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MARCKS as a Negative Regulator of Lipopolysaccharide Signaling

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Myristoylated alanine-rich C kinase substrate (MARCKS) is an intrinsically unfolded protein with a conserved cationic effector domain, which mediates the cross-talk between several signal transduction pathways. Transcription of MARCKS is increased by stimulation with bacterial LPS. We determined that MARCKS and MARCKS-related protein specifically bind to LPS and that the addition of the MARCKS effector peptide inhibited LPS-induced production of TNF-α in mononuclear cells. The LPS binding site within the effector domain of MARCKS was narrowed down to a heptapeptide that binds to LPS in an extended conformation as determined by nuclear magnetic resonance spectroscopy. After LPS stimulation, MARCKS moved from the plasma membrane to FYVE-positive endosomes, where it colocalized with LPS. MARCKS-deficient mouse embryonic fibroblasts (MEFs) responded to LPS with increased IL-6 production compared with the matched wild-type MEFs. Similarly, small interfering RNA knockdown of MARCKS also increased LPS signaling, whereas overexpression of MARCKS inhibited LPS signaling. TLR4 signaling was enhanced by the ablation of MARCKS, which had no effect on stimulation by TLR2, TLR3, and TLR5 agonists. These findings demonstrate that MARCKS contributes to the negative regulation of the cellular response to LPS. The Journal of Immunology, 2012, 188: 3893–3902.

The myristoylated alanine-rich C kinase substrate (MARCKS) family belongs to the unstructured proteins that mediate cross-talk in signaling. MARCKS has been implicated in the regulation of cell migration and adhesion, of endocytosis, exocytosis, and phagocytosis, as well as of neurosecretion and brain development (reviewed in Ref. 1). The MARCKS family consists of two members: MARCKS, a 32-kDa acidic protein, which is expressed ubiquitously, and MARCKS-related protein (synonyms MRP, MLP, MacMARCKS, and F52), a 20-kDa acidic protein expressed mainly in the brain, reproductive tissues, and macrophages (2, 3). MARCKS has an unusual amino acid composition, because 85% of its sequence is composed of only six different amino acid residues with a consequence that it is unstructured with no persistent secondary or tertiary structure (4). The two main regions of MARCKS recognized for their functional importance are the myristoylation site and the effector domain (ED), which governs its interaction with anionic phospholipids of the cell membrane.

The 25-residue ED (residues 151–175), rich in cationic amino acid residues, is central to the function of MARCKS (1). ED is essential for binding of MARCKS to the membrane as the N-terminal myristate alone is insufficient to anchor MARCKS to the membrane (5–7). Through the ED, MARCKS preferentially binds to negatively charged phospholipids such as phosphatidylinositol 4,5-bisphosphate (PIP2) and regulates the level of free PIP2 in the membrane (8) by sequestering PIP2 molecules (9), thus affecting the activity of phospholipase C (10). The ED also binds Ca2+/calmodulin and actin (11, 12), which affects the actin cross-linking (11). Ca2+/calmodulin binding and phosphorylation of ED by protein kinase C (PKC) (12) regulate the binding of MARCKS to the plasma membrane (13, 14). PKC-dependent phosphorylation regulates trafficking of MARCKS between the plasma membrane and LAMP-1–positive lysosomes (15), autophagosomes (16), and mucin granules (17). MARCKS is a key molecule regulating mucin secretion. Binding of MARCKS to mucin granules occurs after phosphorylation by PKCα (18) and is mediated by cysteine string protein and HSP70 chaperones (19), thus influencing mucin secretion by the airway epithelium, which can be blocked by the N-terminal fragment of MARCKS (20). PKC-dependent phosphorylation of MARCKS has also been recently shown to influence lamellipedia formation (21) and cell motility (22). Although most of physiological effects of MARCKS are mediated by the ED, this is not essential for the function of MARCKS in radial glia cell development (23). Such a diversity of binding targets enables MARCKS to mediate cross-talk between several signal transduction pathways.

Transcription of MARCKS and its parologue MRP are strongly increased upon stimulation with bacterial LPS (24–27), but the role...
of this protein family in response to bacterial infection is not well understood. LPS as a constituent of the Gram-negative bacterial cell wall is one of the most potent stimulants of the immune system, imparting a wide range of effects on eukaryotic cells. LPS also induces phosphorylation of MARCKS (28). These properties, along with the binding of MARCKS to acidophilic phospholipids, led us to investigate the effect of MARCKS on the cellular response to LPS. LPS signaling is initiated through its binding to the TLR4/myeloid differentiation-2 (MD-2) complex. LPS/MD-2/TLR4 complex formation occurs at the cell membrane in professional immune cells such as monocytes (29), whereas in epithelial cells, which are constantly exposed to bacteria, or in endothelial cells, most TLR4/MD-2 is confined to the intracellular space and signaling requires the internalization of LPS (30–33).

In this study, we examined the direct interaction between MARCKS or its ED and LPS. High-resolution spectroscopic techniques revealed the structural motif of binding of MARCKS ED to LPS that resembles antimicrobial peptides (34). Confocal microscopy-based fluorescence resonance energy transfer (FRET) experiments showed that MARCKS was in close proximity to the internalized LPS and that it translocated to the endosomes. Enhanced cellular activation by LPS was detected when MARCKS expression was downregulated by small interfering RNA (siRNA) and in MARCKS-deficient mouse embryonic fibroblasts (MEFs), whereas MARCKS overexpression had a strong inhibitory effect. These findings suggest that MARCKS–LPS interaction represents a negative regulator of the cellular response to bacterial infection by sequestration of LPS and repression of the LPS signaling.

Materials and Methods

Reagents

The ED peptide (sequence: KKKKKRFSFKSKLGSFKKNKK), peptide KRFSFKK and scrambled peptide (FRKKSFKK) were amidated at the C terminus. Smooth form LPS (S-LPS) from Salmonella abortus equi (strain HL83) was prepared by the phenol extraction method (35). The rough form LPS (R-LPS) of Salmonella enterica (serovar Minnesota, strain R595) was extracted by the phenol/chloroform/petrol ether method (36). Pam3CSK4, flagellin, and polyinosinic-polycytidylic acid were purchased from InvivoGen.

Cell lines

Embryonic kidney cell line HEK293, epithelial cell line A549 and endothelial cell line ECV304 (ECACC), monocytic cell lines MonoMac 6 and RAW264.7, and primary cell line MEF (a gift from Dr. P. J. Blackshear, National Institute of Health, Bethesda, MD) were used for our experiments. HEK293, A549, and RAW264.7 cells were cultured in DMEM with glutamax (Invitrogen) and in RPMI 1640 medium and 4% human serum), and their number was adjusted to 5 × 105 cells/well. The cultures were incubated for 4 h at 37°C. MRF was adjusted to 5 μM and incubated with MEFs and in MARCKS-deficient mouse embryonic fibroblasts (MEFs), whereas MARCKS overexpression had a strong inhibitory effect. These findings suggest that MARCKS–LPS interaction represents a negative regulator of the cellular response to bacterial infection by sequestration of LPS and repression of the LPS signaling.

RT-PCR analysis

MonoMac 6 cells were seeded at 5 × 107 cells/well and incubated overnight. Cells were stimulated for 2 h with 100 ng/ml LPS. Total RNA was isolated using miRNeasy kit (Qiagen). cDNA was synthesized from total RNA using reverse primers for MARCKS and β2-microglobulin. The following primers were used for cDNA and DNA amplification: MARCKS (forward, 5'-CACGTCTCCAAGACCGCAGC-3'); reverse, 5'-GGTGGCTGAGCATCCTTCC-3'); and β2-microglobulin (forward, 5'-GTGCTGCTTCTCATGTTGTGATGACT-3'; reverse, 5'-TCTTGTTCTCCACCTCCTGAG-3'). Values are indicated as relative quantities to the calibrator sample.

Affinity chromatography of MARCKS on immobilized LPS

S-LPS was conjugated to epoxy-Sepharose (Amersham Pharmacia), according to the manufacturer’s instructions. The brain from a BALB/c mouse was immediately frozen in liquid nitrogen. A mixture of protease inhibitors (Sigma-Aldrich) was added to the thawed brain, which was homogenized in 10 ml buffer containing 0.25 M sucrose, 100 mM Tris-HCl (pH 7.4), 5 mM EGTA, 5 mM EDTA, and 5 mM DTT and centrifuged at 5000 × g for 30 min at 4°C. The resulting supernatant was centrifuged additionally at 30,000 × g for 60 min at 4°C. The supernatant was applied directly to the LPS-Sepharose column and washed with PBS buffer containing 0.3 M NaCl. Bound fractions were eluted with buffer containing 15 mM triethanolamine (pH 11.5), 140 mM NaCl, and 30 mM β-octylglucopyranoside. Detection of MARCKS in the homogenate and eluted fractions was carried out on SDS-PAGE with equal protein loading in all lanes by immunoblot using primary Abs against the MARCKS protein (Calbiochem) and secondary anti-rabbit peroxidase-labeled Abs.

Production of recombinant MRP

The vector for expressing recombinant MRP in Escherichia coli was pET3d-MRP-HIS. The protocol of Schnitz et al. (37) for MRP expression and purification was used with some additional steps. After sonication and centrifugation, the supernatant was heated for 5 min at 80°C and centrifuged again. The supernatant was then loaded onto Ni2+ chelating resin and eluted with buffer containing 250 mM imidazole. Residual endotoxin was removed by repeated extraction with Triton X-114, followed by reversed phase-HPLC separation on an eclipse XDB-C8 column with a gradient of 5–95% acetonitrile in the presence of 0.05% trifluoroacetic acid.

Binding of LPS to MRP using ELISA

Wells in microtiter plates (Maxisorp[P96; Nunc] were coated for 3 h at room temperature with recombinant MRP at various concentrations in 100 μl 50 mM NaHCO3 at pH 9.5. Wells were saturated with albumin. A total of 100 μl 3 μM biotin-LPS was incubated for 2 h at 37°C, followed by incubation with 100 μl 1:1000 streptavidin-peroxidase (Sigma-Aldrich) for 2 h at 37°C. ABTS (Sigma-Aldrich) was used as a substrate, and the absorbance was measured at 405 nm. Recombinant MRP (0.47 μM) was used for coating the wells for the inhibition test with polymyxin B. A total of 3 μM biotin-LPS and various concentrations of polymyxin B were preincubated for 30 min at room temperature and then added to MRP.

Inhibition of the stimulation of human mononuclear cells by LPS

Mononuclear cells were isolated from the heparinized blood of healthy donors as described in Ref. 38. The cells were resuspended in medium (RPMI 1640 medium and 4% human serum), and their number was adjusted to 5 × 105 cells/ml. For stimulation, 200 μM mononuclear cells (1 × 105 cells) were transferred into each well of a 96-well culture plate. R-LPS and R-LPS:peptide mixtures were incubated for 2 h at 37°C and added to the cultures at 20 μl/well. The cultures were incubated for 4 h at 37°C. Supernatants were collected and used for the immunological determination of TNF-α in a Sandwich ELISA using an mAb against TNF-α (clone 6b from Intex AG) as described earlier (39). Data are the mean of three independent experiments (i.e., cells from three individual human donors).

Fluorescence spectroscopy

Fluorescence emission spectra were recorded with an excitation wavelength of 238 nm to monitor fluorescence light from the Phosphores. Binding of LPS to MRP was monitored by the fluorescence emission at 310 nm at 25°C. LPS was added in 0.2 μM increments to 4 μM ED. The dissociation constant for binding of S-LPS to MRP was obtained by measuring the increase in fluorescence of acrylodan-labeled ED upon addition of LPS. A 5-fold molar excess of acrylodan was added, and the mixture was incubated at room temperature for 2 h. DTT was then added to 5 mM to quench the remaining unreaccreted acrylodan. Excess reagents were removed by extraction with ethanol, followed by reversed phase-HPLC separation on an eclipse XDB-C8 column with a gradient of 5–95% acetoni-trile in the presence of 0.05% trifluoroacetic acid. The identity of the product was confirmed by fast atom bombardment mass spectroscopy. A total of 2 μM ED-acrylodan was excited at 365 nm, and emission spectra were measured between 400 and 540 nm. S-LPS was added in 0.2 μM increments. Data were evaluated by integrating the area under the fluorescence curve; a nonlinearity taking into account ligand depletion was performed using the Origin 6.0 program (Microcal).

Nuclear magnetic resonance experiments

A synthetic heptapeptide KRFSFKK-NH2 was dissolved at a concentration of 1 mM in 20 mM sodium phosphate buffer pH 4 in 90% H2O/10% D2O at 30°C. S-LPS was added to the peptide solution in an ~1:10 molar ratio for
transferred nuclear Overhauser effect spectroscopy (NOEY) experiments. Spectra were recorded on a 600 MHz Inova nuclear magnetic resonance (NMR) spectrometer (Varian) equipped with a probe with z-gradients. The spectral width was 12 parts per million with 512 increments in the indirect dimension and 2048 points in the direct dimension. Free induction decays were apodized with a sine square function. NOEY experiments were recorded at mixing times of 50 and 150 ms and TOCSY spectra at mixing times of 50 and 100 ms. Spectra were processed using the Felix2000 program (Accelrys).

Confluent microscopy

pEGFP-N1 MARCKSwt was transfected to A549 and ECV304. Transfected A549 and ECV304 cells were seeded on micro plate culture slides (Lab-tek; Life Technologies) overnight. LPS-rodhamine (100 ng) was added for 15 min. Cells were rinsed twice with PBS prior to fixation with 4% paraformaldehyde for 20 min.

The fluorescence of GFP and rhodamine was detected using appropriate filter sets. FRET was measured by detecting the increase in donor fluorescence as a consequence of complete photobleaching of the acceptor molecule. Rhodamine was bleached by continuous excitation for 2 min. FRET was calculated from the increase in donor fluorescence after acceptor photobleaching by \( (E) = \frac{(GFP\ postbleach) - (GFP\ prebleach)}{(GFP\ prebleach)} \times 100\%\).

Localization of MARCKS and MARCKS A2/G2 in LPS-stimulated cells was detected by either anti-earlly endosome Ag 1 (EEA1)-TRITC Abs and dextran-TRITC or cotransfection with pEGFP-2xFYVE or pmCherry-2xFYVE. Colocalization of MARCKS and TLR4 was detected in HEK293 cells stably expressing TLR4-mCherry, transiently transfected with pDUO MD-2/CD14 and pEGFP-N1 MARCKSwt, and dyed with transferrin-Alexa 633. Cells were either stimulated with unlabeled LPS or LPS-Cy5. Cells were imaged on a Zeiss LSM510 META or Leica TSC SP5 confocal microscopes.

Western blot analysis

For phosphorylation assays, RAW264.7 cells were plated on 24-well plates stimulated with S-LPS for the indicated time points and lysed in radio-immunoprecipitation assay buffer with proteinase inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Calbiochem). A total of 50 µg total cell protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare). Detection of phosphorylated MARCKS was done using specific primary Abs (Ser152/156; Cell Signaling Technology) and appropriate secondary goat anti-rabbit IgG-HRP (Abcam). α/β-Tubulin Abs (Cell Signaling Technology) were used as loading control.

Luciferase assay

HEK293 cells were transiently transfected with 200 ng MARCKS siRNA cassette (5'-TCCTGTGCGTGTGCCTTTTGGTATCCGCAAGCGAAACCGCAGAGA-3') (GenScript) or control siRNA (psiSiRNA h7SKgze sc; InvivoGen), pFLAG-TLR4, pEFBOS-MD-2 expression plasmids and pELAM1-NF-κB–dependent or pGL-IP10 promoter-dependent luciferase, and constitutive Renilla (phRL-TK; Promega) reporter plasmids. According to the manufacturer, the transfection efficiency using Lipofectamine is 99% in HEK cells. Forty-eight hours after transfection, cells were stimulated with 100 ng/ml LPS for 8 h. Cells were lysed and analyzed for reporter gene activities using a dual-luciferase reporter assay system on a Mithras LB940 luminometer (Berthold technologies). The data from luciferase activity were normalized using Renilla luciferase readings. The data shown are representative of four experiments.

In addition, in overexpression experiments, cells were transfected with pEGFP-N1 MARCKSwt (with STOP codon inserted between MARCKS and GFP), pDsRed1-N1 MARCKS A2/G2, or empty plasmid pRK5 instead of siRNA. The data shown are representative of three experiments.

MARCKS wt MEF and MARCKS A2/G2 MEF were seeded in a 96-well plate. Cells were stimulated with two different amounts of ligands (S-LPS, Pam3CSK4, flagellin, and polyinosinic-polycytidylic acid) for 6 h. The supernatants were collected and analyzed for IL-6 production using a mouse IL-6 simplex kit (Bender Medsystems) on an Altra flow cytometer (Beckman Coulter).

Results

Specific binding of MARCKS and MRP to LPS

Transcriptomic data in the NCBI Gene Expression Omnibus database (40), including more than 40 human and 60 murine data sets, overwhelmingly show that both MARCKS and MRP transcripts are strongly increased upon LPS stimulation (41, 42). Upregulation was observed in monocytes, dendritic cells, epithelial cells, brain, spleen, and lung tissue cells. We measured MARCKS mRNA induction after the LPS stimulation of human monocytic cell line. In agreement with transcriptomic results in the database, we observed that after 2 h of stimulation with 100 ng/ml LPS, MARCKS mRNA expression increased 3.5-fold with respect to the control mRNA (Fig. 1A).

We were interested in whether the increased MARCKS expression after the LPS stimulation can have a role in LPS neutralization. To determine whether the endogenous MARCKS is able to bind LPS in the presence of other LPS-binding proteins present in the tissue, we performed a binding study of mouse brain homogenate using the LPS affinity column. We found that MARCKS was enriched in the eluted fraction of proteins that bind to the immobilized LPS (Fig. 1B, right column). Indirect ELISA using purified MRP showed a concentration-dependent binding of LPS to the full-length MRP (Fig. 1C). The specificity of interaction was confirmed by competitive displacement of LPS from the immobilized MRP by polymyxin B, which binds to the lipid A moiety of LPS (Fig. 1D). Significant competition occurred at a relatively high concentration of polymyxin B, indicating strong and specific interaction between MRP and LPS.

Identification of the LPS-binding segment of MARCKS

Because of the net negative charge of the LPS and the positive charge of the ED of MARCKS, we surmised that electrostatic interactions could be important for direct interactions. We identified a cationic sequence, KRFSFKK in MARCKS and KKFSFKK in MRP within the ED segment, both of which are consistent with the consensus LPS-binding motif present in many LPS-binding peptides and observed in the determined tertiary structures of LPS–peptide complexes (34, 43–45). A synthetic ED peptide based on the MARCKS sequence bound to the LPS with a submicromolar affinity (\(K_D = 0.3 \pm 0.03 \mu M\)) (Fig. 1E). Besides cationic aminoacid residues, ED contains all the aromatic residues of MARCKS (five phenylalanine residues). This peptide exhibits an unusual intrinsic fluorescence with a broad, structured emission band between 300 and 320 nm, indicating excimer fluorescence, probably arising from the close proximity of the phenylalanine aromatic side chains in the free peptide in solution (Fig. 1F).

The addition of LPS to the peptide decreased the intrinsic fluorescence of the ED peptide (Fig. 1F, inset), demonstrating the participation of phenylalanine side chains in the interaction with LPS.

The ED peptide was able to neutralize the biological activity of LPS in the Limulus amebocyte lysate assay (data not shown) and on human mononuclear cells (Fig. 1G, open circles). At 6 nM concentration, ED inhibited >90% of the release of TNF-α in mononuclear cells stimulated by LPS, whereas the heptapeptide had lower activity. The most likely reason that the MARCKS ED was more effective than the heptapeptide KRFSFKK (Fig. 1G, filled squares) is that the ED contains two pseudo repeats of the LPS-binding motif. A scrambled peptide with the same amino acid composition as the KRFSFKK heptapeptide was ineffective in the TNF-α release inhibition (Fig. 1G, filled triangles), confirming the importance of the specific peptide sequence motif. The cytotoxicity of peptides against mononuclear cells was checked by trypan blue uptake. We detected 5–10% dead cells at 60 and 200 nM peptide concentrations, identical to the control. At higher concentrations of MARCKS, peptide TNF-α expression started to increase. This effect may be due to the formation of large LPS–peptide aggregates that can be cross-linked by the excessive amount of added peptide and may increase the internalization of LPS. Similar effect on the enhancement of cell activation has been observed for some other cationic antimicrobial peptides (46).

NMR experiments have previously determined that the MARCKS ED adopts an extended conformation (47, 48). We
performed two-dimensional NMR-transferred NOESY experiments of the heptapeptide in the presence of LPS to analyze the interaction between LPS and this peptide. This experiment provides information on the conformation of the peptide in the LPS-bound state. The two-dimensional NOESY spectrum of the free peptide contains only a limited number of weak nuclear Overhauser effects (NOEs) (Fig. 2A). When LPS at 10% of the molar amount of the peptide was added to a solution, a large number of additional cross-peaks appeared in the NMR spectrum, and the signal intensity of several of the existing peptide cross-peaks increased significantly (Fig. 2B), showing that the peptide adopted an induced conformation in complex with LPS. Assignment of the transferred NOE cross-peaks in the NMR spectra is consistent with the extended conformation of the peptide, where both phenylalanine residues are positioned on the same side of the molecule and their neighboring cationic residues are located on the opposite face of the peptide. The family of calculated peptide structures shows the arrangement of the side chains (Fig. 2C). Docking of the peptide to lipid A suggests that the guanidine groups of Arg2 and Lys5 interact with the phosphate groups of the lipid A moiety, thus positioning the phenylalanine side chains close to the aliphatic chains (Fig. 2D) in the energetically most stable conformation of the complex.

From in vitro binding studies, we can conclude that MARCKS and LPS can interact through LPS-binding motif inside the ED portion of the receptor, the intensity of the MARCKSwt-GFP signal increased significantly (35 ± 5%), quantitatively confirming FRET and demonstrating the close proximity between MARCKS and LPS inside living cells.

**Role of membrane attachment of MARCKS on its LPS colocalization**

Because many effects of MARCKS depend on its localization, we wanted to investigate the role of the membrane attachment of MARCKS for its interaction with LPS within cells. The MARCKS A2/G2 mutation disrupts the myristoylation signal. This MARCKS

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**Figure 1.** LPS stimulation increased expression of MARCKS protein and bound to the protein. (A) Quantitative MARCKS mRNA analysis in MonoMac 6 cells stimulated with LPS (100 ng/ml) for 2 h was performed. (B) Mouse brain homogenate was applied to the LPS-Sepharose column. Bound proteins were eluted with β-octyl glucopyranoside. Fractions were analyzed by Western blot using anti-MARCKS Abs. Equal protein loading was applied to both lanes: a brain homogenate loaded to the column (lane 1) and a fraction eluted from the LPS column (lane 2). (C) Wells in the microtiter plate were coated with increasing concentrations of MRP, followed by the addition of biotinylated LPS. Bound LPS was detected with avidin-HRP. (D) Polymyxin B at varying concentrations was added along with LPS (3 μM) to compete with MRP (0.5 μM, adsorbed to wells) for binding of LPS. The amount of bound LPS was detected as in (B). (E) Determination of the dissociation constant for binding of the ED-acrylodan peptide to LPS. The curve represents the best fit for the binding constant of 0.3 μM. (F) Decrease in the intrinsic fluorescence emission of the ED peptide upon addition of LPS. From top to bottom: 0, 0.2, 0.8, 1.4, 2, 2.6, 3.2, and 3.8 μM LPS was added to 2 μM ED peptide. Inset: A titration of the ED peptide with LPS monitoring the fluorescence emission at 312 nm. (G) LPS Re (strain R595; 0.5 ng/ml), preincubated with various concentrations of the ED peptide ( ), the heptapeptide KRFSDKK ( ), and the peptide with the scrambled sequence ( ) were added to human mononuclear cells. The amount of released TNF-α was determined by ELISA.
mutant resided in the cytoplasm in unstimulated cells (Fig. 4A), but when overexpressed a smaller amount associated with the membranes. We expected that this mutation would not affect the ability of MARCKS to bind LPS. In fact, comparable FRET efficiency (28 ± 5%) was determined between MARCKS A2/G2-GFP mutant and LPS-rhodamine as for MARCKSwt. After 15 min of LPS stimulation, MARCKS A2/G2-DsRed was detected inside endosomes. MARCKS A2/G2-DsRed and FYVE-GFP colocalized after LPS stimulation (Fig. 4D, arrow, micrograph). The fluorescence profile across the endosomes in a confocal micrograph demonstrates that the MARCKS mutant was indeed localized in the lumen of vesicles rather than at the cytosolic side of the vesicle membrane. In contrast, MARCKSwt-GFP was embedded in the endosomal membrane after LPS stimulation (Fig. 4B, asterisk, R1 micrograph). MARCKSwt partially colocalized with MARCKS A2/G2 (Fig. 4B, arrow, R2 micrograph) and FYVE (Fig. 4C, arrow, micrograph), indicating that MARCKSwt can be found inside the endosomal compartments as well, where it could interact with the internalized LPS.

**Colocalization of TLR4, MARCKS, and LPS**

TLR4 is the membrane receptor, which mediates LPS signaling. The TLR4 receptor traffics between the plasma membrane and endosomes (49). We found that TLR4 and MARCKS colocalized in transferrin-loaded endosomes after LPS stimulation (Fig. 4E, arrows). Furthermore, when LPS-Cy5 was added, we observed

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**FIGURE 2.** Direct binding of the core ED peptide to LPS. (A) A two-dimensional NOESY spectrum of the heptapeptide KRFSFKK in solution contains only a few NOE signals, while after the addition of 1/10 of the peptide molar ratio of LPS (B), a number of NOEs appear because of the binding and rapid exchange with LPS aggregates. Cross-peak assignments are provided. (C) A family of 10 NMR structures of the peptide calculated from the transferred NOE constraints. The residues are colored according to properties (blue – basic, yellow – polar and gray – hydrophobic). (D) The heptapeptide docked to lipid A (shown as VdW spheres).
endosomes with colocalized MARCKS, TLR4, and LPS (Fig. 4F, arrows), indicating LPS sequestration by MARCKS can be physiologically relevant for the activation of TLR4.

**MARCKS suppresses LPS-induced TLR4 activation**

Next, we evaluated the effect of MARCKS on TLR4-mediated LPS signaling. We used HEK293 cells, which do not express most of TLR receptors, but express downstream signaling molecules. Therefore, this cell line is used to control the expression of selected TLRs in the absence of the background of endogenous receptors. Knockdown of MARCKS by siRNA in cells expressing TLR4/MD-2 receptor complex resulted in an increase of the MyD88-dependent NF-κB luciferase reporter activation after LPS stimulation (Fig. 5A), whereas control siRNA had no effect. This result is consistent with the proposed role of MARCKS as a negative regulator of LPS signaling. In addition, we tested activation of the TRIF-dependent pathway by using the IP-10 promoter-based luciferase reporter and similar results were obtained (Fig. 5B).

MARCKS deficiency is in most cases lethal because of the brain defects in the newborn mice (50), so only MEFs from MARCKS knockout mice could be obtained. MEF cells express several TLRs, so the specific influence of MARCKS on signaling through different TLRs could be analyzed directly by stimulation with their respective ligands. IL-6 responsiveness was significantly increased at 100 ng/ml LPS, whereas even stronger 2-fold enhanced response in comparison with wild-type was observed at higher concentration of LPS (1 μg/ml) (Fig. 5C). MARCKS deficiency had no effect on the signaling of TLR2, TLR5, or TLR3, which share the same signaling pathway with TLR4 (Fig. 5D), which demonstrates the specificity of the effect for TLR4 signaling. We reasoned that if the downregulation of MARCKS increased the response to LPS, its overexpression should suppress it. Indeed, as expected, MARCKSwt overexpression strongly suppressed LPS signaling (Fig. 5E). In addition, the MARCKS A2/G2 mutant had the same inhibitory effect (Fig. 5E), which shows that anchoring of MARCKS to the membrane is not required for this effect. In contrast, overexpression of neither MARCKSwt nor MARCKS A2/G2 inhibited TLR3 activation (data not shown), again demonstrating the specificity of MARCKS for LPS rather than for different polyanions.

**Discussion**

The cellular responsiveness to LPS is regulated at many levels, such as the expression of its cellular receptors, their localization and degradation, by extracellular LPS chaperones such as LBP, CD14, or BPI and by intracellular downstream signaling effectors. Stimulation by LPS strongly upregulates both MARCKS (Fig. 1) and MRP transcripts, with induction of MRP being MyD88-independent (42). Because of its abundance, MARCKS constitutes as much as 90% of all the cellular proteins synthesized in response to LPS stimulation (51). Within the sequence of the MARCKS proteins, we have identified an LPS-binding motif defined by the pattern BHPHB (43), where B, H, and P stand for basic, hydrophobic, and polar amino acid residues, respectively, which is similar to several other LPS binding peptides. We show that MARCKS, as well as the peptide fragments from its ED, bind LPS and neutralize its biological activity.

MARCKS is an intrinsically unfolded protein, which can bind many different ligands. We characterized the molecular structure of the LPS–ED peptide complex, identifying the important role of the aromatic phenylalanine and cationic lysine side chains for this interaction. A similar conclusion with respect to the interaction of MARCKS ED with membranes containing PIP2 has been reached using magic angle spinning NMR experiments (52). The absence of LPS neutralization by the scrambled sequence peptide indicates that LPS–ED interaction is sequence specific and does not occur simply because of the high content of cationic and aromatic residues. The distance between the cationic groups in the extended peptide is close to 15 Å in this structural motif, which matches the distance separating the two phosphate groups of the lipid A (53). The measured affinity is sufficiently high to sequester a large fraction of the internalized LPS or intracellular Gram-negative bacteria within the same cellular compartment as MARCKS, particularly given the high (milimolar) intracellular concentration of MARCKS.
Several processes of the neutralization of the internalized LPS have been described previously. Neutralization of LPS in the apical recycling endosomes by specific IgAs has been reported in epithelial cells (54). Internalized LPS can be deactivated by AOAH in the lysosomal detoxification process (55). We observed complex formation between MARCKS and LPS in the epithelial cells by confocal microscopy, where we found that MARCKS was localized to the endosomes in the presence of LPS (Figs. 3, 4F). This translocation of MARCKS occurred very fast as the colocalization was observed already 15 min after the LPS stimulation. MARCKS and TLR4 partially colocalized in endosomes already in unstimulated cells (data not shown), but the number of vesicles exhibiting colocalization strongly increased after the LPS stimulation. Therefore, LPS stimulation facilitates and increases the translocation of MARCKS to the endosomes. MARCKS translocates from the membrane to the cytosol after phosphorylation (56). The precise mechanism of translocation of MARCKS to the endosomes has yet to be characterized. The localization of MARCKS to intracellular vesicles has been indirectly demonstrated by the requirement for the lysosomal protease cathepsin B for the proteolytic cleavage of MARCKS (57). Spizz and Blackshear (57) proposed that MARCKS contains a lysosome targeting

**FIGURE 4.** MARCKS translocated to TLR4-positive endosomes upon LPS stimulation. (A) MARCKS A2/G2-DsRed is mainly expressed in the cytoplasm in unstimulated HEK293 cells. After LPS stimulation, MARCKS A2/G2-DsRed was detected in endosomes, whereas MARCKSwt-GFP was present in endosome membranes (B, asterisk). In addition, several endosomes were found, where both proteins colocalized inside endosomes (B, arrow). Endosomes containing MARCKSwt (C) or MARCKS A2/G2-DsRed (D) after LPS stimulation were FYVE-cherry or FYVE-GFP positive. Scale bars, 5 μm. Colocalizations are represented in confocal micrographs as fluorescence intensity profiles across the endosomes marked by the arrows and the asterisk. (E) HEK293 cells stably expressing TLR4-mCherry were transfected with MD-2, CD14, and MARCKSwt-GFP, dyed with transferrin-Aexa633 and stimulated with LPS; (scale bars, 7.5 μm) (F) instead of staining with transferrin cells were stimulated with LPS-Cy5. Scale bars, 10 μm.
motif KEE(L,V)Q. An alternative mechanism could be the translocation process based on a cluster of positively charged amino acids similar as in cell-penetrating peptides (58) but also of several antimicrobial peptides (59). Our observation that the MARCKS ED peptide was able to penetrate into macrophages (data not shown) additionally supports this mechanism.

Autophagy might also play a role in the translocation of MARCKS. Although we found that MARCKS and LC3, a marker of autophagosomes, did not colocalize (data not shown), we cannot completely exclude the contribution of autophagy. LPS induces autophagy through activation of TLR4 signaling pathway (60); therefore, the autophagy of MARCKS might contribute to the sequestration of LPS and downregulation of TLR4 signaling. Additional support for this mechanism could be drawn from the decreased LPS responsiveness caused by the inhibition of an autophagy repressor mTOR by rapamycin (61) and increased LPS responsiveness by the inhibitor of PI3K as an activator of autophagy (62).

Phosphorylation of serine residues of MARCKS reduces its association to the plasma membrane. Maximal phosphorylation of MARCKS was detected 40 min after LPS stimulation (Fig. 3B), but the effect of LPS stimulation on MARCKS localization was already observed earlier. We propose that phosphorylation of MARCKS, triggered as an early event of the LPS stimulation, leads to its dissociation from the cell membrane and translocation to the endosomes, where it can bind to LPS and downregulate the excessive activation of TLR4. MARCKS translocation to endosomes may also involve additional chaperones, similarly as translocation to mucin granules mediated by cysteine string protein (63). Efficiency of the myristoylation-deficient A2/G2 mutant of MARCKS, which under the normal conditions resides in the cytoplasm and associates with endosomes upon LPS stimulation, further supports this mechanism.

We showed the physiological relevance of MARCKS–LPS interaction for the LPS signaling by detection of an increased LPS-induced cellular activation in siRNA knockdown and in MARCKS-deficient cells (Fig. 5). The observed inhibition of LPS signaling by overexpression of MARCKS and the rapid increase in MARCKS transcription caused by the LPS stimulation suggest that the role of MARCKS in downregulation of the LPS response...
may be most important at high LPS concentrations in the early stages of the response to the endotoxin, as the inhibitory effect of MARCKS overexpression declined after 24 h (data not shown).

MARCKS interacts with anionic lipid clusters in the membrane through its ED. This might suggest an alternative mechanism based on the interference with TLR4 signaling through competition with the Mal adaptor because Mal contains a cationic motif, essential for its binding to the PIP2 membrane clusters (64). Deletion of this peptide motif prevents association of Mal with the membrane resulting in impaired activation of TLR4 and TLR2. This suggests, however, it is not corroborated by our finding that TLR2 signaling, which also requires Mal, is not affected by the resulting in impaired activation of TLR4 and TLR2. This suggests, however, it is not corroborated by our finding that TLR2 signaling, which also requires Mal, is not affected by the peptide motif prevents association of Mal with the membrane resulting in impaired activation of TLR4 and TLR2. This suggests, however, it is not corroborated by our finding that TLR2 signaling, which also requires Mal, is not affected by the peptide.

Moreover, MARCKS interacts with several cellular proteins, its interference with LPS signaling may have other indirect consequences, in addition to LPS sequestration, and may affect the crosstalk between additional signal transduction pathways.

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The authors have no financial conflicts of interest.

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