that buffer A did not contain methanol.

Expression plasmids. NF-κB reporter Igκ-luciferase and TLR2 expression plasmids were from Alain Israel (Munoz *et al*, 1994) and Marta Muzio (Muzio *et al*, 1998), respectively. Nod2 expression plasmid was from Gilles Thomas (Fondation Jean Dausset/CEPH, Paris, France). TLR1 and TLR6 expression plasmids (pUno hTLR1 and pUno hTLR6) were from Invivogen, and pcDNA3.1 vector was from Invitrogen.

Reporter assays for NF-κB activation. Human embryonic kidney HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. Studies on the synergistic activation of NF-κB by PGs were carried out as described by Inohara *et al* (2002). Briefly, cells were transfected with 75 ng of the reporter plasmid Igκ-luc plus the following vectors: 15 ng Nod2, 300 ng TLR2, TLR1 or TLR6. The pcDNA3.1 vector was used to balance the transfected DNA concentration. PG or LTA preparations were used at 1 μg/ml unless otherwise indicated. Pam_3CysSK_4 (1 μg/ml) and MDP (1 μg/ml) were used as positive controls for TLR2

and Nod2, respectively. In the HK experiments, we added for Gram-negative bacteria 10^8 CFU/ml, for *L. monocytogenes* and *B. subtilis* 10^7 CFU/ml, for *S. pneumoniae* $4–5 \times 10^7$ CFU/ml and for *S. aureus* $4–5 \times 10^6$ CFU/ml, respectively to $\sim 5 \times 10^5$ HEK293T cells per millilitre. This represents a multiplicity of infection ranging from 10 to 200 depending on the bacterial species. The data represent mean ± s.e. of triplicate experiments.

Mice. Female mice (6–10 weeks old) were used for this study. C57BL6/J mice were purchased from Janvier (Le Genest, France). TLR2-deficient mice initially provided by S. Akira (Osaka, Japan) were further backcrossed in C57BL6/J to reach the eighth backcross by Michel Chignard (Institut Pasteur). Mice were submitted to sanitary control tests at the CDTA (Orleans, France) to ensure proper pathogen-free status. All protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and with Public Health Service recommendations.

Cells. Mouse peritoneal macrophages were elicited by injection of 1.5 ml of thioglycolate medium (Bio-Rad, Hercules, CA, USA) in the peritoneal cavity four days before peritoneal lavage with 5 ml of phosphate-buffered saline (PBS) complemented with Heparin Choay (10 U/ml) from Sanofi (Gentilly, France). Cells from five to six mice were pooled and resuspended to 10^6 cells/ml in RPMI/3% FCS in 24-well plates. After 90 min of incubation (37 °C, 5% CO_2), cells were thoroughly washed with PBS, and 500 µl of RPMI/0.2% FCS/penicillin (100 U/ml)/streptomycin (100 µg/ml)/ amphotericin B (250 ng/ml) were added. After 2 h, cells were stimulated in duplicate or triplicate. Unless otherwise indicated in the figure legend, PGs, Pam_3CysSK_4 and MDP were tested at 1 µg/ml and LPS at 100 ng/ml. After 18 h, the supernatants were aliquoted and frozen at −20 °C.

Cytokine dosage. Murine cytokines (TNF-α, IL-6) released into the medium were measured using B-D Pharmingen (San Diego, CA, USA) opt EIA kits. The data represent mean ± s.e. of triplicate experiments.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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