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Toll-like Receptor-2 Mediates *Treponema* Glycolipid and Lipoteichoic Acid-induced NF-κB Translocation*

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Recently Toll-like receptors (TLRs) have been found to be involved in cellular activation by microbial products, including lipopolysaccharide, lipoproteins, and peptidoglycan. Although for these ligands the specific transmembrane signal transducers TLR-4, TLR-2, or TLR-2 and -6 have now been identified, the molecular basis of recognition of lipoteichoic acids (LTAs) and related glycolipids has not been completely understood. In order to determine the role of TLRs in immune cell activation by these stimuli, experiments involving TLR-2-negative cell lines, TLR-expression plasmids, macrophages from TLR-4-deficient C3H/HeJ-mice, and inhibitory TLR-4/MD-2 antibodies were performed. Glycolipids from *Treponema maltophilum* and *Treponema brennaborense*, as well as highly purified LTAs from *Staphylococcus aureus* and *Bacillus subtilis* exhibited TLR-2 dependence in nuclear factor κB activation and cytokine induction; however, *T. brennaborense* additionally appeared to signal via TLR-4. Fractionation of the *T. brennaborense* glycolipids by hydrophobic interaction chromatography and subsequent cell stimulation experiments revealed two peaks of activity, one exhibiting TLR-2-, and a second TLR-4-dependence. Furthermore, we show involvement of the signaling molecules MyD88 and NIK in cell stimulation by LTAs and glycolipids by dominant negative overexpression experiments. In summary, the results presented here indicate that TLR-2 is the main receptor for *Treponema* glycolipid and LTA-mediated inflammatory response.

Translocation of nuclear factor κB (NF-κB)[1] into the nucleus and subsequent activation of responsive genes are important events in immediate cellular immune response in vertebrates and invertebrates (1). Processes involving activation of NF-κB regulate responses to stress, inflammation and viral infection, the communication between cells, embryonic development, and the maintenance of cell type specific expression of genes. Activation of NF-κB is furthermore required for transcriptional activation and subsequent release of many mediators including the pro-inflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (2).

Signaling pathways leading to NF-κB activation, *i.e.* the IL-1-receptor pathway, encompass a cascade of signal transducing proteins. The adapter protein MyD88 is recruited to the receptor complex upon ligand binding (3, 4). MyD88 recruits the downstream IL-1 receptor-associated kinases IRAK, IRAK-2, and IRAK-M to the receptor complex (3, 5, 6). The signal is further directed to the adapter molecule tumor necrosis factor receptor-associated factor-6, the TGF-β-activated kinase 1, and the NF-κB-inducing kinase (NIK) (7–10). NIK activates the IκB kinase complex (IKK1/α, IKK2/β, and NEMO/IKKγ), and phosphorylation of IκB triggers degradation and subsequent nuclear translocation of NF-κB leading to specific gene activation (9, 11–14).

Cellular activation by microbial ligands via members of the family of Toll-like receptors (TLRs) has recently been found to initiate a signaling cascade also resulting in translocation of NF-κB (15–22). The nine currently known human TLRs exhibit homology with the *Drosophila* Toll family also comprising at least nine members (23). The Toll protein was the first member of this protein family described as a key player in embryonic development and contributes to defense against fungal infection in the adult fly (24). Another member of the Toll family in *Drosophila*, 18-wheeler, has been suggested to be involved in antibacterial defense (25). Similar to the IL-1-induced NF-κB induction, TLRs initiated the signaling cascade by the so-called intracellular cytoplasmic Toll/interleukin-1 receptor domain TLRs have in common with the IL-1 receptor (15, 26). The extracellular domains of Toll and TLRs contain leucine-rich repeat motives (26). TLR-4, most likely in connection with the adapter molecule MD-2, is the signal transducing receptor for lipopolysaccharide (LPS) from Gram-negative bacteria (19, 27–30), whereas TLR-2 mediates cellular activation by bacterial lipoproteins, whole Gram-positive bacteria, and yeast (31–39). Recently, peptidoglycan has been shown to be recognized by

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[1] The abbreviations used are: NF-κB, nuclear factor κB; EMSA, electrophoretic mobility shift assay; HIC, hydrophobic interaction chromatography; IκB, inhibitory κB; IKK, inhibitory κB kinase; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MyD88, myeloid differentiation protein; NIK, nuclear factor κB-inducing kinase; OMIZ-Pat, *Treponema* culture medium; PEM, peritoneal macrophage; TLR, Toll-like receptor; TNF, tumor necrosis factor; Ab, antibody; CHO, Chinese hamster ovary; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay.

TLR-2 and -6 (40), whereas TLR-9 mediates detection of bacterial DNA (41).

Lipoteichoic acids (LTAs) are present in the cell wall of most Gram-positive bacteria and are linked to the cytoplasmic membrane (42). Most widespread are poly(glycerophosphate) LTAs such as the ones found in *Staphylococcus aureus* and *Bacillus subtilis* (43, 44). They are macroamphiphiles commonly consisting of hydrophilic polyglycerol phosphate chains and a diacylglycerol lipid anchor (45). LTAs exhibit immunostimulatory activity such as cytokine induction (46, 47). It thus was tempting to speculate that LTAs also utilize TLRs for activation of immune cells. However, results obtained in different cell systems have been controversial, and the role of TLRs in LTA-induced cell stimulation has not been completely understood; *S. aureus* and *Streptococcus sanguis* LTA induced IL-6 and nitric oxide in macrophages from wild-type and TLR-2-deficient mice, but not in macrophages from TLR-4-deficient mice (48). In contrast, overexpression of TLR-2 in HEK293 cells conferred inducibility of NF-κB in response to *B. subtilis*, *Streptococcus pyogenes*, and *S. sanguis* LTAs (38). Recently, an improved isolation procedure employing butanol has been shown to result in pure and biologically highly active LTAs (49).

Previously we have reported on the isolation of two novel *Treponema* species, *T. maltophilum*, associated with peridontitis in humans, and *T. brennaborense*, found in bovine cattle disease (50–52). Isolated membrane glycolipids of both treponemes, which share structural characteristics with LTAs, stimulate mononuclear cells to release TNF-α in a CD14- and lipopolysaccharide-binding protein-dependent fashion (53).

In order to identify the TLRs involved in stimulation of immune cells by *Treponema* glycolipids and LTAs, these two different glycolipids, highly purified butanol-extracted LTAs from *B. subtilis* and *S. aureus*, and, as control, LPS were used for stimulation of cells differing in their TLR expression pattern. Our results confirm our previous findings and indicate that NF-κB activation by these stimuli is induced mainly by TLR-2, however, with additional utilization of TLR-4 potentially depending on the chemical composition of the particular microbial component employed.

EXPERIMENTAL PROCEDURES

Treponema Culture and Preparation of Phenol/Water Extracts—Frozen stocks of *T. brennaborense and T. maltophilum* cells (300 μl, each stored at −80 °C) were inoculated in 3 ml of a culture medium (OMIZ-Pat) as described previously (51). Bacteria were cultured anaerobically (Anaerogen, Oxoid, Germany) at 37 °C for 3–4 days. The cultures were then transferred to a larger volume of OMIZ-Pat (20–100 ml) and further incubated for 1–2 days. Viability of the treponemes and exclusion of contaminating bacteria were assessed by dark field microscopy (400-fold magnification, BH2-RFCA microscope, Olympus, Hamburg, Germany). Sterility controls of the medium were performed by incubating OMIZ-Pat medium under aerobic and anaerobic conditions at 37 °C for 1 week. The pH value of the culture medium was measured repeatedly. Cultures were stopped at pH 6.0 and centrifuged at 12,000 × *g* at 4 °C for 20 min. For some experiments, at this point whole treponeme cells were resuspended in H_2O, washed twice, and frozen.

For glycolipid extraction, aqueous suspensions of treponeme cells were digested with RNase (Sigma, Deisenhofen, Germany), DNase (Merck, Darmstadt, Germany), and proteinase K (Merck). The suspensions were dialyzed and extracted using a hot phenol/water extraction method (54). In brief, the phenol/water extraction was performed by mixing the cell suspension with an equal volume of 90% phenol and stirring at 68 °C for 10 min. After cooling on ice, the mixture was centrifuged at 3,000 × *g* for 10 min at 0 °C, and the upper phase was collected. This procedure was repeated twice, and combined phases were dialyzed and lyophilized. LPS contamination was thoroughly investigated by *Limulus* amoebocyte lysate assay and other methods and could be clearly ruled out. This has been published elsewhere (53). A mock extract including all media and chemicals that were used during extraction was tested in all assays.

Extraction of LTAs from B. subtilis and S. aureus—B. subtilis (DSMZ 1087) and *S. aureus* (DSM20233) were grown in a 8-liter shaker and a 35-liter fermentor, respectively. After harvesting by centrifugation at 4 °C and 8000 rpm, the pelleted bacteria were sonicated (Branson Sonifier, Branson, Schwäbisch-Gemünd, Germany) on ice and extracted with butanol at room temperature (49). The aqueous phase was purified by hydrophobic interaction chromatography (HIC) on octyl-Sepharose. Fractions were screened for phosphorus-rich LTA by a phosphomolybdene blue assay (55); positive fractions were pooled. All LTA-preparations were negative in a chromogenic *Limulus* amoebocyte lysate assay (QCL-1000, Bio Whittaker, Walkersville, MD), *i.e.* they contained less than 30 pg of LPS/mg of LTA from *B. subtilis* and less than 6 pg of LPS/mg of LTA from *S. aureus*. The purity of the preparations was estimated by NMR analysis to be ≥99%.

Stimulation of the Murine Macrophage Cell Line RAW264.7 and Chinese Hamster Ovary (CHO) Cells and Estimation of NF-κB Translocation—1.6×10^6 RAW264.7 cells/well were cultured in six-well tissue culture plates overnight in RPMI 1640 (Life Technologies Ltd, Paisley, United Kingdom) containing 10% fetal calf serum (FCS). After two washing steps with RPMI 1640, cells were starved for 3 h in the absence of FCS. Stimulation with whole treponemes, *Treponema* glycolipids, LTAs from *B. subtilis* and *S. aureus*, *Escherichia coli* 0111:B4 LPS (Sigma), or *Streptococcus minnesota* LPS (Sigma) for 1 h was performed in the presence of 2% non-heat-inactivated FCS. In certain experiments, RAW264.7 cells were incubated with the anti-TLR-4-MD-2 antibody MTS510 (30), at concentrations of 5 μg/ml for 1 h prior to stimulation.

CHO cells transfected with human CD14 (generously provided by L. Hamann, Forschungszentrum Borstel, Borstel, Germany) were cultured with Ham's nutrient medium F-12 (PAA Laboratories GmbH, Linz, Austria) supplemented with 10% FCS and 400 μg/ml hygromycin B (Calbiochem, San Diego, CA). 4×10^5 CHO/CD14 cells were plated in six-well tissue culture plates. At the next morning, cells were starved in FCS-free Ham's medium for 3 h before stimulation with *Treponema* glycolipids, LTAs, or *E. coli* 0111:B4 LPS (Sigma) in the presence of 2% non-heat-inactivated FCS.

After 1 h cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4, and incubated in 150 μl of buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM NaF). After 20 min, cells were harvested mechanically, transferred to 1.5-ml tubes, mixed with 25 μl of Nonidet P-40, and centrifuged at 13,000 × *g* at 4 °C for 1 min. Pellets were resuspended in 50 μl of buffer B (400 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM NaF), incubated for 30 min at 4 °C, spun at 13.000 × *g* at 4 °C for 5 min, and supernatants containing nuclear proteins were collected. The binding activity of NF-κB in the extracts was determined by a standard electrophoretic mobility shift assay (EMSA). Briefly, 4 μg of the extracts were incubated with radiolabeled double-stranded oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′, containing the consensus NF-κB DNA site, and electrophoresis was performed on a 4% polyacrylamide gel.

Stimulation of RAW264.7 and U373MG Cells and Detection of Cytokine Concentration—To assess induction of TNF-α, 5×10^4 cells/well of RAW264.7 cells were cultured overnight in 96-well tissue culture plates. The cells were stimulated with *Treponema* extracts, LTAs, or *E. coli* 0111:B4 LPS (Sigma) in the presence of 2% non-heat-inactivated FCS, and supernatants were harvested after 4 h of incubation. MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 3 μg/ml anti-mTNF Ab (PharMingen, Heidelberg, Germany) in 100 mM Na_3PO_4, pH 6.0. Samples and recombinant mTNF standard (R&D Systems, Wiesbaden, Germany) were incubated at room temperature for 3 h, and detection was performed with 500 ng/ml biotin-conjugated anti mTNF-α Ab (PharMingen), and 1 μg/ml streptavidin-peroxidase with *ortho*-phenylene diphosphate (OPD) as substrate (Sigma).

For measurement of human IL-6, 3.75×10^4 cells/well of U373MG cells (33, 56) were seeded in 96-well tissue culture plates with DMEM containing 10% FCS. In certain experiments, U373MG cells were incubated with the anti-TLR-4 antibody HTA125 (57) at concentrations of 5 μg/ml for 1 h prior to stimulation. On the next day, the cells were incubated overnight with *Treponema* glycolipids, LTAs, or *E. coli* 0111:B4 LPS (Sigma) in the presence of 5% AB serum. MaxiSorp ELISA plates were incubated with 3 μg/ml anti-hIL-6 capture Ab (R&D) in 100 mM Na_2CO_3, pH 8.3. Samples and IL-6 standard (R&D Systems) were incubated at room temperature for 2 h, detection was performed employing 25 ng/ml biotinylated anti hIL-6 Ab (R&D Systems), and substrate reaction was carried out as in the TNF-α ELISA.

Fractionation of Treponema Extracts—Treponema phenol/water ex-

tracts were separated by HIC. HIC was performed on an FPLC system (Amersham Pharmacia Biotech, Freiburg, Germany) using an octyl-Sepharose CL-4B column (Amersham Pharmacia Biotech). An increasing linear gradient (15–60%) of propanol-1 in ammonium acetate buffer (pH 4.7) was used as elution buffer with a flow rate of 0.25 ml/min. 500 μl of each fraction was dried and resuspended in 100 μl of phosphate-buffered saline. For induction of IL-6 in U373MG or TNF-α in RAW264.7 cells, 10 μl of each fraction were used. To induce NF-κB in CHO/CD14 or RAW264.7 cells, 10 μl of each fraction 40–60 or 61–80 were mixed and cells were incubated with 2 μl of the pooled fractions.

Expression Plasmids and Transfection of HEK293 Cells—Expression plasmids for TLR-2 and the dominant-negative mutants of MyD88 and NIK were generated as described (17). Briefly 3×10^5 HEK293 cells (Tularik, San Francisco, CA) were cultured in six-well plates with DMEM (Life Technologies Ltd) supplemented with 10% FCS. HEK293 cells were transfected the following day by the calcium phosphate precipitation method following the manufacturer's instructions (CLONTECH, Palo Alto, CA) with 0.5 μg of ELAM-1 luciferase reporter plasmid, 0.5 μg of Rous sarcoma virus β-galactosidase plasmid to normalize for transfection efficiency, and 0.2 μg of empty vector or TLR-2 expression vector. In some experiments 0.1 μg of dominant-negative mutants of MyD88 or NIK were transfected. On the following day, cells were incubated with *Treponema* glycolipids or LTAs in the presence of 2% non-heat-inactivated FCS for 6 h, and luciferase and β-galactosidase activity was measured by using the Luciferase Reporter-Gene Assay high sensitivity and the β-Gal Reporter-Gene Assay from Roche Diagnostics, Mannheim, Germany.

Preparation and Stimulation of Peritoneal Elicited Macrophages (PEM)—Peritoneal macrophages were isolated from C3H/HeJ or C3H/HeN mice (Charles River, Sulzbach, Germany), by thioglycollate elicidation. Female 7-week-old mice were injected intraperitoneally with 1.5 ml of 3% thioglycollate broth (Sifin, Berlin, Germany). After 3 days (C3H/HeN) or after 5 days (C3H/HeJ), mice were sacrificed and peritoneal macrophages were harvested by injection of 10 ml of ice-cold Hank's Balanced Salt Solution (Life Technologies) intraperitoneally followed by aspiration. Cells were washed twice with RPMI 1640, and 1×10^6 cells were plated in 12-well tissue culture plates in RPMI containing 5% FCS. At the next day, plates were washed twice with RPMI to remove non-adherent cells, and remaining cells were stimulated with *Treponema* glycolipids, LTAs, or *S. minnesota* LPS (Sigma) in RPMI containing 2% non-heat-inactivated FCS for 1 h. Nuclear extracts were prepared and EMSA was performed as described previously.

RESULTS

Induction of NF-κB in the Murine Macrophage RAW264.7 and CHO Cell Line by Treponema Glycolipids and LTAs from B. subtilis and S. aureus—First we examined the ability of glycolipids from *T. brennaborense* and *T. maltophilum*, LTAs from *B. subtilis* and *S. aureus*, and *E. coli* 0111:B4 LPS to induce nuclear translocation of NF-κB in different cell lines. We exposed the murine macrophage cell line RAW264.7 to increasing concentrations of different bacterial components for 1 h and subsequently subjected the nuclear proteins to EMSA. *Treponema* glycolipids, LTAs, and LPS caused NF-κB activation in RAW264.7 cells (Fig. 1). *T. brennaborense* exhibited a slightly increased ability to stimulate these cells as compared with *T. maltophilum*. A mock extract including all media and chemicals used during phenol/water extraction of treponemes failed to exhibit significant stimulation.

To examine the role of TLR-2 in responses to *Treponema* glycolipids and LTAs from *B. subtilis* and *S. aureus*, we used CHO cells transfected with CD14. These cells carry a frameshift mutation in the TLR-2 gene lacking a functional TLR-2 transcript (58). CHO/CD14 cells respond to exposure of increasing concentrations of LPS and *T. brennaborense* by enhanced translocation of NF-κB. In contrast, *T. maltophilum* and both LTAs failed to induce NF-κB even at higher concentrations (Fig. 1).

TLR-dependent Induction of Pro-inflammatory Cytokines by Treponema Glycolipids and LTAs—Next we addressed the influence of TLR-2 expression on cytokine induction by *Treponema* glycolipids and LTAs by employing the TLR-2-negative human astrocytoma cell line U373MG (33). IL-6 concentrations in the supernatants were measured after incubation of U373MG cells with different bacterial components. LPS and *T. brennaborense* were effective in inducing IL-6 production in a dose-dependent manner, whereas *T. maltophilum* caused only marginal IL-6 induction. Both LTAs completely failed to stimulate IL-6 production in U373MG cells (Fig. 2). In contrast, in the murine macrophage cell line RAW264.7, both *Treponema* glycolipids, LTAs, and LPS induced TNF-α production as expected (data not shown).

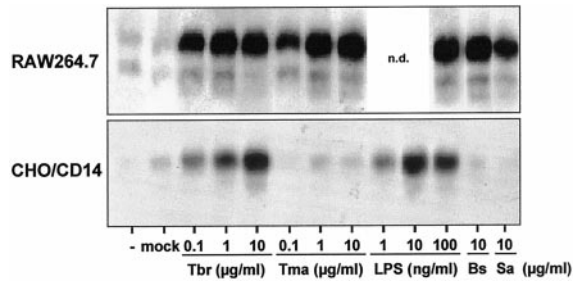

FIG. 1. **Glycolipids and LTAs induce NF-κB translocation in RAW264.7 and CHO/CD14 cells.** RAW264.7 and CHO/CD14 cells were stimulated with concentrations as indicated of glycolipids from *T. brennaborense* (*Tbr*) and *T. maltophilum* (*Tma*) and LTAs from *B. subtilis* (*Bs*) and *S. aureus* (*Sa*), in comparison to mock extract or *E. coli* 0111:B4 LPS in the presence of 2% non-heat-inactivated FCS. Cells were stimulated for 1 h and nuclear extracts were prepared as described under "Experimental Procedures." Extracts were incubated with a radiolabeled DNA probe containing a NF-κB binding site. NF-κB binding activity was determined by EMSA. One representative out of three experiments with similar results is shown (*n. d.*, not determined).

Induction of Pro-inflammatory Cytokines in RAW264.7 and U373MG Cells by Fractions of Treponema Phenol/Water Extracts—In order to further analyze the stimulatory capacity of *Treponema* glycolipids, they were further fractionated using hydrophobic interaction chromatography. Fractions obtained were tested regarding their ability to induce IL-6 in TLR-2-negative U373MG cells and TNF-α in RAW264.7 cells (Fig. 3). Fractions 40–60 from *T. brennaborense* exhibited TLR-2 dependence, indicated by their ability to stimulate RAW264.7 cells and their inability to stimulate U373MG cells for cytokine release (Fig. 3A). However, fractions 61–80 stimulated both cell lines, suggesting the existence of TLR-2-independent stimulatory molecules present in these fractions. Fractions 50–80 from *T. maltophilum* activated RAW264.7 cells, but completely failed to activate U373MG cells.

To confirm the different utilization pattern of TLRs by fractions of *T. brennaborense*, we investigated the influence of these components on NF-κB activation in CHO/CD14 and RAW264.7 cells. To this end we stimulated both cell lines with pooled fractions 40–60 and 61–80. In CHO/CD14 cells only fractions 61–80 induced NF-κB translocation, whereas fractions 40–60 failed to do so (Fig. 4). In RAW264.7 cells both groups of fractions, 40–60 and 61–80, were similarly able to induce NF-κB.

Effect of TLR-2 Overexpression on NF-κB Activation by Treponema Glycolipids and LTAs—To confirm the finding that glycolipids and LTAs stimulate cells via TLR-2, we performed overexpression experiments with the human embryonic kidney cell line HEK293. We transiently transfected HEK293 cells with a NF-κB-dependent ELAM promoter luciferase reporter construct. *Treponema* glycolipids and LTAs from *B. subtilis* and *S. aureus* caused no significant induction of reporter gene activity if a control vector was transfected only (Fig. 5, *A* and *B*). After transient transfection of TLR-2, *Treponema* phenol/water extracts and both LTAs were able to activate the reporter gene (Fig. 5, *A* and *B*).

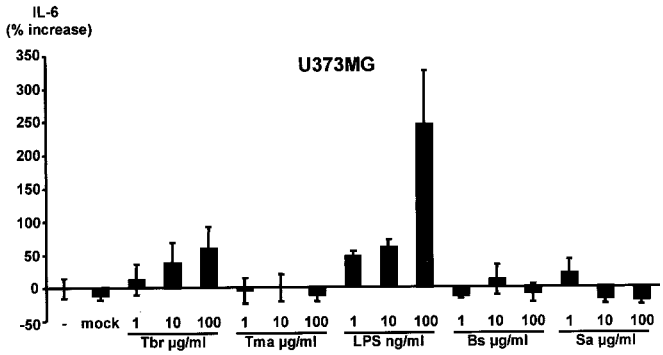

FIG. 2. **Induction of pro-inflammatory cytokines by glycolipids and LTAs.** U373MG cells were stimulated with increasing amounts of *Treponema* glycolipids (*Tbr* and *Tma*), LTAs (*S. aureus* (*Sa*) and *B. subtilis* (*Bs*)) or *E. coli* 0111:B4 LPS in comparison to mock extract. Concentrations of IL-6 in U373MG supernatants were measured by ELISA. Mean ± S.D. is shown for two representative experiments in which each stimulation was performed in three wells.

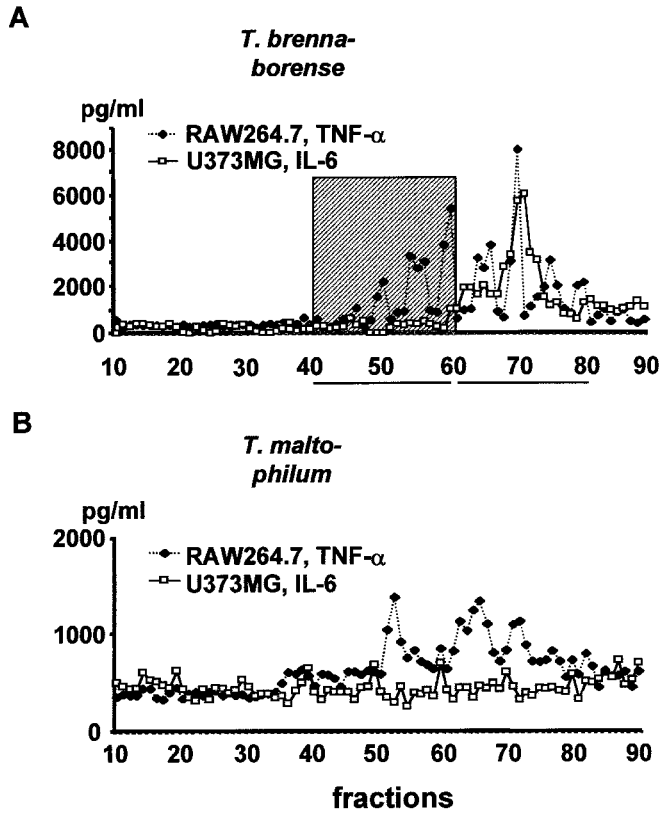

FIG. 3. **Purification of glycolipids from *T. brennaborense* and *T. maltophilum* by octyl-Sepharose column and induction of TNF-α and IL-6 by these fractions.** Treponema phenol/water extracts were fractionated on HIC as described under "Experimental Procedures." *T. brennaborense* (*A*) and *T. maltophilum* (*B*) fractions were collected and tested for their ability to induce IL-6 in U373MG cells and TNF-α in RAW264.7 cells. *T. brennaborense* fractions 40–60 strongly activated RAW264.7 cells, but failed to activate U373MG cells (*hatched area*).

Involvement of MyD88 and NIK in NF-κB Activation by Treponema Glycolipids and LTAs—Following our observation that TLRs are required for cell stimulation caused by *Treponema* glycolipids and LTAs, we investigated the involvement of two downstream signaling molecules of Toll-like receptors, MyD88 and NIK. Vectors containing cDNAs for expression of dominant-negative mutants of the adapter molecule MyD88 or NF-κB-inducing kinase NIK were used in transient cotransfection assays in HEK293 cells. Overexpression of both mutants

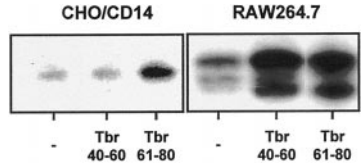

FIG. 4. **Induction of NF-κB translocation in CHO/CD14 and RAW264.7 cells by *T. brennaborense* fractions.** Indicated fractions of *T. brennaborense* were mixed and 2 μl were used to stimulate CHO/CD14 and RAW264.7 cells in the presence of 2% non-heat-inactivated FCS. After 1 h, nuclear extracts were prepared and EMSA was performed as described under "Experimental Procedures."

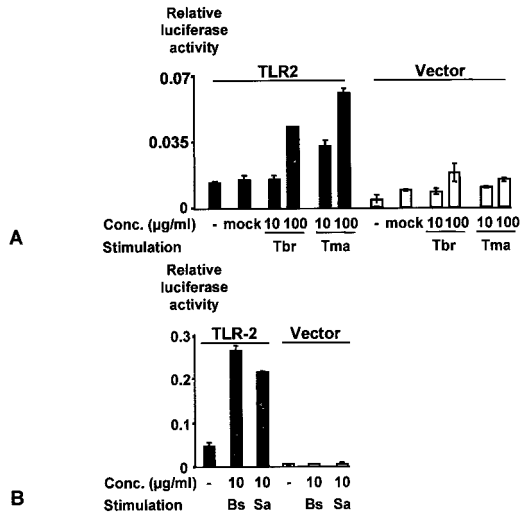

FIG. 5. **Effects of TLR-2 overexpression on NF-κB activation induced by *Treponema* glycolipids and LTAs.** HEK293 cells were transiently transfected with 0.2 μg of empty control vector or TLR-2 expression plasmid, 0.5 μg of NF-κB-dependent ELAM-1 luciferase reporter plasmid, and 0.5 μg of Rous sarcoma virus β-galactosidase plasmid. Cells were stimulated with the indicated concentrations of *Treponema* glycolipids (*Tbr* and *Tma*) (*A*) and LTAs (*B. subtilis* (*Bs*) and *S. aureus* (*Sa*)) (*B*) in the presence of 2% non-heat-inactivated FCS. After 6 h luciferase activities were obtained by luciferase assay and normalized with β-galactosidase activity. Data are shown as mean ± S.E. for one representative experiment of three, with transfection performed in duplicate. *Conc.*, concentration.

inhibited reporter gene activation by *T. maltophilum*, *T. brennaborense*, and LTAs, suggesting their involvement in TLR signaling pathway leading to activation of NF-κB (Fig. 6, *A* and *B*).

Induction of NF-κB by Treponema Glycolipids and LTAs in Peritoneal Macrophages Derived from C3H/HeJ and C3H/HeN Mice—The LPS hyporesponsive C3H/HeJ mice has been shown to bear a single amino acid mutation in the cytosolic domain of TLR-4 (19). In order to analyze a potential TLR-4 utilization in host cell stimulation by *Treponema* glycolipids or LTAs, peritoneal macrophages (PEM) from C3H/HeJ and C3H/HeN mice were stimulated for 1 h, and NF-κB nuclear translocation was assessed. *T. maltophilum* glycolipids led to a comparable induction of NF-κB nuclear translocation in C3H/HeJ and C3H/HeN PEM (Fig. 7). In contrast, *T. brennaborense* induced NF-κB activation in C3H/HeJ macrophages was weaker as compared with C3H/HeN cells, suggesting a TLR-4-dependent stimulatory activity. LTAs from *S. aureus* and *B. subtilis* exhibited a strong stimulatory activity in C3H/HeJ PEM. As expected, *S. minnesota* LPS failed to stimulate PEM derived from C3H/HeJ mice, but strongly induced NF-κB translocation in C3H/HeN cells.

Involvement of TLR-4 in RAW264.7 Cell Stimulation by Treponema Glycolipids and LTAs—We next examined the role of TLR-4 in RAW264.7 cell stimulation by using an inhibitory anti-TLR-4/MD-2 antibody. Therefore, we incubated RAW264.7

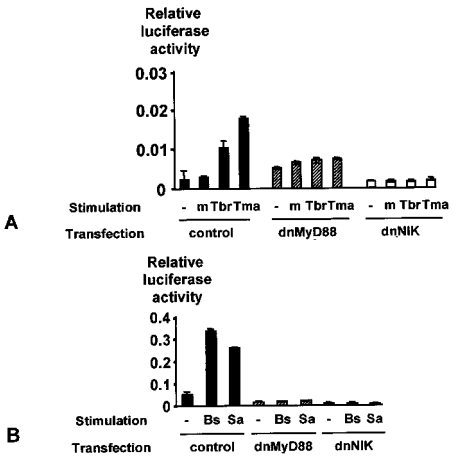

FIG. 6. **Involvement of MyD88 and NIK in NF-κB activation by glycolipids and LTAs.** HEK293 cells were transiently transfected with 0.2 μg of TLR-2 expression plasmid, 0.5 μg of NF-κB-dependent ELAM-1 luciferase reporter plasmid, 0.5 μg of Rous sarcoma virus β-galactosidase plasmid, and 0.2 μg of dominant-negative mutants of MyD88 or NIK. Cells were stimulated with 100 μg of *T. brennaborense* and 100 μg of *T. maltophilum* in comparison to a mock extract (*m*) (*A*) or with 10 μg of LTAs from *B. subtilis* (*Bs*) and *S. aureus* (*Sa*) in the presence of 2% non-heat-inactivated FCS (*B*). After 6 h luciferase activity was monitored as described under "Experimental Procedures" and normalized with β-galactosidase activity. Activity is shown as mean ± S.E. for one representative experiment of three, with transfection performed in duplicate.

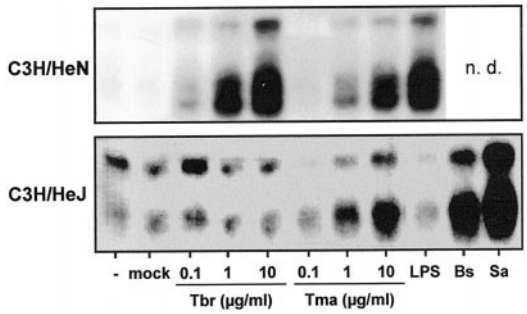

FIG. 7. **NF-κB activation in C3H/HeJ and C3H/HeN macrophages by glycolipids and LTAs.** Peritoneal macrophages from C3H/HeJ mice were stimulated with indicated concentrations of *Treponema* glycolipids, 10 μg/ml LTAs, and 10 ng/ml LPS, and NF-κB activity in the cellular nuclear extracts was determined by EMSA. In comparison, NF-κB activity in C3H/HeN macrophages induced by stimulation with glycolipids or LPS is shown (*n. d.*, not determined). *Bs*, *B. subtilis*; *Sa*, *S. aureus*.

macrophages with the TLR-4/MD-2 antibody MTS510 for 1 h prior to stimulation with glycolipids, LTAs, or *S. minnesota* LPS. RAW264.7 cells were exposed to these stimuli for 1 h, and subsequently nuclear proteins were subjected to EMSA. NF-κB activation caused by *T. brennaborense* and LPS was inhibited by the TLR-4/MD-2 antibody, indicating TLR-4 dependence (Fig. 8). In contrast, TLR-4/MD-2 antibodies failed to impair NF-κB translocation in RAW264.7 cells induced by *T. maltophilum* and LTAs. To further confirm TLR-4 involvement in signaling by *T. brennaborense*, the TLR-2-negative U373MG cell line was stimulated in the presence of an anti-human TLR-4 antibody (HTA125). As expected, blockade of TLR-4 by HTA125 led to inhibition of *T. brennaborense*-induced IL-6 release from U373MG cells (data not shown).

Induction of NF-κB in RAW264.7 Cells by Whole Treponemes—To determine the immunostimulatory potential and TLR utilization pattern of whole treponemes, a first set of experiments with whole bacteria was performed. We incubated RAW264.7 macrophages with *T. brennaborense* and *T. maltophilum* cells, as well as with LPS as control. Cells were pre-

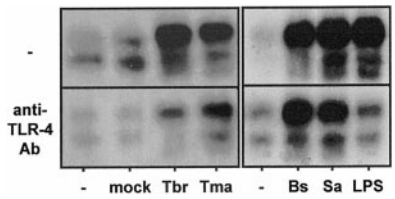

FIG. 8. **Effects of anti-TLR-4 Ab on NF-κB activation by glycolipids and LTAs.** RAW 264.7 cells were incubated with 5 μg/ml anti-TLR-4-MD-2 Ab (MTS510) for 1 h prior to stimulation with 1 μg/ml *Treponema* glycolipids (*Tbr* and *Tma*), 0.1 μg/ml *B. subtilis* LTA (*Bs*), *S. aureus* LTA (*Sa*), or 0.1 ng/ml *S. minnesota* LPS in the presence of 2% non-heat-inactivated FCS. Nuclear extracts were prepared and incubated with a specific probe containing NF-κB binding sites, and NF-κB activity was determined by electrophoretic mobility shift assay. One representative experiment of out of three with similar results is shown.

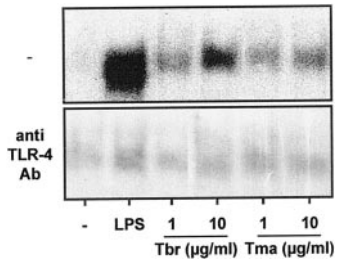

FIG. 9. **Treponeme cells induce NF-κB translocation in RAW264.7 macrophages.** RAW264.7 macrophages were pre-incubated with anti-TLR-4-MD-2 Ab (MTS510) for 1 h, as indicated, and stimulated with two concentrations of whole *T. brennaborense* and *T. maltophilum* cells, and 1 ng/ml *S. minnesota* LPS, in the presence of 2% non-heat-inactivated FCS. After 1 h, NF-κB translocation was measured by EMSA as described under "Experimental Procedures."

incubated with an inhibitory TLR-4/MD-2 antibody (MTS510, kindly provided by Dr. K. Miyake, Saga, Japan) for 1 h, as indicated. After stimulation, the NF-κB translocation was assessed by EMSA. Concentrations of 10 μg/ml *T. brennaborense* cells induced a pronounced NF-κB translocation in RAW264.7 cells (Fig. 9). The anti-TLR-4/MD-2 antibody inhibited the NF-κB induction by *T. brennaborense* cells and LPS, whereas the effect of the antibody on NF-κB translocation brought about by *T. maltophilum* cells was less pronounced.

DISCUSSION

NF-κB has been shown to be activated by several microbial ligands, such as LPS, *Treponema pallidum*, and *Borrelia burgdorferi* lipoproteins and peptidoglycan (59–61). We demonstrate here that whole treponemes, *Treponema* glycolipids, and butanol-extracted LTAs from *S. aureus* and *B. subtilis* are able to activate NF-κB in murine macrophages indicating their proinflammatory capacity. TLR-2 and -4 are members of the Toll-like receptor family that have recently been implicated in LTA signaling. Results obtained by employing different TLR-2-negative cell lines, macrophages from C3H/HeJ mice, and inhibitory anti-TLR-4 antibodies shown here revealed TLR-2 to be crucial for responses to LTAs and *T. maltophilum* glycolipids. In contrast, signaling induced by *T. brennaborense* glycolipids appears to be dependent on both TLR-2 and -4, confirming earlier results from C3H/HeJ and C3H/HeN mice (53).

The results indicating TLR-2 dependence of LTAs support earlier results from TLR-2 overexpression experiments in HEK293 cells (38), but are in contrast to earlier published data indicating TLR-4 dependence of LTAs (48). In this report commercially available LTAs were used, which were additionally purified by hydrophobic interaction chromatography, and which lacked *Limulus* activity (48). The reason for the different results may be that, in this report, peritoneal macrophages from mice were used and it is conceivable that mice TLRs differ

TABLE I
Comparison of Treponema glycolipids and LTAs

Characteristics	*T. brennaborense*	*T. maltophilum*	*B. subtilis*	*S. aureus*
Amount of fatty acids[a]	2.8%	8.3%	~10%	~10%
Repeating units[a]	30–40 repeats of ~5 sugars	~5 repeats of ~20–30 sugars	~25–35 glycerophosphates	~45–50 glycerophosphates
Alanine residues[a]	−	+	+	+
NF-κB in RAW264.7	+++	+++	+++	++
NF-κB in CHO/CD14	++	−	−	−
IL-6 in U373MG	++	−	−	−
NF-κB dep. luciferase in HEK293/TLR-2	+	++	+++	+++
NF-κB induction in C3H/HeN PEM	+++	++	ND[b]	ND
NF-κB induction in C3H/HeJ PEM	+	++	+++	++
NF-κB in RAW264.7 with anti-TLR-4 Ab	+	+++	+++	++
IL-6 in U373 with anti-TLR-4 Ab	−	ND	ND	ND

[a] Chemical analysis for treponemes (53), data for *B. subtilis* and *S. aureus* (43, 44, 49).
[b] ND, not determined.

in their binding activity from human or hamster TLRs. Hashimoto *et al.* (62) showed previously that only minor macromolecular glycolipids separated from *E. hirae* LTA possess cytokine-inducing activity, and that these glycolipids are dependent on both TLR-2 and -4. Controversial results may be due to different bacterial species used and different LTA extraction methods employed.

Furthermore, our results on TLR utilization obtained with purified glycolipids from two *Treponema* strains were confirmed by first experiments with whole treponemes. *T. brennaborense* cells displayed TLR-4 dependence, as shown by using an inhibitory anti-TLR-4 antibody, whereas this effect was less pronounced when using *T. maltophilum* bacteria instead. These results obtained with *T. maltophilum* cells are in line with published data from live *B. burgdorferi* and *T. pallidum* exhibiting TLR-2 dependence (34, 63). Similarly, as indicated by our results with *T. brennaborense* cells, live *Mycobacterium tuberculosis* also activate cells via TLR-2 and TLR-4 (39). In contrast to *M. tuberculosis* and *T. brennaborense* cells, live *Mycobacterium bovis* stimulate cells via TLR-2, but not TLR-4 (39). Our results obtained with whole *T. brennaborense* bacteria present evidence that lipoproteins, acting clearly via TLR-2 only (21), are not the only immuno-stimulatory activity in spirochaetes. Glycolipids acting via TLR-4 as well, as present in *T. brennaborense*, may represent an additional important compound for host-pathogen interaction.

The validity of TLR overexpression experiments in HEK293 cells has been questioned lately. First results leading to the observation of TLR-2 being a potential LPS receptor now have been contradicted by the fact that re-purification of the lipopolysaccharide used in these studies eliminated signaling through TLR-2 in HEK293 cells (17, 64). Several lines of evidence now clearly support the notion that TLR-4, and not TLR-2, is the signal transducer of LPS (65, 66). Regardless of these concerns, the HEK293 cell system still proves to be useful for functional analysis of microbial TLR ligands. In our studies applying TLR-2-transfected HEK293 cells, NF-κB-dependent ELAM luciferase activity was inducible by *Treponema* glycolipids and LTAs. Both LTAs and *T. maltophilum* activated these cells in a stronger fashion than *T. brennaborense*, underlining our findings with CHO/CD14 and U373MG cells. This assay was furthermore helpful in indicating the involvement of MyD88 and NIK in signaling by glycolipids and LTAs leading to activation of NF-κB.

Hydrophobic interaction chromatography has been established for fractionation LTAs according to their size of the hydrophilic chain (67). Fractionation of glycolipids from *T. brennaborense* and subsequent cell stimulation experiments revealed two different peaks of activity, one with a clear TLR-2-dependent activity and one exhibiting TLR-2 independence suggesting TLR-4 utilization.

Chemical analysis of phenol/water extracts from treponemes revealed the lack of components typically found in LPS such as D-*manno*-oct-2-ulosonic acid, heptose, and β-hydroxylated fatty acids (53). On the other hand, the cell membrane components isolated displayed LTA-like elements such as sugar, high phosphate, and alanine similar to that previously identified in *Treponema denticola* (68). This similarity was further supported by isolation and analysis of the dephosphorylated glycosyl part of the repeating units, which exhibited a low number of large repeats composing 20–30 sugars in *T. maltophilum* and a high number of small repeats containing ~5 sugars in *T. brennaborense*. Moreover, in *T. maltophilum* we were able to identify the lipid anchor composed of two monoacetylated diacylglycerols. These chemical data and the similar biological activities of *Treponema* phenol/water extracts and LTAs let us conclude that in *T. maltophilum* and *T. brennaborense* LTA-like glycolipids are the major membrane components responsible for the various biological effects observed in the extracts (Table I) and probably also in the whole cells.

T. maltophilum glycolipids and both LTAs exhibit no TLR-4-dependent activity; since both *Treponema* glycolipids were extracted in a similar way, a relevant endotoxin contamination of all bacterial components can be excluded (53). Little has been known about structural requirements for selective TLR-2 and -4 utilization. TLR-2 is known to be activated by many different ligands like peptidoglycan, bacterial lipoproteins, whole Gram-positive bacteria, and yeast (31–39). Here we show that all types of glycolipids and LTAs employed here are TLR-2 ligands. *T. brennaborense* exhibited additional TLR-4 dependence, confirming the previously reported hypothesis of broad ligand specificity for TLR-2 and a higher selectivity for TLR-4 (34, 35). Lipoproteins/lipopeptides from *B. burgdorferi*, *T. pallidum*, and *Mycoplasma fermentans* were found to activate cells via TLRs. All lipoproteins/lipopeptides activated TLR-2-expressing cells, and acylation of the spirochetal proteins was critical for their activation of TLR-2. It was thus hypothesized that amphipathicity may be important for TLR-2 signaling (34). In agreement with this hypothesis, *T. maltophilum* and both LTAs exhibiting TLR-2 dependence revealed higher fatty acid and less carbohydrate content in our chemical analysis as compared with the TLR-4 and -2 ligand *T. brennaborense*

(Table I). Furthermore, the glycerophosphate chain from *T. maltophilum*, *B. subtilis*, and *S. aureus* carries alanine residues, which have been described to lead to charge compensation (43, 44, 53, 69). On the other hand the more hydrophilic *T. brennaborense* fractions, generated in HIC, seem to stimulate cells exclusively via TLR-2, whereas the more hydrophobic *T. brennaborense* fractions seem to utilize TLR-4. Further structural analyses concerning the biological activity of the different LTAs led us to speculate whether differences in lipid anchors causing different TLR pattern, or possibly, a high number of smaller "repeating units" as found in *T. brennaborense* result in more "LPS-like" structure potentially responsible for a TLR-4 utilization. A more detailed chemical analysis currently performed in our laboratories will enable us to prove or disprove this interpretation. In summary, our data provide evidence that TLR-2 is the major receptor for *Treponema* glycolipids and LTAs. Additional TLR-4 involvement may depend on structural elements present in the glycolipids of *T. brennaborense*.

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REFERENCES

1. Ghosh, S., May, M. J., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
2. Baeuerle, P. A., and Baltimore, D. (1996) *Cell* **87,** 13–20
3. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) *Immunity* **7,** 837–847
4. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., and Janeway, C. A., Jr. (1998) *Mol. Cell* **2,** 253–258
5. Muzio, M., Ni, J., Feng, P., and Dixit, V. M. (1997) *Science* **278,** 1612–1615
6. Wesche, H., Gao, X., Li, X., Kirschning, C. J., Stark, G. R., and Cao, Z. (1999) *J. Biol. Chem.* **274,** 19403–19410
7. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) *Nature* **383,** 443–446
8. Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998) *J. Exp. Med.* **187,** 2097–2101
9. Malinin, N. L., Boldin, M. P., Kovalenko, A. V., and Wallach, D. (1997) *Nature* **385,** 540–544
10. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398,** 252–256
11. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) *Nature* **388,** 548–554
12. Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., Cao, Z., and Rothe, M. (1997) *Cell* **90,** 373–383
13. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Agou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93,** 1231–1240
14. Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999) *Science* **284,** 316–320
15. Gay, N. J., and Keith, F. J. (1991) *Nature* **351,** 355–356
16. Medzhitov, R., Preston-Hurlburt, P., and Janeway, C. A., Jr. (1997) *Nature* **388,** 394–397
17. Kirschning, C. J., Wesche, H., Merrill Ayres, T., and Rothe, M. (1998) *J. Exp. Med.* **188,** 2091–2097
18. Yang, R. B., Mark, M. R., Gray, A., Huang, A., Xie, M. H., Zhang, M., Goddard, A., Wood, W. I., Gurney, A. L., and Godowski, P. J. (1998) *Nature* **395,** 284–288
19. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282,** 2085–2088
20. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 115–122
21. Aderem, A., and Ulevitch, R. J. (2000) *Nature* **406,** 782–787
22. Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J. L., and Tong, L. (2000) *Nature* **408,** 111–115
23. Tauszig, S., Jouanguy, E., Hoffmann, J. A., and Imler, J. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10520–10525
24. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996) *Cell* **86,** 973–983
25. Williams, M. J., Rodriguez, A., Kimbrell, D. A., and Eldon, E. D. (1997) *EMBO J.* **16,** 6120–6130
26. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 588–593
27. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752
28. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189,** 1777–1782
29. Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) *J. Exp. Med.* **189,** 615–625
30. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., and Miyake, K. (2000) *J. Immunol.* **164,** 3471–3475
31. Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Bleharski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J., and Modlin, R. L. (1999) *Science* **285,** 732–736
32. Aliprantis, A. O., Yang, R. B., Mark, M. R., Suggett, S., Devaux, B., Radolf, J. D., Klimpel, G. R., Godowski, P., and Zychlinsky, A. (1999) *Science* **285,** 736–739
33. Hirschfeld, M., Kirschning, C. J., Schwandner, R., Wesche, H., Weis, J. H., Wooten, R. M., and Weis, J. J. (1999) *J. Immunol.* **163,** 2382–2386
34. Lien, E., Sellati, T. J., Yoshimura, A., Flo, T. H., Rawadi, G., Finberg, R. W., Carroll, J. D., Espevik, T., Ingalls, R. R., Radolf, J. D., and Golenbock, D. T. (1999) *J. Biol. Chem.* **274,** 33419–33425
35. Underhill, D. M., Ozinsky, A., Smith, K. D., and Aderem, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14459–14463
36. Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R., and Golenbock, D. (1999) *J. Immunol.* **163,** 1–5
37. Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M., and Aderem, A. (1999) *Nature* **401,** 811–815
38. Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999) *J. Biol. Chem.* **274,** 17406–17409
39. Means, T. K., Wang, S., Lien, E., Yoshimura, A., Golenbock, D. T., and Fenton, M. J. (1999) *J. Immunol.* **163,** 3920–3927
40. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13766–13771
41. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) *Nature* **408,** 740–745
42. Lambert, P. A., Hancock, I. C., and Baddiley, J. (1977) *Biochim. Biophys. Acta* **472,** 1–12
43. Iwasaki, H., Shimada, A., Yokoyama, K., and Ito, E. (1989) *J. Bacteriol.* **171,** 424–429
44. Polotsky, V. Y., Fischer, W., Ezekowitz, R. A., and Joiner, K. A. (1996) *Infect. Immun.* **64,** 380–383
45. Fischer, W., and Rosel, P. (1980) *FEBS Lett.* **119,** 224–226
46. Bhakdi, S., Klonisch, T., Nuber, P., and Fischer, W. (1991) *Infect. Immun.* **59,** 4614–4620
47. De Kimpe, S. J., Kengatharan, M., Thiemermann, C., and Vane, J. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10359–10363
48. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 443–451
49. Morath, S., Geyer, A., and Hartung, T. (2001) *J. Exp. Med.* **193,** 393–398
50. Choi, B. K., Paster, B. J., Dewhirst, F. E., and Göbel, U. B. (1994) *Infect. Immun.* **62,** 1889–1895
51. Wyss, C., Choi, B. K., Schupbach, P., Guggenheim, B., and Göbel, U. B. (1996) *Int. J. Syst. Bacteriol.* **46,** 745–752
52. Schrank, K., Choi, B. K., Grund, S., Moter, A., Heuner, K., Nattermann, H., and Göbel, U. B. (1999) *Int. J. Syst. Bacteriol.* **49,** 43–50
53. Schröder, N. W. J., Opitz, B., Lamping, N., Michelsen, K. S., Zähringer, U., Göbel, U. B., and Schumann, R. R. (2000) *J. Immunol.* **165,** 2683–2693
54. Westphal, O., and Jann, K. (1965) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., ed) 5th Ed., p. 83, Academic Press, New York
55. Schnittker, H., Papenberg and E. Ganse. (1959) *Biochem. Z.* **332,** 167–185
56. Schumann, R. R., Pfeil, D., Freyer, D., Buerger, W., Lamping, N., Kirschning, C. J., Göbel, U. B., and Weber, J. R. (1998) *Glia* **22,** 295–305
57. Akashi, S., Ogata, H., Kirikae, F., Kirikae, T., Kawasaki, K., Nishijima, M., Shimazu, R., Nagai, Y., Fukudome, K., Kimoto, M., and Miyake, K. (2000) *Biochem. Biophys. Res. Commun.* **268,** 172–177
58. Heine, H., Kirschning, C. J., Lien, E., Monks, B. G., Rothe, M., and Golenbock, D. T. (1999) *J. Immunol.* **162,** 6971–6975
59. Müller, J. M., Ziegler-Heitbrock, H. W., and Baeuerle, P. A. (1993) *Immunobiology* **187,** 233–256
60. Gupta, D., Kirkland, T. N., Viriyakosol, S., and Dziarski, R. (1996) *J. Biol. Chem.* **271,** 23310–23316
61. Norgard, M. V., Arndt, L. L., Akins, D. R., Curetty, L. L., Harrich, D. A., and Radolf, J. D. (1996) *Infect. Immun.* **64,** 3845–3852
62. Hashimoto, M., Imamura, Y., Morichika, T., Arimoto, K., Takeuchi, O., Takeda, K., Akira, S., Aoyama, K., Tamura, T., Kotani, S., Suda, Y., and Kusumoto, S. (2000) *Biochem. Biophys. Res. Commun.* **273,** 164–169
63. Sellati, T. J., Bouis, D. A., Caimano, M. J., Feulner, J. A., Ayers, C., Lien, E., and Radolf, J. D. (1999) *J. Immunol.* **163,** 2049–2056
64. Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. (2000) *J. Immunol.* **165,** 618–622
65. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) *J. Biol. Chem.* **274,** 10689–10692
66. Yang, H., Young, D. W., Gusovsky, F., and Chow, J. C. (2000) *J. Biol. Chem.* **275,** 20861–6
67. Leopold, K., and Fischer, W. (1992) *Anal. Biochem.* **201,** 350–355
68. Schultz, C. P., Wolf, V., Lange, R., Mertens, E., Wecke, J., Naumann, D., and Zähringer, U. (1998) *J. Biol. Chem.* **273,** 15661–15666
69. Greenberg, J. W., Fischer, W., and Joiner, K. A. (1996) *Infect. Immun.* **64,** 3318–3325