Cellular Recognition of Tri-/Di-palmitoylated Peptides Is Independent from a Domain Encompassing the N-terminal Seven Leucine-rich Repeat (LRR)/LRR-like Motifs of TLR2*

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Toll-like receptors (TLRs) mediate microbial pattern recognition in vertebrates. A broad variety of agonists has been attributed to TLR2 and three TLRs, TLR4, TLR2, and TLR3, have been demonstrated to bind microbial products. Distinct agonists might interact with different subdomains of the TLR2 extracellular domain. The TLR2 extracellular domain sequence includes 10 canonical leucine-rich repeat (LRR) motifs and 8–10 additional and potentially functionally relevant LRR-like motifs. Thus, the transfection of TLR2 LRR/LRR-like motif deletion constructs in human embryonic kidney 293 cells and primary TLR2-deficient mouse fibroblasts was performed for analysis of the role of the regarding domains in specific pattern recognition. Preparations applied as agonists were highly purified soluble peptidoglycan, lipoteichoic acid, outer surface protein A from Borrelia burgdorferi, synthetic mycoplasmal macrophage-activating lipoprotein-2, tripalmitoyl-cysteinyl-seryl-(lysyl)3-lysine (P3CSK4), dipalmitoyl-CSK4, and monopalmitoyl-CSK4 (PCSK4), as well as lipopolysaccharide and inactivated bacteria. We found that a block of the N-terminal seven LRR/LRR-like motifs was not involved in TLR2-mediated cell activation by P3CSK4 and P2CSK4 ligands mimicking triacylated and diacylated bacterial polypeptides, respectively. In contrast, the integrity of the TLR2 holoprotein was compulsory for effective cellular recognition of other TLR2 agonists applied, including PCSK4. The formation of a functionally relevant subdomain by a region including the N-terminal seven LRR/LRR-like motifs rather than by single LRRs is suggested by our results. They further imply that TLR2 contains multiple binding domains for ligands that may contribute to the characterization of its promiscuous molecular pattern recognition.

Immune responses toward microbes are preceded by their recognition. Pathogen-associated molecular patterns (PAMPs)†
TLR2 agonist largely resemble TLR4 agonist effects but differ in some aspects (4, 14). In addition, cooperation between TLR1 and TLR2 in recognition of triacylated peptides, as well as cooperation between TLRs 6 and 2 for diacylated mycoplasmal peptides, has been reported previously (15–17). Whether heteromerization is obligatory for cellular recognition of specific PAMPs such as acylated proteins or whether it is required for all TLR2-mediated effects remains unknown. In addition, specific TLR homodimers/heterodimers might associate with further receptor chains such as CD14, MD-2, and/or MD-1/Ip105 as has been demonstrated for TLR4 (18, 19).

Because the extracellular domain of TLR2 is considered to interact with various PAMPs (see above) (15), the question arises whether different parts of the ECD interact with these various ligands. TLR ECD sequences include arrays of leucine-rich repeat (LRR) motifs. The LRR consensus sequence encompasses 24–29 amino acid residues containing a highly conserved core region (LXXLXX/NL/XXXLXXXL) and is implicated in protein–protein interaction (5, 20). Crystal structures of multi-LRR domains of proteins such as ribonuclease inhibitor and internalin potentially provide a model of TLR ECD structure (20, 21). The ribonuclease inhibitor and internalin crystal structures revealed that the LRR motifs are composed of β strand-helix modules with the β strands being oriented in parallel and positioned in close proximity. Based on these structural considerations, it might be expected that mutations of the extracellular domain of TLR2 could influence susceptibilities to infections. This has been implicated for a polymorphism of the TLR2 intracellular domain (ICD), which correlates with altered functionality of the receptor (22).

Accordingly, we generated TLR2 ECD deletion mutants and compared the ability of the resulting protein constructs to mediate recognition of a variety of TLR2-specific PAMPs. We have found that cell activation by distinct TLR2-specific PAMPs requires different subdomains of the TLR2 ECD.

EXPERIMENTAL PROCEDURES

Reagents—Bacillus subtilis (DSMZ, 1087) and Escherichia coli (DH5α, Invitrogen) were cultured in standard brain-heart medium overnight at 37°C. Bacterial cells were adjusted to a concentration of 1 × 10⁹ colony-forming units/ml. Bacterial suspensions were heat-inactivated at 56°C for 45 min and adjusted to a concentration of 1 × 10⁶ colony-forming units/ml in cell culture experiments (heat-inactivated B. subtilis and heat-inactivated E. coli). Borrelia burgdorferi inactivated through sonication was kindly provided by Dr. Weis (University of Utah, Salt Lake City, UT) and applied at a concentration of 1.9 μg/ml. LPS from E. coli 0111:B4 (Sigma) was generally applied at a concentration of 0.1 μg/ml. sPGN was prepared from Staphylococcus aureus by vancomycin affinity chromatography (23) and applied at a concentration of 10 μg/ml as indicated. Highly purified LTA from B. subtilis (DSMZ 1087) was applied at a concentration of 5 μg/ml (24). Synthetic mycoplasmal macrophage-activating lipoprotein (R-MALP-2) was from Dr. Mühlradt (GBF Brunswick, Germany) and applied at a concentration of 1.3 ng/ml or as indicated (17). Synthetic N-palmitoyl-S-his (palmitoylthiopeptide), S-histidinylmycolic (P-CSK), S-histidinylpalmitoyl (P-CSK), and N-palmitoyl-CSK (P-CSK) were purchased from ECHAZ microcollections (Tübingen, Germany) and applied at a concentration of 0.1 μg/ml if not indicated otherwise. Lipidated OspA, a tripalmitoylated lipoprotein from B. burgdorferi, was from Dr. Dunn (Brookhaven National Laboratory, Upton, NY) and applied at 4.5 μg/ml (26). Highly purified recombinant chlamydial HSP60 (OspA, Sigma) (27) and applied at a concentration of 8 μg/ml. Zymosan and phorbol 12-myristate 13-acetate (PMA) were from Sigma and applied at concentrations of 50 μg/ml and 0.1 μg/ml, respectively.

Mutagenesis—A wild-type human TLR2 expression plasmid lacking the original leader sequence in favor of a 5'-terminally fused trypsin leader sequence followed by a FLAG tag coding sequence (pFLAGCMV-1, Sigma) was employed as template in overlap-PCR based mutagenesis (QuickChange Kit, Stratagene, Amsterdam, Netherlands). Deletion mutants lacking the following internal peptides as determined from the TLR2 cDNA sequence (GenBank™ accession number HSU86878) were generated as follows: Mut1 (ΔS48-F170), Mut2 (ΔF170-D201), Mut3 (ΔT202-T241), Mut4 (ΔS244-N533), Mut5 (ΔL173-S198), Mut6 (ΔL173-V220), MutC (ΔL123-F170), MutD (ΔL123-V220), MutE (ΔL123-N274), MutF (ΔS48-K121), MutG (ΔS48-S196), MutH (ΔS48-V220), MutI (ΔS48-T262), and MutJ (ΔS48-C287) as well as MutCK (ΔK19-N533), MutCD (ΔK19-V578), TLR2ICD (ΔK19-V578), and TLR2ICD-L1M1 (ΔH60). Positioning of deletion termini was performed by application of the Peptide software program (Chromatix, Reading, UK). Minimal changes of the secondary structure and line up of LRR β sheet subdomains as revealed from computer based calculation served as main criterion.

Cell Culture—The human embryonic kidney cell line (HEK293) as well as TLR2-/- embryonic fibroblasts (MEFs) were applied for protein expression and function analysis. TLR2-/- MEFs were provided by Tularik Inc. (San South Francisco, CA) (28). TLR2-/- mouse embryonic fibroblasts (MEFs) were generated from embryos isolated at day 12 post-fertilization. Cells were grown under regular mammalian cell culture conditions in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Roche Applied Science), standard antibiotics (Invitrogen, Auckland, Scotland), and 50 μg/ml thiglycolic acid (Sigma). Cells were passaged and expanded for five times. Frozen stocks were thawed and cultured for experiments.

Reporter Gene Assay—3 × 10⁶ HEK293 cells or TLR2-/- MEFs were cultured on single wells of 96-well plates. HEK293 cells were cotransfected with an NF-κB-recruiting endothelial-leukocyte adhesion molecule-1 (ELAM-1) promoter luciferase construct and a Rous sarcoma virus promoter-β-galactosidase reporter plasmid (29) as well as a cytomegalovirus promoter-regulated expression plasmid for human TLR2 by the calcium phosphate precipitation method (10, 30). For equilibration of expression levels, DNA amounts used were adjusted and expression levels were analyzed by immunoblot analysis (data not shown). TLR2-/- MEFs were transfected by electroporation at 960 microfarads and 260 mV (Gene Pulser II system, Bio-Rad). 7 h after medium change, preparations of bacterial products or analogues were added to transfected cells for 16 h. Cells were lysed for measurement of luciferase and β-galactosidase activities using reagents from Promega (Madison, WI) and Promega Biosystems (Bedford, MA). Luciferase activities were related to β-galactosidase activities for normalization.

Enzyme-linked Immunosorbent Assay—TLR2-/- MEFs and HEK293 cells were cultured on 96-well plates (2 × 10⁵ cells/well) with bacterial components for 16 h as indicated. Culture supernatants were applied to enzyme-linked immunosorbent assay (R&D systems, Minneapolis, MN) for measurement of murine IL-6 as well as human IL-8 concentrations, respectively, by enzyme-mediated colorimetry (Megellan, Tecan, Crailsheim, Germany) according to supplier protocols.

Immunoblot Analysis—HEK293 cells were lysed upon protein overexpression and stimulation for 30 min. Lysates from 2.5 × 10⁶ cells or immune complexes prepared from 3 × 10⁶ cells for each sample were prepared and analyzed by immunoblot analysis as described previously (29). 30 min after stimulation, 3 μl of total lysates (approximately 7.5 × 10⁶ cells) were applied. Rabbit polyclonal anti-antibodies specific for phosphorylated p38, ERK1/2, JNK, or Akt/protein kinase B were used (Cell Signaling). Specific epitopes were visualized by enhanced chemiluminescence (Western lightning, PerkinElmer Life Sciences).

Electromobility Shift Assay (EMSA)—1 × 10⁶ HEK293 cells were stimulated for 2 h in Dulbecco’s modified Eagle’s medium serum containing 2% fetal calf serum. Cells were washed with ice-cold phosphate-buffered saline and lysed (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5% Nonidet P-40, 0.1 mM EDTA, 0.2 mM mg/ml leupeptin and aprotonin, 0.5 mM phenylmethylsulfonfluoride, and 1 mM dithiothreitol). Nuclei were obtained by centrifugation at 10,000 g for 90 min at 4°C. Nuclei were sonicated (40 s on Ice, 10 s at 4°C, and 10 s off Ice) and antibiotics were added (0.1 mg/ml leupeptin and aprotonin, 1 mM phenylmethylsulfonfluoride, and 1 mM dithiothreitol). Debris was pelleted, and supernatant recovered (32). 5 μg of protein was applied to EMSA with a radioactively labeled double-stranded DNA oligonucleotide (5′-GATGCC ATTGGG GATTCC TCTTCT ACTG-3′) representing an NF-κB recognition element as described above (see above) (29) and applied at a concentration of 0.02 μM. Results were visualized by PhosphorImager (Storm 840, Abrams-Biosciences) aided signal detection.

Intracellular Staining—Pools of transfected HEK293 cell clones were grown on polylysine-coated glass covers each with eight culture dishes with removable walls (BD Biosciences). Cells were washed with PBS (pH 7.4) and peptide-treated saline and fixed with 2% formalin for 20 min at room temperature. Cells were washed...
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and blocked with phosphate-buffered saline containing 0.2% saponin and 3% bovine serum albumin for 30 min at room temperature. A first antibody, either anti-FLAG polyclonal rabbit antiserum (3 μg/ml) from Sigma or mouse monoclonal anti-human TLR2 2.1 (5 μg/ml) provided by Dr. Lien was applied prior to washing after 30 min of incubation. As a second antibody, Alexa Fluor 546-conjugated goat anti-rabbit/mouse IgG (4 μg/ml) was applied for 30 min (Molecular Probes) and washed. Cells were sealed in the presence of mounting fluid (Chromaidia pneumoniae micro-IP, Labsystems Oy, Helsinki, Finland) for analysis with a laser-scanning microscope with documentation unit (LSM510, Carl Zeiss, Oberkochen, Germany).

Immunoprecipitation—For immunoprecipitation of transiently overexpressed proteins, 10 μg of total expression plasmid DNA for the expression of the respective two proteins was transfected into 3 × 10^5 HEK293 cells seeded on 100-mm dishes by the calcium phosphate precipitation method (30). Mutant constructs and controls applied were overexpressed as FLAG-tagged hybrid proteins while the co-expressed protein was Myc-tagged. FLAG mAb M2 beads were used for precipitation (Sigma) (31). Immunocomplexes were analyzed by application of polyclonal anti-Myc tag agisermun for immunoblot analysis (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Mutagenesis—Structural information about TLR ECDs is restricted to sequence-based domain assignment at this stage. Using this method, the presence of 19 LRRs in TLR2 has been described previously (5). In addition to the 10 canonical LRR motifs, we have assigned 10 LRR-like motifs to the TLR2 ECD previously (9). We deleted single or groups of LRR/LRR-like motifs from the TLR2 ECD with the assumption that the removal of entire LRR subdomains would not alter overall protein structure (Fig. 1). We used the resulting constructs for potential identification of domains distinctively involved in cellular PAMP recognition. All of the TLR2 constructs were expressed at similar levels as revealed by anti-FLAG tag immunoblot analysis of total lysates of HEK293 cells following transfection of equal amounts of specific expression plasmid DNA preparations. The sizes observed were in agreement with expected mutant protein sizes. DNA amounts were adjusted for transfection, and expression levels were controlled by immunoblot analysis (data not shown).

Functional Analysis of TLR2 ECD Mutant Constructs—For all of the preparations of bacterial products used, wild-type TLR2 conferred NF-κB dependent reporter gene activation and release of IL-8 in HEK293 cells (Tables 1 and 2). The mean values displayed in both tables represent the results of at least three independent experiments. The data in Table 1 were normalized by calculation of the ratio of NF-κB-dependent and constitutive reporter gene activity. The significance of all of the values listed in the tables was analyzed through application of the Student’s t test for unconnected samples upon relation to vector controls. sPGN and all of the additional non-tripalmitoylated TLR2 agonists used did not induce cellular activation through any of the TLR2 mutant constructs overexpressed at equal levels. Examples are constructs Mut2 to Mut4, which cover three-fourths of the entire LRR-rich region (Fig. 1 and Tables 1 and 2). Mut1, however, mediated a weak signal upon application of P3CSK4 (Tables 1 and 2). In contrast, MutA and MutB lacking the LRRs adjacent to the Mut1 deletion, single LRR6, or LRRs 6 and 7, respectively, were not functional. This was also true for MutC carrying a deletion that was limited to the C terminus of Mut1 (LRRs 4 and 5) and two constructs carrying C-terminally extended deletions, namely MutD (LRRs 4–7) and MutE (LRRs 4–9) (Fig. 1 and Tables 1 and 2). Deletion of the N-terminal three LRRs abrogated cell activation as well (MutF). Notably, C-terminal extension of a deletion represented by Mut1 resulted in a successively increasing cell activation upon application of P3CSK4, OspA, and inactivated B. subtilis lipoprotein through MutG (LRRs 1–6) and MutH (LRRs 1–7), respectively (Fig. 1 and Tables 1 and 2). However, further C-terminal extension of the deletion abrogated cell activation as revealed from overexpression and analysis of the constructs MutI and MutJ, which lack the eight and nine N-terminal LRRs, respectively (Fig. 1 and Tables 1 and 2). Results from analysis of transiently and stably transfected HEK293 cells were similar (data not shown).

We further analyzed whether increased expression levels of MutH or other mutants would enable cellular recognition of a wider variety of PAMPs. Transfection of 50-fold amounts of expression plasmid for MutH as compared with wild-type TLR2 partially conferred cellular activation by the diacylated peptide R-MALP 2 in a dose-dependent manner. However, activation by application of other TLR2 agonists such as sPGN was barely detectable even upon application of very high amounts of stimulants (Fig. 2A). None of the other mutants mediated responsiveness following either increased expression or through application of ligands at high concentrations (Fig. 2A).

To assess the role of single palmitoylations for recognition of tripalmitoylated peptides by TLR2, two P3CSK4 derivatives, P3CSK4 and PCSK4, were used to challenge transiently transfected HEK293 cells expressing each of the TLR2 mutants that were generated (Fig. 2B). Wild-type TLR2 conferred NF-κB activation upon application of all three derivatives. The constructs MutG and MutH mediated response to P3CSK4 as well as P3CSK4 to different degrees. In the case of MutH, P3CSK4 induced a more robust NF-κB activation compared with P3CSK4 (Fig. 2B). PCSK4, albeit clearly activating cells expressing wild-type TLR2, did not elicit a significant signal through any of the mutant constructs analyzed (Fig. 2B). Similar results were obtained upon transfection of 50-fold amounts of mutant expression plasmid as compared with wild-type TLR2 plasmid as well as application of very high amounts of stimulants (Fig. 2C). Cotransfection of both wild-type TLR2 and each of the mutant DNA constructs in a ratio of 1:50 was performed for analysis of mutant effects on wild-type TLR2-mediated cell activation. TLR2 deletion mutants inhibited wild-type TLR2-mediated cell activation when transfected cells were stimulated with heat-inactivated B. subtilis or P3CSK4 with the exception of MutH (data not shown). Consistent with results from analysis of transfection of HEK293 cells, over-
pression of wild-type human TLR2 restored responsiveness toward LTA as well as P3CSK4 in TLR2−/− MEFs as indicated by NF-κB-dependent reporter gene activation (Fig. 3A) and release of IL-6 (Fig. 3B). In contrast, MutH-mediated cell activation was restricted to P3CSK4 stimulation and further mutants such as MutJ were inactive (Fig. 3, A and B).

Cellular Localization of Wild-type and Mutant TLR2—Pools of six cell clones overexpressing wild-type TLR2, Mut1, MutF, MutG, MutH, or MutJ were analyzed immunocytochemically. Concavalin A was used for staining of the cell membrane. Although control HEK293 cells did not express a FLAG epitope, overexpression of wild-type FLAG-TLR2 revealed the localization of the tagged protein specifically at the cell membrane (Fig. 4). Overexpressed wild-type TLR2 and all of the mutant proteins analyzed were located at the cellular membrane and not within the cell as revealed by comparison with overexpressed FLAG-tagged IL-1 receptor-associated kinase 1, which represents a cytoplasmically located protein (Fig. 4 and data not shown). We further applied an anti-human TLR2 monoclonal antibody (mAb 2.1) for analysis. The presence of wild-type TLR2, MutH, and MutJ at the cell membrane was confirmed. None of the other mutants used was recognized by mAb 2.1 to a detectable degree, although most of them inevitably carried the domain forming the respective epitope in wild-type TLR2 and MutH (data not shown).

DNA Binding of NF-κB and Phosphorylation of Cellular Kinase Akt as Well as That of Mitogen-activated Protein Kinases p38, ERK1/2, and JNK Mediated by TLR2 and Mutant Receptors—Controls as well as HEK293 cell clone pools stably expressing wild-type TLR2, MutH, or MutJ were subjected to molecular analyses of cell activation. Nuclear extracts as well as total lysates of cells were prepared 2 h or 30 min after the start of stimulation, respectively, with sPGN, P3CSK4, or PMA. Nuclear extracts were applied to EMSA and total lysates for analyses of cellular kinase Akt as well as mitogen-activated protein kinases p38, ERK1/2, and JNK phosphorylations by immunoblot analysis. EMSA revealed nuclear translocation and binding of NF-κB to a canonical NF-κB DNA recognition element as well as kinase phosphorylation in all of the clones upon PMA stimulation as compared to unstimulated cells (Fig. 5, A and B). NF-κB activation and phosphorylation of kinases analyzed upon stimulation with sPGN depended on the expression of wild-type TLR2 but was also observed in cells overexpressing MutH lacking the N-terminal seven LRRs. Constitutive NF-κB activation was also observed in cells overexpressing MutH lacking the N-terminal seven LRRs. Control HEK293 cells as well as cells overexpressing mutant J did not respond to challenge with P3CSK4 as revealed from NF-κB EMSA and analysis of kinase phosphorylation (Fig. 5, A and B).

Interaction of Wild-type TLR2 with Mutant TLR2 Constructs—To analyze the role of the TLR2 ECD in homologous or heterologous interaction of TLR2, we performed immunoprecipitation experiments. FLAG-tagged wild-type TLR2, Mut1, Mut2, Mut3, Mut4, MutH, MutJ, MutCK, MutCD, or vector as negative control were cotransfected with Myc-tagged wild-type TLR2 or TLR1. FLAG-tagged mutant as well as wild-type proteins as indicated all coprecipitated with the Myc-tagged wild-type TLR2, MutH, or MutJ were subjected to molecular analyses of cell activation. Nuclear extracts as well as total lysates of cells were prepared 2 h or 30 min after the start of stimulation, respectively, with sPGN, P3CSK4, or PMA. Nuclear extracts were applied to EMSA and total lysates for analyses of cellular kinase Akt as well as mitogen-activated protein kinases p38, ERK1/2, and JNK phosphorylations by immunoblot analysis. EMSA revealed nuclear translocation and binding of NF-κB to a canonical NF-κB DNA recognition element as well as kinase phosphorylation in all of the clones upon PMA stimulation as compared to unstimulated cells (Fig. 5, A and B). NF-κB activation and phosphorylation of kinases analyzed upon stimulation with sPGN depended on the expression of wild-type TLR2 but was also observed in cells overexpressing MutH lacking the N-terminal seven LRRs. Control HEK293 cells as well as cells overexpressing mutant J did not respond to challenge with P3CSK4 as revealed from NF-κB EMSA and analysis of kinase phosphorylation (Fig. 5, A and B).

### Table I

**TLR2 ECD mutant-mediated NF-κB dependent reporter gene activation in HEK293 cells**

| Rel. Luc. activity | Vector | Wild-type | Mutant |
|-------------------|--------|-----------|--------|
|                   |        | 1 2 3 4 A B C D E F G H I J |        |
| Unstim.           | 2.1    | 1.6       | 0.01   |
| s. B.b.           | 21.2a  | 2.1a      | 1.4    |
| h.i.c.            | 2.1    | 1.11b     | 2.9    |
| h.i.b.s.          | 5.3    | 32.5a     | 7.5    |
| Zymos.            | 9.1a   |           | 1.4    |
| LPS               | 1.5    | 16.0a     | 4.1    |
| sPGN              | 1.2    | 20.2a     | 4.3    |
| LTA               | 1.3    | 22.0a     | 4.3    |
| HSP60             | 1.5    | 10.7a     | 4.3    |
| OspA              | 1.9    | 27.1a     | 4.3    |
| P3CSK4            | 1.7    | 34.9a     | 4.3    |
| MALP-2            | 8.5a   | 8.2a      | 4.3    |
| PMA               | 0.24a  | 0.29a     | 4.3    |

* p < 0.001; significance as revealed from Student’s t test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vector-untreated value).

### Table II

**TLR2 ECD mutant mediated IL-8 release from HEK293 cells upon PAMP application**

| IL-8 ng/ml | Vect. | Wild-type | Mutant |
|------------|-------|-----------|--------|
|            | 1 2 3 | 4 A B C D E F G H I J |        |
| Unstim.    | 0.01  | 0.01      | 0.01   |
| s.B.b.     | 0.26  | 0.01      | 0.01   |
| h.i.c.     | 0.01  | 0.01      | 0.01   |
| h.i.b.s.   | 0.26  | 0.38      | 0.07   |
| Zymos.     | 0.55a |           | 0.01   |
| LPS        | 0.45a |           | 0.01   |
| sPGN       | 1.3a  | 0.27      | 0.10   |
| LTA        | 1.1a  | 0.13      | 0.10   |
| HSP60      | 0.30a |           | 0.10   |
| OspA       | 0.15a |           | 0.10   |
| P3CSK4     | 0.03  | 0.13a     | 0.08   |
| MALP-2     | 0.28a |           | 0.28a  |
| PMA        | 0.24a | 0.29a     | 0.28a  |

* p < 0.05; significance as revealed from Student’s t test for unconnected samples by relation to vector control for the regarding stimulant (PMA induction values were related to the vector-untreated value).
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DISCUSSION

Comparative mutational analysis of mouse and human TLR4 and analysis of TLR5 implicated particular ECD domains in species-specific recognition of LPS modifications and binding of flagellin, respectively (33, 34). TLR2 and/or TLR4 binding of glucuronoxylomann capsules of *Cryptococcus neoformans* as well as of LPS and sPGN have been reported previously (35–37). To date, evidence for direct binding of PAMPs to TLRs as well as recognition of a relatively large variety of PAMPs particularly through TLR2 is compelling and may imply the existence of different binding sites of various specific ligands. Here we used mutagenesis of the TLR2 ECD for its functional analysis.

We speculated that in addition to the 10 canonical LRR, 8–10 LRR-like motifs present in the TLR2 ECD sequence might represent functionally relevant subdomains (5, 9). The LRRs are evenly distributed throughout the TLR2 ECD, and we deleted them in four blocks, each containing five motifs (Fig. 1). We then focused on the 10 N-terminal LRRs by successive deletion of internal regions. In total, 14 mutant ECD TLR2 constructs were generated (Fig. 1). Specifically, we asked which of the ligands within a representative group of known agonists were able to induce cell activation through mutant constructs. We identified one class of agonists inducing signaling in the absence of the seven N-terminal LRRs. As such, our results

**FIG. 2.** TLR2 mutant-mediated stimulus and NF-κB-dependent reporter gene activation in HEK293 cells. Cells were cotransfected with reporter gene constructs as well as CD14 and wild-type or mutant TLR2 expression plasmds. 24 h after transfection started, cells were stimulated with the microbial products or synthetic derivatives as indicated for 16 h and lysed. Cell activation was measured as luciferase reporter activity in the lysates. Experiments were repeated at least twice. (A) dose kinetics of agonists applied to cells transfected with 50-fold amounts of expression plasmds for TLR2 mutants MutH and MutJ as compared to wild-type TLR2 (IL-1α as positive control). Amounts of agonists applied increased successively: P3CSK4, 1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μg/ml; sPGN, 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml; MALP-2, 10 pg/ml, 0.1 ng/ml, 1 ng/ml, and 10 ng/ml; and IL-1α, 20 ng/ml.

**FIG. 3.** TLR2 wild-type/mutant-mediated stimulus-dependent reporter gene activation in TLR2−/− MEFs and IL-6 release from TLR2−/− MEFs. Results upon overexpression of wild-type human TLR2 or TLR2 mutant constructs in TLR2−/− MEFs in terms of NF-κB-dependent reporter gene activation (A) and IL-6 release (B) are illustrated. Tumor necrosis factor α was applied for stimulation as positive control.

or the LRR-rich domain only (MutCK) coprecipitated with the wild-type TLRs 2 and 1 (Fig. 6 and data not shown).

**FIG. 3.** TLR2 wild-type/mutant-mediated stimulus-dependent reporter gene activation in TLR2−/− MEFs and IL-6 release from TLR2−/− MEFs. Results upon overexpression of wild-type human TLR2 or TLR2 mutant constructs in TLR2−/− MEFs in terms of NF-κB-dependent reporter gene activation (A) and IL-6 release (B) are illustrated. Tumor necrosis factor α was applied for stimulation as positive control.

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imply interaction of dipalmitoylated/tripalmitoylated peptides with the C-terminal region of the TLR2 ECD (38).

A trend of mutant activity became evident at equal expression levels of each TLR2-derived construct. Only MutG and MutH conferred cell activation to significant degrees upon challenge with P3CSK4, P2CSK4, or OspA but not upon application of any of the other PAMPs and analogues as indicated (Fig. 2 and Tables 1 and 2). Tripalmitoylation is a typical characteristic of bacterial proteins eliciting host responses through TLR2 (4, 39). Additionally, increased overexpression in combination with application of increased amounts of synthetic R-MALP-2 rendered MutH-expressing cells responsive, whereas sPGN-induced cell activation was barely detectable (Fig. 2A). None of the mutants encompassing Mut2 to Mut4, MutA to MutF, as well as MutI and MutJ (Fig. 1) mediated activation of the signaling pathways analyzed upon application of any TLR2-specific agonist (Tables 1 and 2).

TLR2 dependence of cell activation upon challenge with P2CSK4, P3CSK4 lacking the amide-linked fatty acid, and PCSK4 missing the two ester-bound fatty acids was in line with a recent report describing primary immune cell responses to two of these ligands ex vivo as TLR4-independent (39). Although stimulating activities of P2CSK4 and P3CSK4 were almost equal, those of PCSK4 were only 30–50% (Fig. 2B). On the other hand, palmitoylation of a peptide at the amino group of a terminal cysteine was sufficient for recognition through TLR2, yet dipalmitoylation increased the stimulatory potential of the peptide considerably. These results suggest that triacylation is not obligatory for TLR2-dependent stimulatory activity. It also raises questions for further aspects of structural properties, which recently have been addressed in the case of a diacylated peptide such as R-MALP-2 (17). Notably, the levels of P2CSK4-induced cell activation were similar when mediated through MutH or wild-type TLR2. However, PCSK4 did not induce cell activation through any of the mutant TLR2 constructs (Fig. 2B). Thus, the additional palmitoylation of PCSK4 confers independence of cellular recognition from the N-terminal third of the TLR2 LRR-rich domain. This was confirmed upon increased TLR2 mutant expression and amounts of stimulants applied (Fig. 2C). Interestingly, recognition of diacylated R-MALP-2 through MutH was detectable only at increased expression levels (Fig. 2, A and B).

Overexpression of mouse TLR2 (15) as well as that of human TLR2 (Fig. 3, A and B) complemented cellular responsiveness...
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with wild-type or mutant TLR2 lacking the entire ECD (MutCD) was evident, whereas only a TLR2∆ICD construct did not coprecipitate with the tagged cytoplasmic TLR2 domain (TLR2ICD, signal sequence deleted, data not shown). These results further indicate a role of the transmembrane domain rather than that of the TLR ECD in receptor dimerization/oligomerization as has been proposed also by others previously (38, 39) and that might be mediated by unknown proteins within a receptor complex.

Our data imply that the N-terminal 7 of 18–20 LRRs are not involved in cellular recognition of triacylated and diacylated microbial polypeptides through TLR2. The activity of a respective TLR2 mutant (MutH) was only slightly diminished when compared with wild-type TLR2 (Tables 1 and 2 and Fig. 2B). In accordance with the findings of Mitsuzawa et al. (38) who demonstrated the involvement of the domain Ser-40 to Ile-64 as an sPGN-binding domain, our results suggest involvement of the N-terminal third of the TLR2 ECD in cellular recognition of sPGN and other TLR2 agonists applied (38). Thus, potential binding domains most probably differ for tripalmitoylated and diacylated polypeptides as compared with those of the other TLR2 ligands tested. These conclusions might contribute to elucidation of the molecular basis of TLR-mediated PAMP recognition including possible differences in cell activation triggered by distinct ligands or different doses of one ligand via one receptor (43, 44). One possible explanation of our findings could be that there exist yet unknown recognition proteins for LTA and other TLR2 agonists, which differ from a potential P3CSK4 recognition protein. If so, both types of endogenous proteins might function serum independently (39) and mediate cell activation by interacting with distinct regions of the TLR2 ECD. Future PAMP-TLR-binding and structural analyses will further clarify the perspective on pattern recognition receptor function of TLRs.

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