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Cellular Recognition of Trimyristoylated Peptide or Enterobacterial Lipopolysaccharide via Both TLR2 and TLR4*[S]

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Evidence for specific and direct bacterial product recognition through toll-like receptors (TLRs) has been emphasized recently. We analyzed lipopeptide analogues and enterobacterial lipopolysaccharide (eLPS) for their potential to activate cells through TLR2 and TLR4. Whereas bacterial protein palmitoylated at its N-terminal cysteine and N-terminal peptides derived thereof are known to induce TLR2-mediated cell activation, a synthetic acylhexapeptide mimicking a bacterial lipoprotein subpopulation for which N-terminal trimyristoylation is characteristic (Myr_3CSK_4) activated cells not only through TLR2 but also through TLR4. Conversely, highly purified eLPS triggered cell activation through overexpressed TLR2 in the absence of TLR4 expression if CD14 was coexpressed. Accordingly, *TLR2*$^{-/-}$ macrophages prepared upon gene targeting responded to Myr_3CSK_4 challenge, whereas *TLR2*$^{-/-}$/*TLR4*$^{d/d}$ cells were unresponsive. Through interferon-γ (IFNγ) priming, macrophages lacking expression of functional TLR4 and/or MD-2 acquired sensitivity to eLPS, whereas TLR2/TLR4 double deficient cells did not. Not only *TLR2*$^{-/-}$ mice but also *TLR4*$^{-/-}$ mice were resistant to Myr_3CSK_4 challenge-induced fatal shock. D-Galactosamine-sensitized mice expressing defective TLR4 or lacking TLR4 expression acquired susceptibility to eLPS-driven toxemia upon IFNγ priming, whereas double deficient mice did not. Immunization toward ovalbumin using Myr_3CSK_4 as adjuvant was ineffective in *TLR2*$^{-/-}$/*TLR4*$^{-/-}$ mice yet effective in wild-type, *TLR2*$^{-/-}$, or *TLR4*$^{-/-}$ mice as shown by analysis of ovalbumin-specific serum Ig concentration. A compound such as Myr_3CSK_4 whose stimulatory activity is mediated by both TLR2 and TLR4 might constitute a preferable adjuvant. On the other hand, simultaneous blockage of both of the two TLRs might effectively inhibit infection-induced pathology.

Promiscuous specificity of pattern recognition receptors (PRRs)[2] for microbial and viral products named pathogen-associated molecular patterns (PAMPs) carrying species-specific modifications supports high effectiveness of invader recognition by the innate immune system through a small set of PRRs (1). Lipopolysaccharide from pathogenic enterobacterial bacteria (eLPS) has been applied in numerous experimental models of infection in which PRRs such as LPS-binding protein (LBP) and CD14 have central roles (2–4). Lipoprotein is an immunostimulatory PAMP as well (5–7). Challenge with immunostimulatory Gram-negative or Gram-positive bacterial constituents overactivates the immune system and elicits symptoms that in part are characteristic for sepsis in the course of acute infection (8). On the other hand, immune activation through bacterial challenge is deployed therapeutically in vaccinology and oncology (9).

Toll-like receptors (TLRs) include a subgroup of PRRs and transduce activatory signals upon specific PAMP challenge (10). Originally, TLR4 has been demonstrated to induce NF-κB activation in mammalian cells (1, 11), as well as to recognize eLPS (3). TLR2 has been implicated in cellular recognition of a variety of PAMPs such as specific LPS and lipoproteins (12, 13). Tripalmitoylated proteins and dipalmitoylated proteins have been assigned as agonists to TLR2/TLR1 and TLR2/TLR6 dimers, respectively (12, 14, 15). Most known germ line-encoded cellular receptors mediate cellular recognition of endogenous ligands such as cytokines. However, activity of innate TLRs, specific lectins, and cytoplasmic leucine-rich repeat-containing molecules depends upon direct interaction with PAMPs (16, 17).

Like LPS, *Escherichia coli* outer membrane protein (OMP) has been identified as PAMP whose immune stimulatory

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1S–6S.

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[2] The abbreviations used are: PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; eLPS, enterobacterial lipopolysaccharide; TLR, toll-like receptor; Pam_3CSK_4, tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine; Myr_3CSK_4, trimyristoyl-cysteinyl-seryl-(lysyl)3-lysine; PGN, peptidoglycan; LTA, lipoteichoic acid; D-GalN, D-galactosamine; PMB, polymyxin B; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; IFNγ, interferon-γ; PBMC, peripheral blood mononuclear cells; ODN, oligodeoxynucleotide; LBP, LPS-binding protein; OVA, ovalbumin; OMP, outer membrane protein; TNF, tumor necrosis factor; FL, Flt3 ligand; DC, dendritic cells; h, human; m, murine; IL, interleukin.

capacity depends on specific acylations. *E. coli* OMP is triacylated at an N-terminal cysteine. Although one fatty acid is amide-bound, thio-esterification of cysteine with glycerol provides two hydroxyls to esterify with the two additional fatty acids (5). This finding was validated by chemical synthesis and subsequent biological analysis of a palmitoylated analogue of the dominant fraction of an OMP preparation (7). However, different protein acylations have been identified originally. Total fatty acids contained in the lipoprotein fraction isolated from total bacterial protein were analyzed by gas chromatography and mass spectrometry without or upon differential cleavage of ester-bound fatty acids with sodium methylate. 53% of fatty acids were found to carry 16 carbon atoms identifying them as palmitic acid. Also, longer fatty acids carrying up to 19 carbon atoms were found in total fatty acids of the lipoprotein fraction. Notably, 3.1% of all fatty acids, 1.5% of the ester-bound, and 2.4% of the amide-bound fatty acids were saturated and carried 14 carbon atoms (5), which is characteristic of myristic acid. Specificity of TLRs for lipoproteins other than palmitoylated polypeptides has not yet been analyzed comparatively to our knowledge.

We analyzed responsiveness of *TLR2*$^{-/-}$, *TLR4*$^{d/d}$ (C3H/HeJ, expressing defective TLR4), or *TLR4*$^{-/-}$ as well as *TLR2*$^{-/-}$/*TLR4*$^{d/d}$ and *TLR2*$^{-/-}$/*TLR4*$^{-/-}$ mice or immune cells derived thereof to acylated TLR ligands lipopeptide and eLPS. We now report on a synthetic lipopeptide carrying a trimyristoylated cysteine at its N terminus that activated TLR4 aside from TLR2. We also report activation of TLR2 by highly purified eLPS if CD14 was coexpressed or upon IFNγ priming of mice lacking TLR4 expression.

EXPERIMENTAL PROCEDURES

Reagents—LPS from *E. coli* strain O111:B4 (smooth, carrying a long polysaccharide chain) purified by phenol extraction and gel filtration was from Sigma. *E. coli* O111:B4 LPS (used only for experiments illustrated in supplemental Fig. 1S) and *Salmonella enterica* serovar Minnesota strain R595 (Re mutant) LPS (rough, composed of lipid A and two additional sugars only, both from List, Campbell, CA; highly purified according to Ref. 18), as well as *S. enterica* serovar Friedenau (smooth) LPS were used also. The latter LPS was extracted from dried bacterial cells of *S. enterica* Friedenau by the hot phenol water procedure, purified by repeated ultracentrifugation, calcium chloride precipitation, and reextraction with phenol/chloroform/petrol ether, as well as converted by electrodialysis into the uniform sodium salt (19). Contamination with nucleic acid (detection limit 0.3%) was not detectable by gas chromatography of alditol acetates. 2.83 μg of protein detected in 8 mg of *S. enterica* Friedenau LPS by Bradford microassay (Biomol, Hamburg, Germany) corresponds to 0.035% of LPS by weight in accordance with UV spectrometric analysis of the LPS solubilized in 0.1 M NaOH (1 mg/ml), which showed a single maximum at a wavelength of 220 nm. Synthetic 506 type *E. coli* lipid A was as described (20). D-Galactosamine (D-GalN), polymyxin B (PMB), and ovalbumin (OVA) were purchased from Sigma. Highly pure lipoteichoic acid (LTA) from *Bacillus subtilis* and *Staphylococcus aureus* was prepared by propanol extraction (21). Soluble peptidoglycan (PGN) was purified from *S. aureus* by vancomycin affinity chromatography (22). Tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (Pam_3CSK_4) and trimyristoyl-cysteinyl-seryl-(lysyl)3-lysine (Myr_3CSK_4) synthesized under exclusion of LPS contamination applying validated synthesis procedures, as well as analyzed by electrospray ionization-mass spectrometry, were from ECHAZ Microcollections (Tübingen, Germany) (23). Recombinant IFNγ was from PeproTech (London, UK).

Cell Culture and Blocking Antibody Application—For *ex vivo* experiments, thioglycolate-elicited peritoneal macrophages as well as bone marrow-derived macrophages and dendritic cells (DC) were applied (24). Macrophages were primed with 50 ng/ml IFNγ overnight. DC were generated with Flt3 ligand (FL) supplemented bone marrow cultures and challenged with 10 μg/ml TLR2 and TLR4 agonists (25). Cells were challenged with thiolated DNA oligodeoxynucleotides for positive control (1668, TCCATGACGTTCCTGATGCT, or 2216, GGGGGACGATCGTCGGGGGG) at 2 μM (TIB MOLBIOL, Berlin, Germany) (25). Antibody T2.5 antagonizing mTLR2 and hTLR2 or mTLR2-specific isotype control T2.13 were applied to cell cultures at a concentration of 10 μg/ml 30 min prior to challenge with TLR agonists (26). Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and cultured in RPMI 1640 medium containing 5% calf serum (PAA GmbH, Pasching, Austria) under regular conditions.

NF-κB-driven Reporter Gene and Proliferation Assay—NF-κB-dependent luciferase assay was performed as described (26). For analysis of pattern recognition serum dependence, adherent cells were washed upon DNA plasmid transfection, and serum-free medium or medium containing 10% of fetal calf serum was added. Murine splenocytes were isolated and erythrocytes removed. 2×10^5 cells were plated per well of a 96-well plate (triplicate plating per sample) and pulsed by the addition of [^{3}H]thymidine carrying an activity of 1.5 μCi (Hartmann Analytik, Braunschweig, Germany) for 6 h upon challenge with microbial constituents for 48 h. Cells were harvested, and scintillation of immobilized DNA was analyzed using a filter counter (Canberra Packard, Dreteich, Germany) (24).

PMB Application—eLPS or lipopeptides were incubated with the antibiotic PMB solubilized in PBS for 30 min at room temperature within 0.1 volume of the final cell culture volume. PMB concentration was 10 μg/ml after addition of the solution to cell culture. Control samples were treated in the absence of PMB.

Analysis of Supernatants and Sera by ELISA—Murine macrophages or hPBMC were cultured on 96-well plates (2×10^5 cells per well) and challenged. For analysis of serum cytokine concentrations, mice were anesthetized upon systemic challenge by intraperitoneal injection of bacterial products or synthetic analogues. Blood was collected from the retrobulbar plexus (27). Culture supernatants and mouse sera were analyzed by TNFα and IL-6-specific ELISA (R&D Systems, Minneapolis, MN).

Preparation of Inactivated Bacteria Suspensions—B. subtilis (DSMZ 1087) and *E. coli* (DH5α, Invitrogen) were cultured at 37 °C in regular brain-heart medium overnight, washed once in PBS, and adjusted to a titer of 1×10^{10} colony-forming units/ml

by resuspension in PBS. Suspensions were either used for infection or bacteria were heat-inactivated at 64 °C for 50 min. Alternatively, *B. subtilis* suspension was incubated at 100 °C for 20 min and sonicated for 5 min subsequently. Soluble material (supernatant) was sampled upon 20 min of tabletop centrifugation for subsequent application. The sediment was resuspended in PBS. 10 mM $MgCl_2$, 5 mM $CaCl_2$, 20 μg/ml DNase I, 50 μg/ml RNase A (Sigma), and 1× proteinase inhibitor mixture (Roche Applied Science) were added. The suspension was shaken at 37 °C overnight, incubated at 100 °C for 20 min, and then incubated overnight upon addition of 7.5 mg/ml proteinase K (Sigma) at 37 °C. Finally, the suspension was incubated at 100 °C again for 10 min. Nonsoluble fraction (pellet) was separated from the supernatant by tabletop centrifugation and resuspended in PBS, and samples were frozen prior to subsequent application.

$TLR2^{-/-}$, $TLR2^{-/-}/TLR4^{d/d}$, $TLR2^{-/-}/TLR4^{-/-}$, $TLR4^{-/-}/MD\text{-}2^{-/-}$, $MyD88^{-/-}$, and $TNFRI/p55^{-/-}$ Mice and Systemic Challenge—A portion of the open reading frame within exon 3 of *TLR2* spanning from base residue C1362 to G1778 encoding the C-terminal ectodomain and the N-terminal transmembrane domain corresponding to amino acid residues Cys-454 to Gly-593 (of immature protein) was replaced by a neomycin cassette in Sv129 embryonic stem cells to generate $TLR2^{-/-}$ mice. Two positive embryonic stem cell clones aggregated independently in C57BL/6 mice. Sequences of primers applied for genotyping were as follows: P1, CTTCCTGAATTTGTCCAGTACAGG; P2, TCGACCTCGATCAACAGGAGAAGGG; and P3, GGGCCAGCTCATTCCTCCCACTCAT. PCR product sizes were 499 bp (P1 and P2, wild-type allele) and 334 bp (P1 and P3, $TLR2^{-/-}$ allele). $TLR2^{-/-}$ mice back-crossed toward the C57BL/6 background (9-fold), those 9-fold back-crossed toward the C3H/HeJ background carrying a point mutation in *TLR4* (designated *d*/*d*) and $TLR2^{-/-}/TLR4^{d/d}$, as well as $TLR4^{-/-}$ and $TLR2^{-/-}/TLR4^{-/-}$ mice 6-fold backcrossed toward the C57BL/6 background were used (28). $MD\text{-}2^{-/-}$ mice were crossed with the above named $TLR4^{-/-}$ mice to yield $TLR4^{-/-}/MD\text{-}2^{-/-}$ mice (28, 29). $MyD88^{-/-}$ and $TNFRI/p55^{-/-}$ mice were as described (30, 31).

100 μg of Pam_3CSK_4 was administered intraperitoneally for analysis of TLR2 and time-dependent IL-6 and TNFα release to the serum by ELISA (R&D Systems, Minneapolis, MN). For shock experiments, TLR agonist preparations were applied alone or upon sensitization using D-GalN alone or in combination with IFNγ. For IFNγ priming, 50 μg/kg IFNγ was injected intravenously 45 min prior to application of eLPS, synthetic lipid A, or LTA intraperitoneally at the amounts indicated. TLR agonists listed above, suspensions of heat-inactivated bacteria, or 200 μg of Myr_3CSK_4 were injected intraperitoneally together with 800 mg/kg D-GalN if indicated (27). Lethality was monitored within 72 h in respect to high dose application (no sensitization) and within 16 h upon low dose application (sensitization by D-GalN treatment alone or additional IFNγ priming). Mice were under observation twice daily for 7 days upon challenge.

Flow Cytometry of Macrophages and DC—Upon 17 h of stimulation with TLR agonists, Flt3 ligand-induced bone marrow cells (FL-DC) were labeled with a combination of anti-CD45RA-PE, anti-CD11c-APC, and anti-CD40-FITC (all from BD Biosciences) (32). Cells were >90% CD11c-positive, and 30–40% of cells were CD45RA high thus displaying a plasmacytoid phenotype. FL-DC were analyzed on a FACSCalibur flow cytometer (BD Bioscience) for CD40 expression.

Electromobility Shift Assay—Peritoneal macrophages were challenged by application of bacterial products or synthetic analogues in RPMI 1640 serum containing 2% fetal calf serum (PAA GmbH, Pasching, Austria) for 2 h, and nuclear proteins were analyzed for NF-κB recognition element-specific DNA binding (33).

Immunization—Mice were challenged by injection of 200 μg of Myr_3CSK_4 and 50 μg of OVA solubilized in 400 μl of PBS intraperitoneally. The first injection was followed by a second injection after 3 weeks and the third injection upon an additional 4 weeks and 3 days when serum was collected. Amounts of OVA-specific Ig contained in serum samples were monitored by ELISA using a murine Ig-specific antibody (Dianova, Hamburg, Germany).

Immunoblot Analysis—Total lysates of 5×10^5 primary macrophages per lane were analyzed by using a purified polyclonal rabbit antiserum raised against a 28-mer peptide representing a subdomain of mTLR2 ectodomain (34).

RESULTS

We analyzed lipopeptide analogues differing in the numbers of intramolecular palmitoylations or identities of triacyl moieties (33, 35) for their potential to activate TLR2 and TLR4. Although NF-κB-driven reporter gene activation by five of six different lipopeptides was TLR2-dependent (data not shown), Myr_3CSK_4 induced NF-κB-dependent reporter gene activation not only through TLR2 but also via murine (m)TLR4/MD-2 or human (h)TLR4/MD-2 (Fig. 1*A*). Overexpression of hTLR4 or mTLR4 did not confer responsiveness to Myr_3CSK_4 in the absence of MD-2. Notably, a mixed species TLR4-MD-2 complex did not transduce a detectable signal if challenged with Myr_3CSK_4, whereas eLPS used as positive control was recognized readily through the complex (Fig. 1*A*).

S. *enterica* Minnesota R595 LPS activated otherwise unresponsive HEK293 cells if applied at a high concentration of 10 μg/ml upon transfection of TLR2 and CD14 expression plasmids, whereas LPS of *E. coli* O111:B4 was detectable already at a concentration of 1 ng/ml (Fig. 1*B* and supplemental Fig. 1S). Overexpressed TLR4/MD-2 heteromer, however, mediated detection of these two eLPS species and *S. enterica* Friedenau eLPS at concentrations as low as 100 pg/ml or 1 ng/ml (Fig. 1*B*, supplemental Fig. 1S, and data not shown). Lipopeptide analogue Pam_3CSK_4 was detectable at concentration of 10 ng/ml and activated cells in a TLR2-specific manner (Fig. 1*B* and supplemental Fig. 2S). Cellular recognition of synthetic lipid A, in contrast, was mediated by TLR4 exclusively (Fig. 1*B*).

To bypass a potential caveat of protein overexpression, we used $TLR2^{-/-}$ mice generated by gene targeting (Fig. 2*A*). Northern blot, genotype, flow cytometric, and immunoblot analyses indicated *TLR2*-specific mutation of the murine wild-type genome (data not shown). Although responding normally to eLPS challenge, $TLR2^{-/-}$ splenocytes did not proliferate upon challenge with PGN or LTA (Fig. 2*B*). Systemic challenge

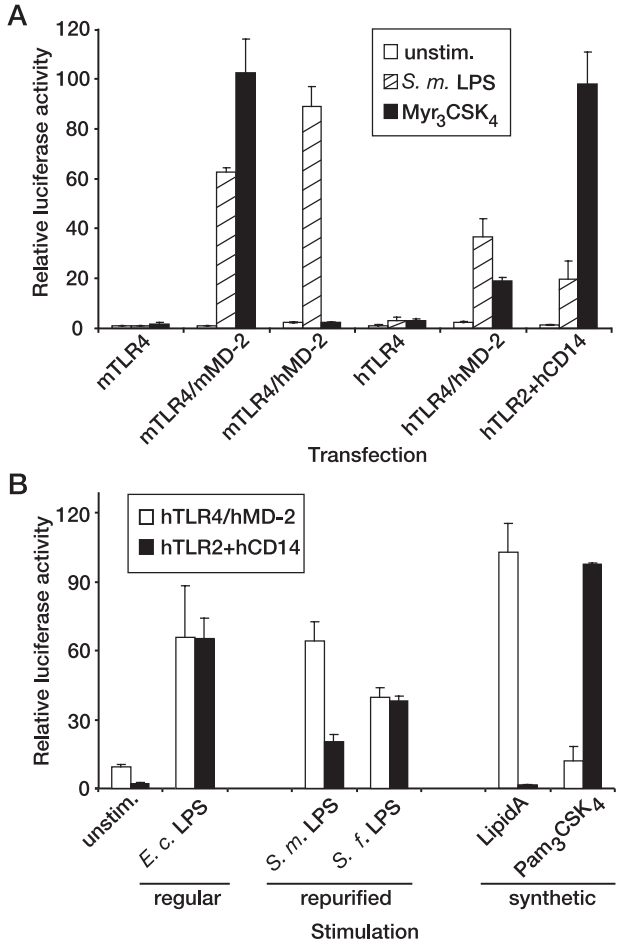

FIGURE 1. **NF-κB-dependent reporter gene activation through ectopically expressed TLR2 or TLR4 upon cellular challenge.** *A* and *B,* HEK293 cells were transfected with reporter plasmids, as well as plasmids mediating expression of receptors indicated. Cells were challenged with 10 μg/ml of the compounds indicated (*unstim.*, untreated). After 16 h, cells were lysed for analysis of intracellular luciferase activity. *E. c.*, *E. coli* O111:B4; *S. m.*, *S. enterica* serovar Minnesota strain R595 (Re mutant); *S. f.*, *S. enterica* serovar Friedenau; LPS preparations accorded to regular protocol or were repurified as indicated; Pam_3CSK_4 and Myr_3CSK_4, synthetic as indicated.

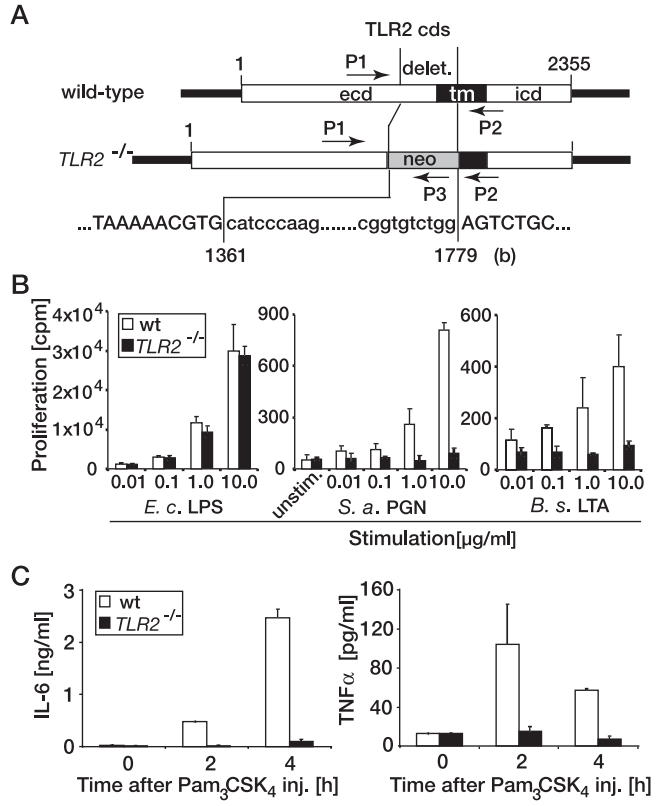

FIGURE 2. **TLR2 targeting.** *A*, schematic comparison of wild-type and mutant ($TLR2^{-/-}$) allele; *ecd*, extracellular domain; *tm*, trans-membrane domain; *delet.*, deletion; *icd*, intracellular domain; *neo,* neomycin resistance cassette; *cds*, coding sequence. DNA sequence transition from coding region (*capital letters*) to inserted neo-cassette (*lowercase letters*), as well as terminal positions of coding sequences are indicated by base (*b*) numbers; *P1*, *P2*, and *P3*, positions of genotyping primer sequences. *B*, comparative analysis of splenocyte proliferation upon challenge with agonists indicated. *wt*, wild-type; *unstim.*, untreated; *E. c.*, *E. coli*; *S. a.*, *S. aureus*; *B. s.*, *B. subtilis*; *cpm*, counts/min, tritium radioactivity correlating with cell proliferation. *C*, serum IL-6 and TNFα levels were determined upon peritoneal injection (*inj.*) of 100 μg of Pam_3CSK_4 ($n = 3$ for each genotype).

with the bacterial lipopeptide analogue Pam_3CSK_4 failed to induce IL-6 and TNFα in $TLR2^{-/-}$ mice, whereas substantial amounts of serum IL-6 and TNFα in wild-type mice were noted (Fig. 2*C*).

We used FL-DC of the four genotypes wild-type, $TLR2^{-/-}$, $TLR4^{d/d}$, and $TLR2^{-/-}/TLR4^{d/d}$ to compare specificity of both of the TLRs for different eLPS preparations. Analysis of IL-6 release as well as regulation of cell surface CD40 and CD62L expression as parameters of cell activation confirmed TLR2 dependence and TLR4 independence of Pam_3CSK_4 recognition (Fig. 3 and data not shown). Notably, a rough eLPS preparation failed to trigger TLR4-deficient FL-DC, whereas a smooth eLPS activated FL-DC in a TLR4- and TLR2-dependent fashion (Fig. 3).

Next, we analyzed primary macrophages either untreated or exposed to IFNγ prior to TLR-specific challenge. Release of TNFα, IL-6, and NO_2^- as well as activation of NF-κB upon challenge with microbial products or synthetic analogues was dose-dependent (Fig. 4, *A–C*, and data not shown). We observed that robust cell activation upon CpG DNA challenge (positive con-

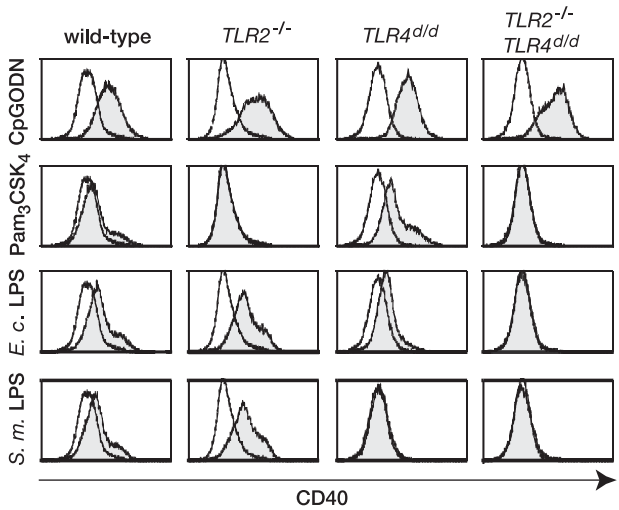

FIGURE 3. **Genotype-dependent FL-DC activation upon TLR-specific challenge.** Bone marrow-derived FL-DC of the four genotypes indicated were challenged for 17 h as indicated. Surface expression of CD40 was analyzed by flow cytometry. *E. c.*, *E. coli* strain O111:B4; *S. m.*, *S. enterica* serovar Minnesota strain R595 (Re mutant); *CpGODN*, stimulatory oligodeoxynucleotide 2216; *unfilled area*, unstimulated; *gray area*, stimulated.

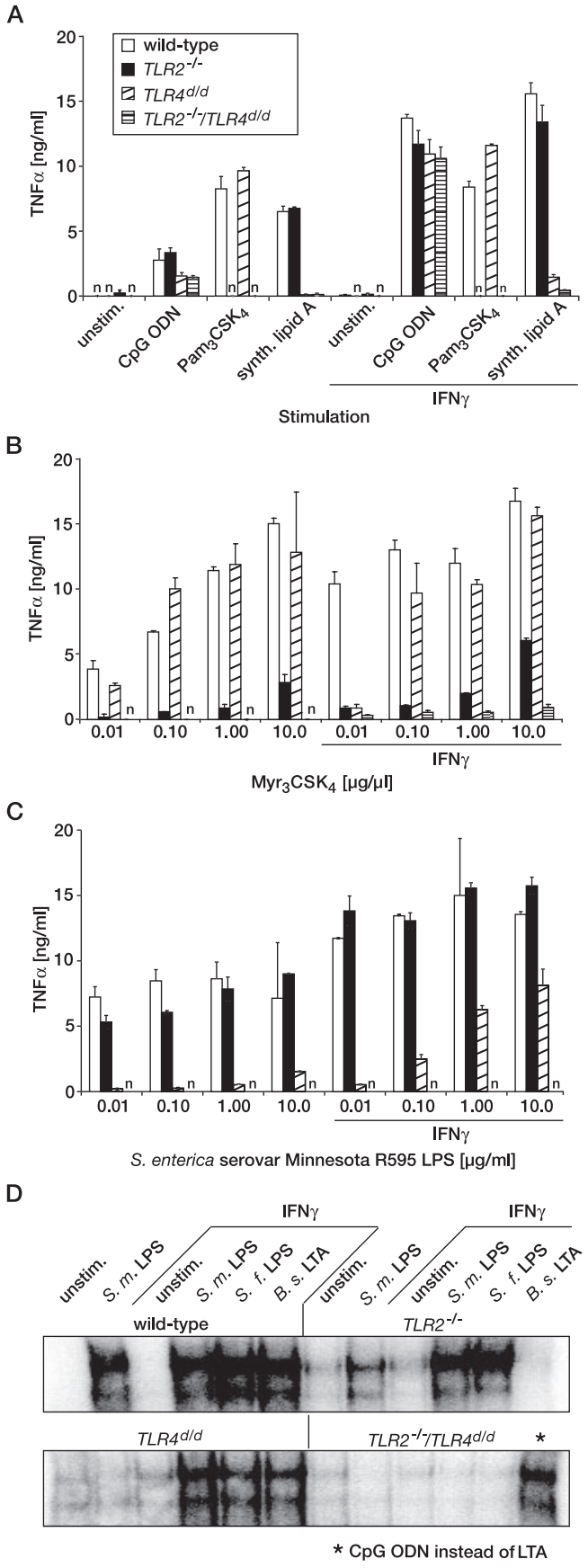

FIGURE 4. **TNFα release and NF-κB activation of primary macrophages upon IFNγ priming and TLR-specific challenge.** *A–C*, legend in *A* applies to *B* and *C* also; challenge of bone marrow-derived macrophages without or upon IFNγ priming with microbial products or analogues as indicated at a concentration of 10 μg/ml except for oligodeoxynucleotide 1668 (*CpG ODN*, 2 μM) or as indicated (*n*, not detectable; *synth.*, synthetic). *D*, peritoneal macrophages of the four genotypes indicated were challenged for 2 h as indicated. Nuclear extracts were analyzed by NF-κB-specific *EMSA*, electromobility shift assay; *unstim.*, untreated; *B. s.*, *B. subtilis*; *S. m.*, *S. enterica* serovar Minnesota strain R595 (Re mutant); *S. f.*, *Salmonella enterica* serovar Friedenau. LPS as well as LTA from *B. subtilis* were applied at a concentration of 10 μg/ml; *CpG ODN*, stimulatory oligodeoxynucleotide 1668, applied at 2 μM.

trol) depended on IFNγ priming in cells of the four genotypes tested (Fig. 4*A*). This was observed in the case of Pam_3CSK_4-induced cell activation through TLR2 (Fig. 4*A*) and for TLR4- and TLR2-dependent cellular activation driven by Myr_3CSK_4 (Fig. 4*B*) to a small degree only. Notably, high dose Myr_3CSK_4 challenge induced activation of macrophages lacking TLR2 expression but not of those lacking expression of both TLR2 and TLR4 (Fig. 4*B*). Furthermore, IFNγ priming rendered *TLR4*$^{d/d}$ or *TLR4*$^{-/-}$ macrophages sensitive to challenge with LPS of *S. enterica* Minnesota R595 and *S. enterica* Friedenau to both of which unprimed TLR4-deficient macrophages did not respond. The unresponsiveness of macrophages lacking both TLR2 and TLR4 even when IFNγ primed supported implication of TLR2 as secondary eLPS signal transducer (Fig. 4, *C* and *D* and data not shown). Synthetic lipid A-induced cell activation, however, was TLR4-specific (Fig. 4*A*).

Myr_3CSK_4-induced cell activation was inhibited by the antagonistic monoclonal antibody T2.5 to an equally high degree as compared with Pam_3CSK_4-induced activity if TLR2 was expressed in the absence of TLR4/MD-2 (Fig. 5*A*). Dose dependence of inhibition indicated partial TLR2 dependence of Myr_3CSK_4-induced cell activity also in the presence of TLR4/MD-2 (Fig. 5*B*). However, cell activation upon Myr_3CSK_4 challenge was inhibited to a substantially smaller extent by preincubation with T2.5 in the presence of endogenous hTLR4/MD-2 or mTLR4/MD-2 expression in PBMC or macrophages, respectively. Accordingly, levels of released TNFα were similar to those observed upon application of T2.13 used as isotype control upon application of Myr_3CSK_4 at high dose (Fig. 5*B*). TLR2 blockage had analogous effects on IL-6 release (data not shown).

The effectiveness of agonist application in shock models for analysis of TLR2 function *in vivo* relied on concomitant host sensitization. Accordingly, fatal shock induction upon application of 1 mg of Pam_3CSK_4 depended on simultaneous sensitization with D-GalN, whereas 100 μg of Pam_3CSK_4 induced lethality only upon pre-sensitization with IFNγ in addition to D-GalN treatment (Table 1). Notably, 150 μg of *Salmonella* LPS preparations elicited lethal toxemia in TLR4-deficient mice, whereas TLR2/4 double deficient mice were resistant. Up to 500 μg of LTA did not induce shock if administered intraperitoneally to D-GalN-sensitized wild-type mice (data not shown). However, IFNγ priming and sensitization using D-GalN rendered wild-type and *TLR4*$^{d/d}$ mice susceptible to lethal shock upon application of 150 μg of LTA from *B. subtilis* or *S. aureus*. In contrast, *TLR2*$^{-/-}$ and *TLR2*$^{-/-}$/*TLR4*$^{d/d}$ mice were resistant to such IFNγ/LTA/D-GalN challenge (Table 1).

Next, we sought to analyze systemic effects of Myr_3CSK_4 challenge. First, application of Myr_3CSK_4 according to a low

dose protocol (D-GalN sensitization) to wild-type mice induced lethal shock. In contrast to wild-type mice, mice of the three other genotypes ($TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR2^{-/-}/TLR4^{-/-}$) were resistant (Fig. 6*A*). Second, immunization against OVA using Myr_3CSK_4 as potential adjuvant was successful in wild-type, $TLR4^{-/-}$, and $TLR2^{-/-}$ mice, although OVA-specific Ig levels in sera of the latter mice were decreased by 50% approximately. Notably, OVA-specific Ig concentration in sera of $TLR2^{-/-}/TLR4^{-/-}$ mice resembled the level in naive wild-type mice (Fig. 6*B*).

To analyze whole bacteria recognition by host organisms in respect to potential TLR2 and TLR4 dependence, we monitored the viability of mice challenged with heat-inactivated bacteria. Although susceptibilities of wild-type and $TLR2^{-/-}$ mice to *S. aureus* or *Listeria monocytogenes* challenge did not differ, $TLR2^{-/-}$ but not wild-type and $TLR4^{d/d}$ mice were resistant to *B. subtilis* induced shock-like syndrome (Table 2 and data not shown). Notably, $TNFRI/p55^{-/-}$ mice were resistant to a low dose *B. subtilis* challenge. The soluble fraction (supernatant) of a lysate from 5×10^9 colony-forming units of *B. subtilis* did not induce detectable symptoms of toxemia, whereas sediment carried full stimulatory activity despite specific enzymatic treatment. In contrast to preparations of heat-inactivated Gram-positive bacteria other than *B. subtilis*, which induced toxemia irrespective of the absence of expression of both TLR2 and TLR4, heat-inactivated *E. coli* induced fatal toxemia in wild-type, $TLR2^{-/-}$, $TLR4^{d/d}$, and $TLR4^{-/-}$ mice but not in $TLR2^{-/-}/TLR4^{-/-}$ mice (Table 2 and data not shown).

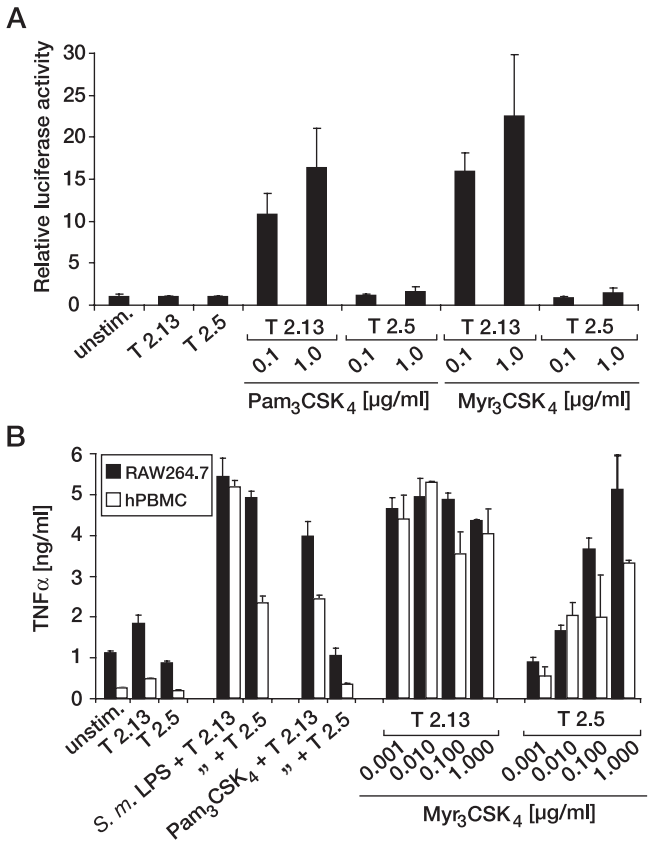

FIGURE 5. **Antagonistic TLR2-specific antibody-dependent inhibition of lipopeptide-induced cell activation.** Cells were left untreated (*unstim.*), pretreated with anti-TLR2 monoclonal antibody (*T2.5*) or isotype control mTLR2-specific antibody (*T2.13*) at a concentration of 10 μg/ml for 30 min. *A*, HEK293 cells transfected with NF-κB reporter and control reporter plasmids, as well as plasmids mediating expression of human TLR2 and CD14, were challenged with different lipopeptides as indicated. Lysates were lysed 18 h thereafter for analysis of luciferase activity. *B*, murine RAW264.7 macrophages or human PBMC were challenged with compounds at a concentration of 1 μg/ml or as indicated. Supernatants were recovered after 24 h for subjection to ELISA. *S. m.*, *S. enterica* serovar Minnesota.

DISCUSSION

According to a commonly held view, bacterial lipopeptides are TLR2 ligands, whereas eLPS is a TLR4 ligand (3, 12). Here we report data that extend this view. Challenge with a trimyristoylated analogue of bacterial lipopeptide induced activation of murine and human cells through TLR4/MD-2, whereas eLPS activation was not only TLR4-dependent but was mediated by TLR2 also. Therefore, our results of analyses *in vitro*, *ex vivo*, and *in vivo* indicate specificity of both of the two TLRs for both of the two agonists.

We found that the synthetic lipopeptide analogue Myr_3CSK_4 mimicking a subpopulation of acylated *E. coli* OMP (5) activated cells not only through TLR2 but also through TLR4/MD-2 (Fig. 1*A* and Fig. 4*B*). Conversely, eLPS did activate HEK293 cells expressing TLR2 together with CD14 (Fig. 1*B*). Notably, a mixed species complex of mTLR4 and hMD-2 mediated cellular eLPS recognition but failed to confer responsiveness to Myr_3CSK_4 (Fig. 1*A*). Furthermore, PMB did not inhibit Myr_3CSK_4 activity (supplemental Fig. 2S). Both latter findings like serum independence of lipopeptide recognition (supplemental Fig. 3S) indicated absence of LPS from Myr_3CSK_4 preparations.

To compare functions of endogenous TLR2 and TLR4, a mouse strain lacking TLR2 expression was generated. Proliferation of $TLR2^{-/-}$ splenocytes upon eLPS challenge but not upon PGN or LTA challenge as well as the lack of systemic cytokine release in response to systemic Pam_3CSK_4 challenge were largely accorded with a report on the properties of another $TLR2^{-/-}$ mouse strain (36). These findings and resistance of $TLR2^{-/-}$ but not of $TLR4^{d/d}$ mice to LTA-induced shock indi-

TABLE 1

Survival of mice upon challenge with TLR agonists upon sensitization (D-GalN) and additional IFNγ priming

For survivors/total mouse number, all deaths occurred within 72 h of treatment. Two matched control mice injected with IFNγ and D-GalN alone survived.

IFNγ, i.v., 45 min	D-GalN i.p.	Challenge i.p.	Amount/mouse	Wild type	$TLR2^{-/-}$	$TLR4^{d/d}$	$TLR2^{-/-}/TLR4^{d/d}$
−	+	Pam_3CSK_4	1 mg	0/3	3/3		
+	+	Pam_3CSK_4	100 μg	0/3	3/3	0/3	3/3
+	+	*S. f.*[a]	150 μg	0/3	0/3	0/3	3/3
+	+	*S. m.*[a]	150 μg	0/3	0/3	0/3	3/3
+	+	*B. s.* LTA[a]	150 μg	0/3	3/3	0/3	3/3
+	+	*S. a.* LTA[a]	150 μg	0/3	3/3	0/3	3/3

[a] LPS of *S. enterica* serovar Friedenau (*S. f.*) or *S. enterica* serovar Minnesota strain R595 (*S. m.*). *B. s.* indicates *B. subtilis*; *S. a.* indicates *S. aureus*; Pam_3CSK_4 indicates palmitoylated lipopeptide analogue; i.v. indicates intravenous injection; i.p. indicates intraperitoneal injection; − indicates not applied; + indicates applied.

cated the absence of contaminating LPS and of ligands of other TLRs from both of these two TLR2 ligand preparations (Fig. 2*B* and Table 1). This conclusion was supported by the unresponsiveness of cells overexpressing TLR4/MD-2 to both of the two Gram-positive components, as well as serum independence of its cellular perception (data not shown). An *S. aureus* PGN preparation of the quality used herein has been analyzed upon muramidase digest and did not contain detectable lipoprotein (22). Equality of the biological activities of a synthetic *S. aureus* preparation (37) and the purified preparations from *S. aureus* and *B. subtilis* indicated the purity of the latter LTA preparations as well (21) (data not shown).

Here we asked whether IFNγ influences the responsiveness of primary innate immune cells to specific TLR agonists (38). Notably, IFNγ priming did not alter lipopeptide responsiveness to a significant degree *in vitro*, irrespective of whether Pam_3CSK_4 or Myr_3CSK_4 was applied (Fig. 4, *A* and *B*). On the one hand, effectiveness of TLR2 blockage in respect to both Pam_3CSK_4 and Myr_3CSK_4 challenge in the absence of TLR4 expression suggested that TLR2 provides an epitope to which both of the two lipopeptides might bind (Fig. 5*A*). On the other hand, several lines of evidence implicated TLR4 as additional receptor for Myr_3CSK_4. First, $TLR2^{-/-}$ macrophages but not double deficient macrophages responded to Myr_3CSK_4 challenge (Fig. 4). Second, partial responsiveness of primary human PBMC and murine macrophages to Myr_3CSK_4 challenge upon TLR2 blockage was observed (Fig. 5*B*). Third, we noted differential toxicity as indicated by the necessity for IFNγ priming to induce lethal toxemia upon challenge with Pam_3CSK_4 at a dose at which Myr_3CSK_4 challenge induced lethal shock in the absence of IFNγ priming (100 μg range; Table 1 and Fig. 6*A*). Furthermore, the latter finding might indicate rise of TLR2-dependent but not TLR4-dependent toxicity upon IFNγ priming. Resistance of $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR2^{-/-}/TLR4^{-/-}$ mice to otherwise lethal Myr_3CSK_4 challenge might indicate proportionality of toxicity grade and the number of TLRs employed by a specific challenge (Fig. 6*A*). Finally, residuary production of 50% of OVA-specific Ig by $TLR2^{-/-}$ mice as compared with wild-type mice upon immunization using Myr_3CSK_4 as adjuvant, as well as absence of OVA-specific Ig from serum of functional TLR2/TLR4 double deficient mice, indicated a Myr_3CSK_4 signal transducer role of TLR4 (Fig. 6*B*).

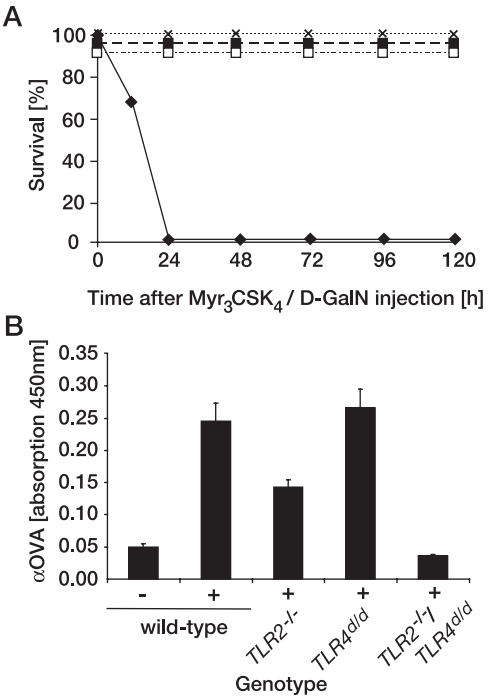

FIGURE 6. **Systemic Myr_3CSK_4 challenge induced lethality and ovalbumin-specific immunization.** *A*, mice of genotypes wild-type (*filled diamond*), $TLR4^{-/-}$ (*unfilled square*), $TLR2^{-/-}$ (×), or $TLR2^{-/-}$/TLR4$^{-/-}$ (*filled square*) were challenged by intraperitoneal injection of Myr_3CSK_4 and D-GalN, and viability was monitored ($n = 3$ for each group). *B*, mice of the genotypes indicated ($n = 2$ for each group) were challenged by intraperitoneal injection of Myr_3CSK_4 and ovalbumin (*OVA*, +) for three times consecutively or left untreated (−). Content of OVA-specific Ig in serum was monitored by ELISA (α, specific Ig).

Partial gain of eLPS responsiveness of otherwise eLPS-resistant $TLR4^{d/d}$ mice upon infection or IFNγ priming has been reported recently (39–41). Furthermore, invariable eLPS resistance of C57BL/10ScCr mice has been shown to base on both the lack of TLR4 expression and mutation of the IL-12 receptor (42). Accordingly, induction of IFNγ, which primes macrophages in an MyD88-dependent manner, is largely impaired in this mouse strain (42, 43). Consistently, responsiveness of TLR4-deficient macrophages to rough eLPS depended on IFNγ priming, whereas TLR2-TLR4/double deficient macrophage unresponsiveness was immutable (Fig. 4, *C* and *D*). IFNγ induced the increase of TLR2 expression in an MyD88-independent manner (supplemental Fig. 4S) (43) by involving it as a mechanism underlying the enhancement of TLR2-specific pattern recognition, which corresponded with *E. coli* LPS binding to both of the two TLRs *in vitro* (44). The potential increase of LBP, CD14, and/or CD36 expression upon IFNγ priming might contribute to TLR2-specific sensitivity (45, 46).

Although an *E. coli* O111:B4 LPS preparation of the kind used by us was separable into a non-TLR2- and a TLR2-specific fraction, contaminating protein was not detectable by gold

TABLE 2

Survival of mice upon challenge with suspensions of heat-inactivated bacteria or specific fractions of bacterial lysate

For survivors/total mouse number, all deaths occurred within 72 h of treatment; two out of two control mice challenged with D-GalN alone survived.

D-GalN	Challenge (i.p.)	CFU/mouse[a]	BL/6 wild type	$TLR2^{-/-}$	$TLR2^{-/-}/TLR4^{-/-}$	C3H/wild type	$TLR4^{d/d}$	$TNFRI/p55^{-/-}$
−	sup. *B. s.*	1×10^9	3/3	3/3				
−	pell. *B. s.*	1×10^9	0/2	3/3				
−	h. i. *B. s.*	1×10^9	0/3	3/3		0/3	0/3	
+	h. i. *B. s.*	1×10^7	0/8	4/4		0/3	0/3	4/4
−	h. i. *E. c.*	1×10^{10}	0/4	0/4	4/4	0/4	0/4	

[a] CFU indicates colony-forming units; BL/6 indicates C57BL/6; − indicates not applied; + indicates applied; *B. s.* indicates *B. subtilis*; *E. c.* indicates *E. coli*; i.p. indicates intraperitoneal injection in 500 μl of PBS; h. i. indicates heat-inactivated at 64 °C in PBS; sup. indicates supernatant, soluble fraction after boiling and sonication of *B. subtilis* suspensions; pell. indicates sediment after boiling, sonication, and enzymatic treatment of *B. subtilis* suspension.

staining (27, 47). Transition of a TLR2 stimulatory activity into the organic phase and a contaminating lipoprotein in a different eLPS preparation have been shown (47–49). However, isolation of a separable "LPS-like" TLR4-specific and TLR2-specific *E. coli* O111:B4 LPS fraction has been reported lately. Its cellular recognition was PMB-sensitive and serum-dependent (50, 51). Accordingly, *E. coli* and *Salmonella* LPS-induced TLR2 activity was inhibited by PMB preincubation and depended on the presence of serum containing LBP and soluble CD14 (52) in cell culture medium. On the contrary, lipopeptide-dependent cell activation was not inhibited by preincubation with PMB and was serum-independent (supplemental Figs. 2S and 3S).

Although lipopeptides, including Myr_3CSK_4 and lipoproteins, activated TLR2-deficient cells overexpressing a hTLR2 construct lacking the N-terminal seven leucine-rich repeat motifs, both *E. coli* O111:B4 and *S. enterica* Minnesota R595 LPS preparations failed to do so (supplemental Fig. 5S) (33). It is relevant on the subject of LPS purity that 500 ng of Pam_3CSK_4 mimicking the major fraction of an *E. coli* lipoprotein (5) did not induce shock if applied to wild-type mice upon sensitization with D-GalN and IFNγ priming (data not shown). 500 ng is by an order of magnitude above the amount of a protein contamination (0.035%) within 150 μg of *S. enterica* Friedenau LPS (Table 1, see "Experimental Procedures"). These findings might be indicative of distinct properties such as specific acylations and/or glycosylations of different eLPS subfractions, as well as the distinguishability of different intramolecular eLPS moieties in respect to their perceptibility through TLR4 or TLR4 and TLR2 (18, 47, 51). Our conclusions imply the lack of a demonstrable role of a proteinaceous eLPS contamination as independent immunostimulatory PAMP and indicate intrinsic TLR2-specific activity of eLPS. Reports on a capacity of TLR2 to mediate recognition of nonenterobacterial LPS species, namely assignment of both *Porphyromonas gingivalis* and *Leptospira interrogans* LPS or its lipid A moieties as agonists to TLR2 or TLR4 (27, 53–56), support our implication of TLR2 in eLPS recognition.

Previously, a contributive role of the saccharide chain bound to the lipid A moiety in eLPS perceptibility has been reported (57, 58). We observed relatively low sensitivity of TLR2 for rough eLPS and stronger dependence of rough eLPS recognition through TLR2 on CD14 expression as compared with that of smooth eLPS recognition (Fig. 3, supplemental Figs. 1S and 5S, and data not shown). Although contrasting with a recent report implicating TLR2 in lipid A recognition by γδT cells (59), synthetic lipid A activated cells TLR4/MD-2 specifically (Fig. 1*B* and 4*A*). Therefore, we hypothesize that the polysaccharide chain might contribute to eLPS recognition through TLR2. Of note, *MD-2*$^{-/-}$ and TLR4/MD-2 double deficient macrophages responded to eLPS upon IFNγ priming (supplemental Fig. 6S), thus negating a prominent role of MD-2 in TLR2-dependent eLPS recognition *ex vivo* (60).

It remains largely inexplicable why Myr_3CSK_4 recognition is TLR2-independent partially and why IFNγ priming might be required for TLR4-independent recognition of eLPS through TLR2. In any case, substantially higher amounts of both of the agonists were needed for "cross-activation" as compared with requirements for cell activation through TLR2 or TLR4 by challenge with lipopeptides or eLPS, respectively. Perhaps low affinity of TLR4 for Myr_3CSK_4 and low affinity of TLR2 for eLPS are because of different numbers of acylations in eLPS and Myr_3CSK_4. Low affinity of hTLR2 to a lauroylated lipopeptide as compared with high affinity of mTLR2 to this lipopeptide exemplifies distinctiveness of TLR affinities (35).

B. subtilis (Gram-positive)-induced shock depended on expression of TLR2, as well as expression of TNFα receptor I indicating the importance of consecutive activation of both of the two receptors (Table 2) (31). The TLR2-stimulatory activity of *B. subtilis* was contained in the nonsoluble and specific enzyme treatment-inert fraction. The latter finding and resistance of *TLR2*$^{-/-}$ mice to challenge with *B. subtilis* LTA (Table 1) support the implication of LTA as a major immunostimulatory activity of *B. subtilis* (61). In contrast, host responses to heat-inactivated *E. coli* (Gram-negative) depended on expression of both TLR2 and TLR4 (Table 2) providing evidence for perception of major immunostimulatory constituents of *E. coli* through both of the two receptors.

Taken together, we describe here formerly unreported aspects of TLR2 and TLR4 function. TLR4 contributed to cellular recognition of the lipopeptide Myr_3CSK_4 possibly explaining its higher toxicity as compared with that of Pam_3CSK_4, whereas TLR2 contributed to cellular recognition of eLPS. Upon priming with IFNγ, TLR2-dependent host sensitiveness to PAMPs such as LTA and eLPS was also enhanced *in vivo*. It follows that simultaneous usage of TLR4 and TLR2 antagonists might inhibit systemic responses to lipoproteins or eLPS more largely as compared with blockage of one of the two TLRs.

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