THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 13, Issue of March 30, pp. 9713–9719, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Activation of Mitogen-activated Protein Kinases p42/44, p38, and Stress-activated Protein Kinases in Myelo-monocytic Cells by *Treponema* Lipoteichoic Acid*

Received for publication, October 2, 2000, and in revised form, December 20, 2000
Published, JBC Papers in Press, December 28, 2000, DOI 10.1074/jbc.M008954200

Nicolas W. J. Schröder‡, Dagmar Pfeil‡, Bastian Opitz‡, Kathrin S. Michelsen‡§, Johannes Amberger‡, Ulrich Zähringer¶, Ulf B. Göbel‡, and Ralf R. Schumann‡‖

From the ‡Institut für Mikrobiologie und Hygiene, Universitätsklinikum "Charité," Medizinische Fakultät der Humboldt-Universität zu Berlin, Dorotheenstrasse 96, D-10117 Berlin, Germany and the ¶Labor für Immunchemie, Forschungszentrum Borstel, Parkallee 22, D-23845 Borstel, Germany

We have shown previously that phenol/water extracts derived from two novel *Treponema* species, *Treponema maltophilum*, and *Treponema brennaborense*, resembling lipoteichoic acid (LTA), induce cytokines in mononuclear cells. This response was lipopolysaccharide binding-protein (LBP)-dependent and involved Toll-like receptors (TLRs). Here we show that secretion of tumor necrosis factor-α induced by *Treponema* culture supernatants and extracted LTA was paralleled by an LBP-dependent phosphorylation of mitogen-activated protein kinases (MAPKs) p42 and p44, and p38, as well as the stress-activated protein kinases c-Jun N-terminal kinases 1 and 2. Phosphorylation of p42/44 correlated with an increase of activity, and tumor necrosis factor-α levels were significantly reduced by addition of inhibitors of p42/44 and p38, PD 98059 and SB 203580, respectively. Treponeme LTA differed from bacterial lipopolysaccharide regarding time course of p42/44 phosphorylation, exhibiting a prolonged activation of MAPKs. Furthermore, MAPK activation and cytokine induction failed to be strictly correlated. Involvement of TLR-4 for phosphorylation of p42/44 was shown employing the neutralizing anti-murine TLR-4 antibody MTS 510. In TLR-2-negative U373 cells, the compounds studied differed regarding MAPK activation with *T. maltophilum* leading to a stronger activation. In summary, the data presented here show that treponeme LTA are able to activate the MAPK and stress-activated protein kinase pathway involving LBP and TLR-4.

Spirochetes are involved in a number of chronic inflammatory diseases, *i.e. Treponema pallidum* causing syphilis and *Borrelia burgdorferi* causing Lyme disease. These diseases are characterized by certain inflammatory reactions of the host evoked by the pathogens (1, 2). A possible explanation for these findings is the presence of cell wall compounds, which, after being released by the bacteria, induce the secretion of pro-inflammatory cytokines by host cells, such as macrophages (3). Such pathways are well established for a range of bacterial compounds, including lipopolysaccharide (LPS)[1] of Gram-negative bacteria, or LTA and peptidoglycan of Gram-positive bacteria (4–7). Lipoglycans isolated from spirochetes have been found to be chemically distinct from LPS (8, 9), and the recent completion of the genome analysis of *T. pallidum* revealed the absence of any known LPS synthesis genes (10). Other investigators implicated outer membrane lipoproteins of *T. pallidum* and *B. burgdorferi* to be responsible for activation of host cells (11–13). During the last years, it has been shown by others and ourselves that certain treponeme species are associated with periodontitis (14–19). This chronic inflammatory disease is characterized by an inflammatory reaction followed by extensive loss of tissue. Macrophages are assumed to be responsible for the inflammatory reactions seen in the host (20), and cell wall compounds released by spirochetes have been shown to mediate the production of pro-inflammatory cytokines, *i.e.* TNF-α (12, 21).

In previous studies we have investigated the biological characteristics of phenol/water preparations of the outer membrane of *T. maltophilum* (TM), a spirochete found in periodontal lesions, and *Treponema brennaborense* (TB), a spirochete associated with dermatitis digitalis in cattle, a chronic inflammation of the heels associated with cachexia (17). We were able to chemically characterize these preparations by gas liquid chromatography-mass spectroscopy as lipoglycans due to the presence of a diacylglycerol lipid anchor in TM, and the presence of repeating carbohydrate units in both strains (22), resembling LTA of Gram-positive bacteria. These data are in agreement with a recent study analyzing outer membrane lipoglycans of *Treponema denticola* (23). We could show that these compounds, which are released by bacteria during cell growth, are able to elicit strong cytokine release by myelo-monocytic cells involving CD14, LBP, and the Toll-like receptor (TLR) family (22). This pathway resembles that known for LPS of Gram-negative bacteria (4, 24–26), but chemical analysis of the treponeme preparations revealed a structure being clearly distinct from LPS (22).

* This work was supported in part by Deutsche Forschungsgemeinschaft Grant Schu 828/1-5 (to R. R. S.), and by Bundesministerium für Bildung und Forschung Grants 01 KI 94750 (to R. R. S.) and 01/KI 9318 (to U. B. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Boehringer Ingelheim Foundation.

‖ To whom correspondence and reprint requests should be addressed. Tel.: 49-30-2093-4747; Fax: 49-30-2093-4704; E-mail: ralf.schumann@charite.de.

[1] The abbreviations used are: LPS, lipopolysaccharide; ERK, extracellular stress-related kinase; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; LBP, lipopolysaccharide-binding protein; LTA, lipoteichoic acid; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular stress-related kinase kinase; NF-κB, nuclear factor κB; OMIZ-Pat, treponeme culture medium; rmLBP, recombinant murine LBP; SAPK, stress-activated protein kinase; TB, *Treponema brennaborense*; TLR, Toll-like receptor; TM, *Treponema maltophilum*; TNF, tumor necrosis factor; Ab, antibody; mAb, monoclonal antibody; GST, glutathione *S*-transferase; FCS, fetal calf serum; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

The aim of this study was to elucidate the signaling pathways leading to cytokine induction in myelo-monocytic cells caused by treponeme LTA. It is well established that activation of macrophages caused by LPS is mediated by LBP, which transfers it to its cellular receptor consisting of CD14, TLR-4, and the MD-2 molecule (27–29). LPS apparently initiates signaling via TLR-4 (30–32), while peptidoglycan, as well as bacterial lipoproteins, and lipoarabinomannan initiate immune responses via TLR-2 (33–36). It has remained controversial, however, whether LTA stimulates cell via TLR-2 or -4 (37, 38). Downstream signaling elements activated by LPS include Ras (39, 40), which, as well as protein kinase C, leads to activation of the protein kinase Raf1 (39, 41–45), which in turn activates mitogen-activated protein kinase (MAPK) kinases. MAPKs p42 and p44 also referred to as "extracellular stress-related kinase" (ERK)-1/ERK-2 are activated by MAPK/ERK kinase 1 (MEK1) (46–49). p38, a kinase with high homology to high osmolarity glycerol response protein 1 of *Saccharomyces cerevisiae* (50–53), has been shown to be activated in response to LPS (54), mediated by a MAPK kinase homologue, MAPK kinase 3 (55). Another family of proteins closely related to MAPKs are the stress-activated protein kinases (SAPKs), a member of which is the c-Jun N-terminal kinases (JNKs). They are involved in a range of cellular responses, including UV irradiation (56), as well as LPS activation (57). These three groups of kinases lead to cytokine production by activating a number of transcription factors, including c-Jun (activated by JNK), ATF 2 (activated by p38 and JNK), and Elk-1 (activated by p42/44) (58–60).

In this study, we show that treponeme LTA share the ability of parallel activation of multiple tyrosine-kinase cascades with LPS. Furthermore, involvement of TLR-2 and -4 in activation of these pathways, employing the neutralizing anti-murine TLR-4 antibody MTS 510 and the TLR-2-negative human astrocytoma cell line U373, was investigated.

EXPERIMENTAL PROCEDURES

Treponeme Culture and Processing of Culture Supernatants—Frozen stocks of a suspension of TB and TM (300 μl, stored at −80 °C) were inoculated in 3 ml of a culture medium (OMIZ-Pat) as described previously (19) and cultured under anaerobic conditions (Anaerogen, Oxoid, Germany) at 37 °C for 3–4 days. The cultures were then transferred to 20–100 ml of OMIZ-Pat and incubated for another 1–2 days. For phenol/water extraction of whole cells, these cultures were transferred to 500 ml of OMIZ-Pat. Viability of the treponemes and the exclusion of contaminating bacteria were assessed by dark field microscopy (400-fold magnification; BH2-RFCA microscope, Olympus, Hamburg, Germany). Sterility controls of the medium preparation 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, and the cultures were stopped at pH 6.0. The protein concentration of the supernatants was determined employing the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Concentrations ranged from 1.2 to 1.5 μg/ml (TB), and from 0.4 to 0.6 μg/ml (TM), respectively. Cultures were centrifuged at 12,000 × *g* for 20 min at 4 °C and passed through 0.2-μm sterile filters (Schleicher & Schuell, Dassel, Germany). For some studies, culture supernatants were heat-inactivated at 100 °C for 20 min and passed again through 0.2-μm sterile filters. OMIZ-Pat medium, treated similarly, served as control.

Extraction of Whole Treponema Cells—For an extraction of treponeme whole cells, aqueous suspensions of treponeme cells were digested with RNase (Sigma, Deisenhofen, Germany), DNase (Merck, Darmstadt, Germany), and proteinase K (Merck). These suspensions were dialyzed and further extracted employing a hot phenol/water extraction method (61). 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 3000 × *g* for 10 min at 0 °C, and the upper phase was collected. This procedure was repeated twice; combined phases were dialyzed and lyophilized. A mock extract, including all enzymes and chemicals used, but no bacteria, was also prepared.

Stimulation of the Murine Macrophage Cell Line RAW 264.7 and the Human Astrocytoma Cell Line U373—To assess induction of TNF-α, 5×10^4 cells/well of RAW 264.7 cells were cultured overnight in 96-well tissue culture plates using RPMI 1640 containing 10% FCS. After two washing steps with RPMI 1640, stimulation was performed in the absence or presence of 1 μg/ml recombinant murine LBP in a total volume of 100 μl. Supernatants were harvested after 4 h of incubation, and cells were stained with trypan blue. For some experiments, cells were incubated with the MEK inhibitor PD 98059 (Calbiochem, Schwalbach, Germany), or the p38 inhibitor SB 203580 (Alexis, Läufingen, Switzerland) at 37 °C for 1 h prior to stimulation. In other experiments, as indicated, the inhibitory monoclonal anti-TLR-4 antibody MTS510 (62), kindly provided by Dr. Miyake (Saga, Japan), was added 30 min prior to stimulation. To investigate phosphorylation and activity of tyrosine kinases, 1.6×10^6 cells/well were cultured in six-well tissue culture plates over night in RPMI 1640 containing 10% FCS. After two washing steps with RPMI 1640, cells were starved for 3 h in the absence of serum. Stimulation was done in the absence or presence of 1 μg/ml rmLBP. After 20 min, supernatants were removed and cells were washed three times with ice-cold PBS containing 1 mM Na_3VO_4, followed by addition of 150 μl of lysis buffer, containing 1% Triton X-100 (Roth, Karlsruhe, Germany), 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethanesulfonyl fluoride, 0.2 mM leupeptin, and 0.15 unit/ml aprotinin. After 15 min of incubation at 4 °C, cells were scraped off the plates and centrifuged for 30 min at 12,000 × *g* at 4 °C. The postmitochondrial supernatant was recovered, protein content was determined employing the Bio-Rad protein assay, and the remaining lysates were stored at −80 °C. For some experiments cells were stimulated for a longer period, as indicated.

The previously described lack of TLR-2 on U373 cells (34) was confirmed in our laboratory by reverse transcription-polymerase chain reaction. For stimulation experiments, U373 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Eggenstein, Germany) containing 10% FCS. For studies on activation of p42/44, 3×10^5 cells/well or 6×10^4 cells/well were cultured overnight in 6- or 24-well tissue culture plates, respectively. Prior to stimulation, cells were starved in Dulbecco's modified Eagle's medium without FCS for 2 h, followed by stimulation and lysis as described above. For studies on interleukin-6 (IL-6) secretion, 1×10^4 cells/well were cultured overnight in 96-well tissue culture plates at 37 °C followed by stimulation. After incubating for 24 h, supernatants were assayed for IL-6 content as described below.

Detection of Murine TNF-α and Human Interleukin-6 (IL-6)—For detection of murine TNF-α, MaxiSorp enzyme-linked immunosorbent assay plates were coated with 3 μg/ml anti-mTNF-α Ab (PharMingen, Heidelberg, Germany) in 100 mM Na_3PO_4, pH 6.0. Samples and recombinant mTNF-α standard (R&D Systems, Wiesbaden, Germany) were incubated at room temperature for 3 h, and detection was performed employing a biotin-conjugated anti mTNF-α Ab (PharMingen), and streptavidin-peroxidase with *ortho*-phenylene diphosphate as substrate. The detection limit was ~15 pg/ml. For quantification of IL-6 plates were coated with anti-human IL-6 antibody (R & D Systems). Samples and recombinant human IL-6 (R & D Systems) were detected employing a biotin-labeled monoclonal anti-human IL-6 antibody (R & D Systems). The detection limit was 16 pg/ml. Shown are mean values ± S.D.

Anti-phosphotyrosine Immunoblotting—Samples of 50 μg/ml protein were mixed with sample buffer to obtain a final concentration of 50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, 10% glycerol, and 0.1% bromphenol blue, and heated for 5 min at 95 °C. Samples were loaded onto 12% SDS-polyacrylamide gels, and separated employing a Tris buffer system. After electrophoresis, gels were immersed in transfer buffer containing 25 mM Tris-HCl, 0.2 M glycine, and 20% methanol, and transferred to Hybond-C extra membranes (Amersham Pharmacia Biotech, Braunschweig, Germany) by semidry blotting (Hölzel GmbH, Dorfen, Germany). For detection of phosphorylated p42/44 and p38 (pp42/44, pp38) membranes were blocked with PBS containing 5% skim milk and 0.1% Tween 20. After washing, blots were incubated either with rabbit anti-pp38 mAb (Santa Cruz, Heidelberg, Germany) or rabbit anti-pp42/44 mAb (New England Biolabs, Schwalbach, Germany), diluted 1:1000 in PBS, 5% bovine serum albumin (BSA), 0.05% Tween 20 for 3 h. After washing, membranes were further incubated with goat anti-rabbit Ab, conjugated with horseradish peroxidase (Biogenes, Berlin, Germany), diluted 1:5000 in PBS, 5% BSA), 0.05% Tween 20 for 90 min. After a final washing step, blots were detected employing the ECL-system (Amersham Pharmacia Biotech) according to the manufacturer's protocol, and visualized on Hyperfilm ECL-films (Amersham Pharmacia Biotech). Membranes were stripped

by incubation with 0.1 M glycine, 0.1 M NaCl, pH 2.5, for 30 min at room temperature, and reprobed. For detection of phosphorylated JNK, membranes were incubated with a mouse mAb directed against phosphorylated JNK1 and JNK2 (Santa Cruz) diluted 1:100 in Tris-buffered saline containing 1% BSA, 1% skim milk, and 0.05% Tween 20, for 3 h. Blocking of membranes and incubation with a goat anti-mouse IgG Ab conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) diluted 1:1000, for 90 min, was achieved applying the same buffer. Blots were visualized as described above employing the ECL system.

Estimation of p42/44 Activity—Cell lysates were investigated for p42/44 activity using the Biotrak system (Amersham Pharmacia Biotech). In brief, cell lysates were incubated with a peptide derived from epidermal growth factor receptor highly selective for p42/44, in the presence of 50 mM [γ-^{33}P]ATP (3, 7 $\times$ 10^3 Bq/test) for 30 min at 36 °C. After separating the peptide from unincorporated activity, incorporated ATP was measured in a scintillation counter. Shown are mean values ± S.D.

Expression and Purification of mLBP—Murine LBP was expressed in a baculovirus system as described (63). Briefly, the murine LBP-cDNA was inserted into the baculovirus expression vector pAcGHLT-B (PharMingen) containing the baculovirus polyhedrin promoter for high level expression in insect cells, and the glutathione *S*-transferase (GST) gene for purification of the resulting fusion protein. Sf-9 insect cell culture, virus amplification, and expression of recombinant murine LBP (rmLBP)-GST fusion protein were performed according to the manufacturer's protocol. Cell lysates were incubated with GST-Sepharose 4B (Amersham Pharmacia Biotech), and bound fusion protein was incubated with thrombin (ICN Biochemicals, Eschwege, Germany) to release mLBP.

RESULTS

Treponema Culture Supernatants Cause Phosphorylation of p42/44, p38, and JNK1/2—In previous studies, we have demonstrated that lipoglycans from TB and TM are released by treponemes during cell growth. Culture supernatants exhibited a similar stimulation pattern in comparison to phenol/water extracts of whole cells. To assess whether the stimulation observed involves phosphorylation of MAPKs, we incubated RAW 264.7 cells with the supernatants and analyzed the cell lysates for phosphorylation of these kinases. We observed an LBP-dependent phosphorylation and activation of p42/44 (Fig. 1, *A* and *B*), as well as phosphorylation of p38 (Fig. 2*A*). Involvement of the SAPK pathway was analyzed by assessment of JNK1/2 phosphorylation (Fig. 2*B*). Here also, an LBP-dependent phosphorylation induced by the culture supernatants was observed. However, phosphorylation caused by TM-derived culture supernatants was clearly stronger as compared with TB, although TNF-α induction was similar. Activity of p42/44 was related to the state of phosphorylation as revealed by the Biotrak p42/44 activity assay (Fig. 1*A*).

Treponema LTA Extracted from Whole Cells Reveals a Similar Phosphorylation Pattern—Next we performed experiments with LTA extracted from whole treponeme cells employing a hot phenol/water method. Cells were stimulated either with treponeme compounds or with LPS in the presence or absence of recombinant murine LBP for 20 min. Immunoblotting of the cell lysates revealed a strong phosphorylation of p42/44 caused by both treponeme LTA and LPS. These reactions were clearly LBP-dependent (Fig. 3*B*). The lysates were also assayed for their state of activation employing the Biotrak p42/44 system, showing a clear correlation between phosphorylation and activation of the investigated kinases (Fig. 3*A*). For p38 and JNK, lysates of RAW 264.7 stimulated as described were investigated for phosphorylation revealing a strong, LBP-dependent phosphorylation of p38 (Fig. 4*A*) as well as JNK1 and JNK2 (Fig. 4*B*) upon stimulation by treponeme extracts. However, in these experiments, the level of phosphorylation and activation of kinases failed to differ between the two strains. In all experiments performed, the "mock" extract did not cause phosphorylation of the kinases investigated.

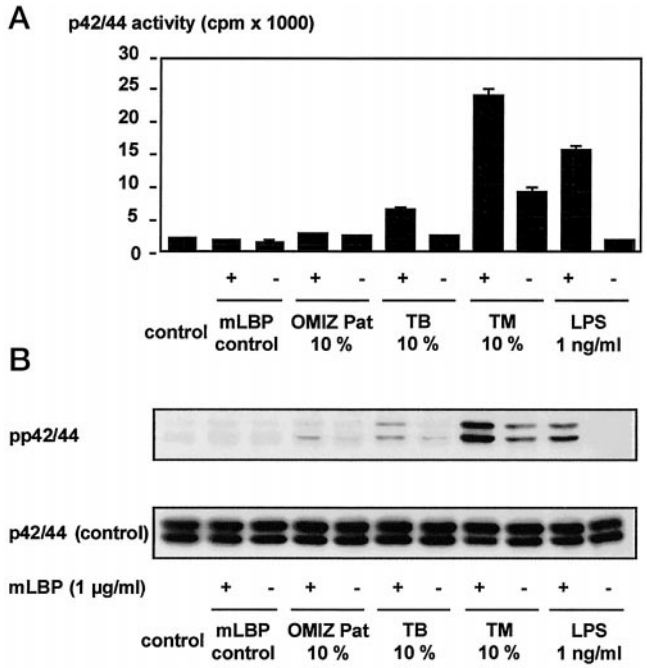

FIG. 1. **Phosphorylation and activation of p42/44 by treponeme culture supernatants.** RAW 264.7 cells were stimulated with 10% treponeme culture supernatants or 10 ng/ml LPS (*Escherichia coli* 0111:B4) for 20 min in the presence or absence of rmLBP. Cells were lysed, and 5 μg of the lysates were assayed for p42/44 activity employing a Biotrak kinase activity assay (*A*). Counts per minute (cpm) on the *y* axis represent p42/44 activity. This assay was performed in triplicate. 50 μg of protein were further analyzed by immunoblotting employing an anti-phospho-p42/44 Ab (*B*). Blots were stripped and detected with an antibody directed against unphosphorylated p42 and p44 as a loading control. Shown is one representative experiment of four with similar results.

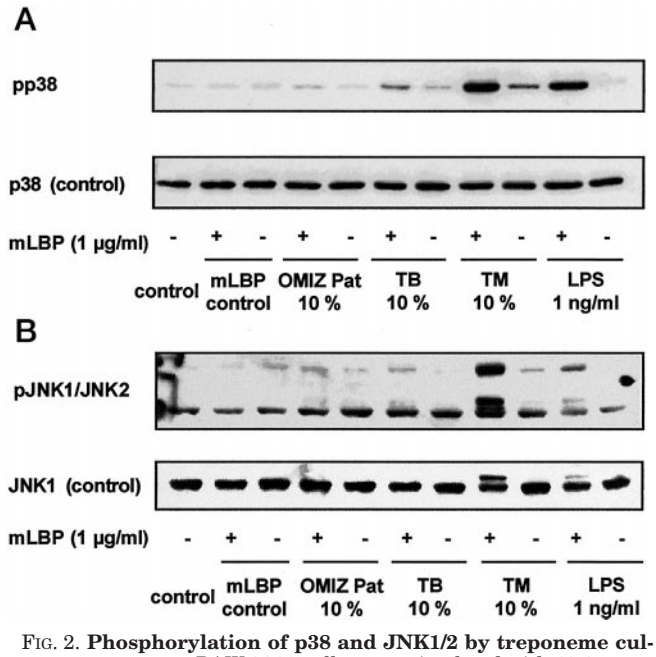

FIG. 2. **Phosphorylation of p38 and JNK1/2 by treponeme culture supernatants.** RAW 264.7 cells were stimulated with 10% treponeme culture supernatants, 10% culture medium alone, or 10 ng/ml LPS (*E. coli 0111:B4*) for 20 min with or without rmLBP as indicated. Cell lysates (50 μg for p38, 100 μg for JNK1/2) were blotted, and detection of phosphorylated p38 (*A*) and JNK1/2 (*B*) was performed employing antibodies against their phosphorylated isoforms as described under "Experimental Procedures." Antibodies against unphosphorylated p38 and JNK1/2 served as loading controls. Shown is one representative of two experiments.

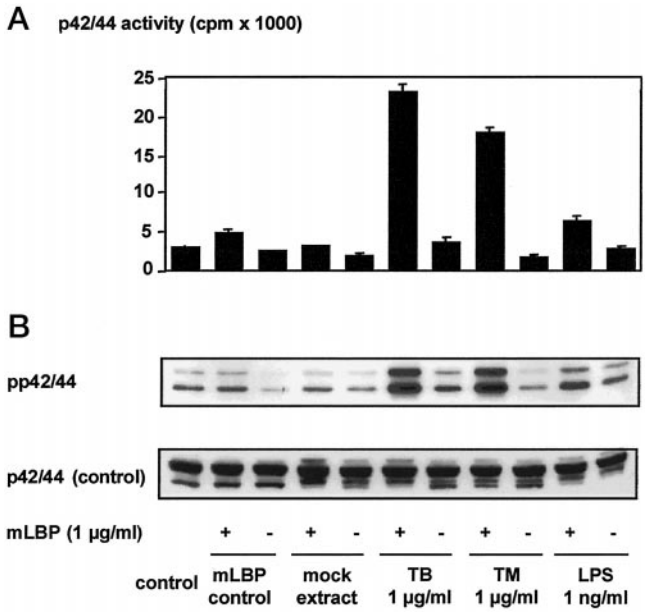

FIG. 3. ***Treponema* LTA derived from whole cells induces phosphorylation and activation of MAPKs p42/44.** RAW 264.7 cells were stimulated with LTA derived from *T. brennaborense* or *T. maltophilum* (1 μg/ml), with a mock extract, or with LPS (*E. coli* 0111:B4, 10 ng/ml) for 20 min. rmLBP was added at a concentration of 1 μg/ml as indicated. Phosphorylation of p42/44 (*B*) was assessed via immunoblotting of 50 μg of protein derived from cell lysates. Shown is one representative experiments out of four. Activation of p42/44 was assessed using 5 μg of protein derived from the same lysates (*A*). The assay was performed in triplicate. Shown is one representative of four experiments.

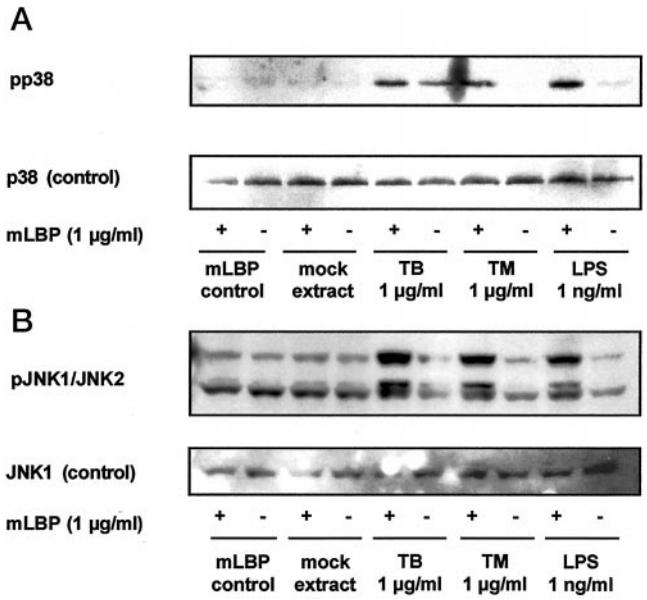

FIG. 4. **Phosphorylation of p38 and JNK1/2 by treponeme LTA.** RAW 264.7 cells were stimulated with treponeme LTA (1 μg/ml), mock extract, or LPS (*E. coli 0111:B4*, 10 ng/ml) for 20 min with or without rmLBP. Cell lysates (50 μg for p38, 100 μg for JNK1/2) were blotted, and detection of phosphorylated p38 (*A*) and JNK1/2 (*B*) was performed employing antibodies against their phosphorylated isoforms. Antibodies against unphosphorylated p38 and JNK1/2 served as a loading control. Shown is one representative of two experiments.

Dose Response and Kinetics of Phosphorylation of MAPKs Caused by Treponeme LTA and LPS in Comparison to TNF-α Release—In previous studies treponeme phenol/water extracts were found to be less potent regarding cytokine induction as

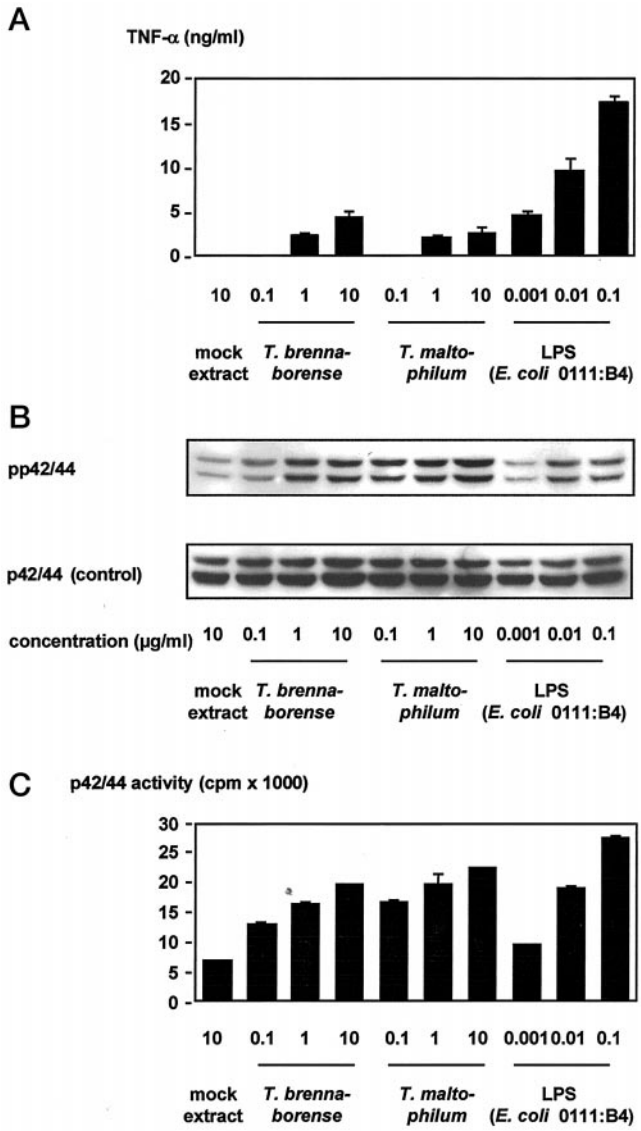

FIG. 5. **Dose-dependent phosphorylation of p42/44 and TNF-α release caused by treponeme LTA and LPS.** RAW 264.7 cells were stimulated with increasing concentrations of LTA derived from *T. brennaborense*, *T. maltophilum*, mock extract, or 100 ng/ml LPS (*E. coli* 0111:B4) in the presence of rmLBP (1 μg/ml). Cell lysates (5 μg of protein) were assayed for activation of p42/44 (*A*). This assay was performed in duplicate. 50 μg of lysates were examined for phosphorylation of MAPKs (*B*); shown is one representative of two experiments. In parallel, RAW 264.7 cells were stimulated with the same compounds for 4 h and TNF-α content of the supernatants was assessed as described under "Experimental Procedures" (*C*). Shown is one representative of three experiments.

compared with LPS. To obtain TNF-α concentrations comparable to those elicited by LPS, the treponeme preparations were concentrated ~1000-fold. To compare the dose dependence of MAPK activation with that found in TNF-α release, cells were stimulated with treponeme LTA or LPS for assessment of p42/44 phosphorylation, or TNF-α release, respectively. TNF-α release induced by LPS was significantly stronger as compared with treponeme LTA-induced activation (Fig. 5*A*). For phosphorylation studies, cells were stimulated with LPS or treponeme LTA, and activation of p42/44 was assessed. LPS exhibited a stronger activation of kinases; however, at the concentrations used, all stimuli exhibited a comparable state of activation (Fig. 5, *B* and *C*). It has been shown that LPS-induced activation of MAPKs reaches its maximum after 15 min (64). To compare kinetics of MAPK activation induced by treponemes and LPS,

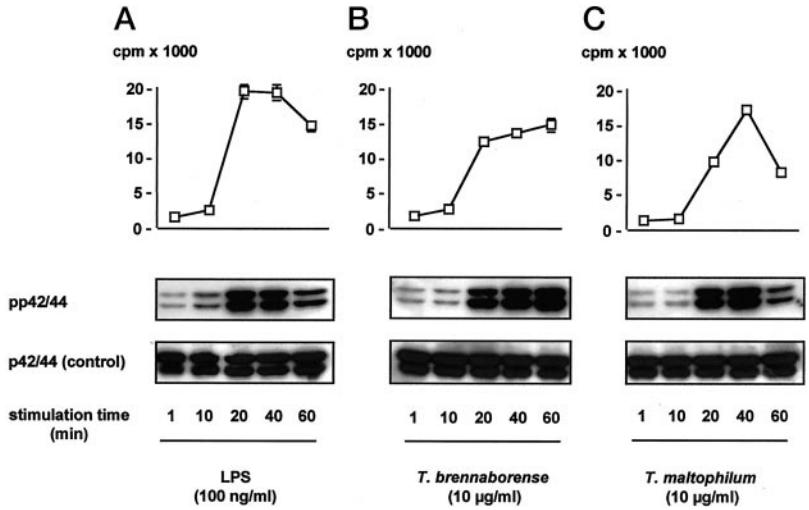

FIG. 6. **Time course of phosphorylation of p42/44 induced by treponeme LTA in comparison to LPS.** RAW 264.7 cells were stimulated with a mock extract (*A*), LPS (*E. coli* 0111:B4, 100 ng/ml, *B*), or with LTA derived from *T. brennaborense* (10 μg/ml, *C*) or *T. maltophilum* (10 μg/ml, *D*) for 1, 10, 20, 40, and 60 min in the presence of rmLBP (1 μg/ml). Cells were lysed, and 50 μg of protein were examined for phosphorylation of p42/44 by immunoblotting. Activation of MAPKs was assessed employing the Biotrak activity assay. Shown is one representative of two experiments. The activity assay was performed in duplicate.

we stimulated RAW 265.7 for different time periods ranging from 10 to 60 min. In these experiments LPS-induced phosphorylation and activation of MAPKs reached a maximum at 20 min (Fig. 6*B*). In contrast, treponeme LTA exhibited a different time pattern of phosphorylation and activation of p42/44; for TB activation was observed after 20 min, but further increased up to 60 min after stimulation (Fig. 6*C*). TM exhibited an activation of p42/44 with a maximum at 40 min (Fig. 6*D*).

Inhibition of MEK and p38 Leads to Decreased Cytokine Release—To determine a crucial involvement of MAPKs p42/44 and p38 in the events leading to release of TNF-α, RAW 264.7 were incubated with inhibitors of MEK1 and p38 prior to stimulation, and TNF-α-concentrations were measured after 4 h. MEK1 has been shown to activate MAPK p42/44, and inhibition of this kinase leads to suppression of MAPKs activity (65, 66). Cytokine induction caused by both treponeme preparations and LPS was strongly reduced in the presence of both inhibitors (Fig. 7). When both inhibitors were used simultaneously, TNF-α levels further decreased. Higher concentrations did not further influence cytokine induction (data not shown).

Activation of MAPKs p42/44 by Treponema LTA and LPS in the U373 Astrocytoma Cell Line, and Inhibition of MAPK Activation in RAW 264.7 Cells by the Anti-murine TLR-4 Antibody MTS 510—TLR-4 has been shown repeatedly to be associated with LPS signaling, while its role regarding activation of the MAPK pathway still remains unclear (29). As we have obtained evidence for a distinct role of TLR-2 and -4 for cellular activation by the two compounds studied, with TM exhibiting a TLR-2-dependent NF-κB translocation and NO induction (21), we used two *in vitro* systems to investigate the role of TLRs in treponeme LTA-induced MAPK activation. The TLR-2-negative U373 astrocytoma cell line was stimulated with both treponeme preparations and LPS, and MAPK activation was assessed (Fig. 8). In contrast to previous observations, TM caused a clear MAPK activation while TB failed to do so. IL-6 production induced by TM, however, appeared to be TLR-2-dependent as it was less pronounced as compared with TB (data not shown).

To investigate a potential link between TLR-4 and MAPK activation, we performed experiments employing the anti-murine TLR-4 antibody MTS 510 known to inhibit LPS effects. Activation of p42/44 in RAW 264.7 caused by both preparations, as well as by LPS, was clearly decreased in the presence of the MTS 510 antibody (Fig. 9). The inhibitory capacity of MTS 510 appeared to be more pronounced in regard to TB-induced MAPK activation, as compared with TM, when low stimulatory concentrations were employed. However, TNF-α induction in RAW 264.7 caused by the same stimuli was not reduced in the presence of MTS 510 (data not shown).

DISCUSSION

In previous studies we have shown that treponeme phenol/water extracts interact with LBP and CD14, as well as with endotoxin neutralizing protein and polymyxin B, structures commonly viewed as specific binding partners of LPS (22). Here we demonstrate that treponeme compounds are able to elicit parallel phosphorylation of the three major tyrosine kinase cascades, the p42/44, p38, and the SAPK pathway. Our data therefore indicate biological similarities between treponeme phenol/water extracts and LPS, although a chemical analysis of the investigated compounds revealed a structure being clearly different from that of LPS while resembling LTA of Gram-positive bacteria, with TM containing a diacylglycerol lipid anchor, which is also proposed for TB. This is in concordance with an analysis of a lipoglycan derived from *T. denticola*, another spirochete associated with periodontitis (23).

The stimulating activity of the two treponeme species analyzed was also found within culture supernatants. We therefore assume that treponemes release LTA during cell growth provoking signaling events in host cells involving similar pathways as LPS. *T. maltophilum* is found in lesions of patients suffering from periodontitis (17–19), and compounds released by these bacteria may be the cause for the constant and severe inflammatory reactions seen in the host. This reaction pattern also has been proposed as a key pathogenic principle for *Porphyromonas gingivalis*, a Gram-negative bacterium containing LPS, which is also associated with periodontitis (67). *T. brennaborense* has been found in lesions of the heels of cattle suffering from dermatitis digitalis, a chronic inflammatory disease of the foot. Although this disease does not affect any other organs, it coincides with severe cachexia, causing extensive losses in milk production with severe economic consequences worldwide (15). This cachexia may be caused by increased production of TNF-α in the host, elicited by LTA via signaling pathways proposed here. The findings observed here may also apply for other diseases caused by spirochetes, such as syphilis (caused by *T. pallidum*, closely related to the strains investigated here (Ref. 19)), or Lyme disease.

According to the results presented here, treponeme LTA and LPS differ in the time course of phosphorylation of MAPKs. This could indicate the potential activation of other yet undefined pathways, leading to a prolonged state of activation of kinases. The result of this prolonged phosphorylation is yet unclear but fails to be associated with an increase of cytokine

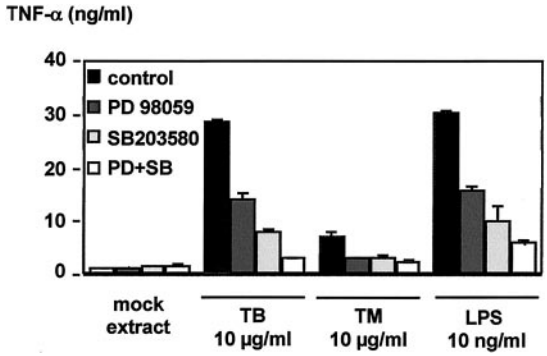

FIG. 7. **Reduction of TNF-α release by inhibitors of p42/44 and p38.** RAW 264.7 cells were incubated with the MEK inhibitor PD 98059 (50 μM) or the p38 inhibitor SB 203580 (10 μM) for 1 h at 37 °C prior to stimulation. Cells were stimulated with treponeme LTA (10 μg/ml) or LPS (*E. coli* 0111:B4, 10 ng/ml), and supernatants were harvested after 4 h followed by detection of mTNF-α. Shown is one representative of three experiments; measurements were performed in quadruplicate.

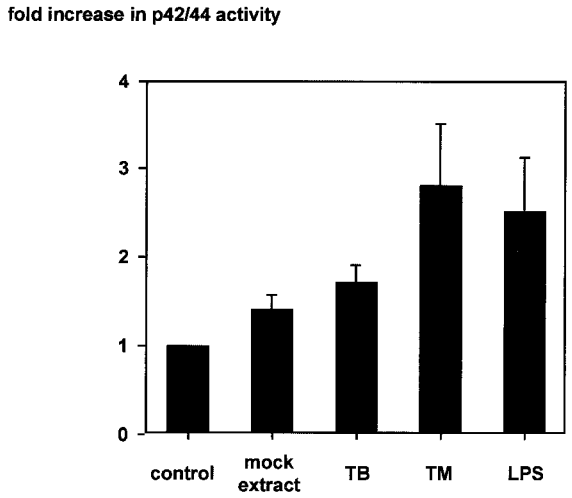

FIG. 8. **Activation of MAPKs p42/44 in the human astrocytoma cell line U373.** U373 cells were incubated with 10 μg/ml *Treponema* LTA, mock extract, or 100 ng/ml LPS, respectively, for 20 min at 37 °C followed by lysis. 10 μg of lysates were assayed for p42/44 activity employing the Biotrak kinase activity assay. Experiments were performed in triplicate; shown are combined results of two separate experiments.

induction. Our data provide evidence that treponeme LTA, although being recognized by the host as indicated by a substantial initiation of signaling cascades, induces a weak cytokine production. TNF-α induction was particularly weak when *T. maltophilum*-derived preparations were employed; however, LTA of both strains induced signaling events in a comparable manner.

Within the last 2 years, the importance of TLRs for host cell activation by pathogens has become evident. First experiments revealed a close link between TLR activation and NF-κB translocation in different cell systems leading to a rapid induction of pro-inflammatory cytokines (68, 69). In contrast, few data exist establishing a clear link between TLRs and the MAPK/SAPK pathways with one study describing divergent pathways for SAPKs (70). Here we show for the first time that inhibiting ligand binding to TLR-4 reduces activation of MAPK p42/44. This is evidence for a connection of TLR-4 and MAPKs and may, furthermore, indicate a link between the well established TLR-NF-κB cascade and the MAPKs. The inhibition experiments presented here confirm our previous observations of a divergent use of TLRs by the two different compounds investigated. Although TB in experiments employing macrophages

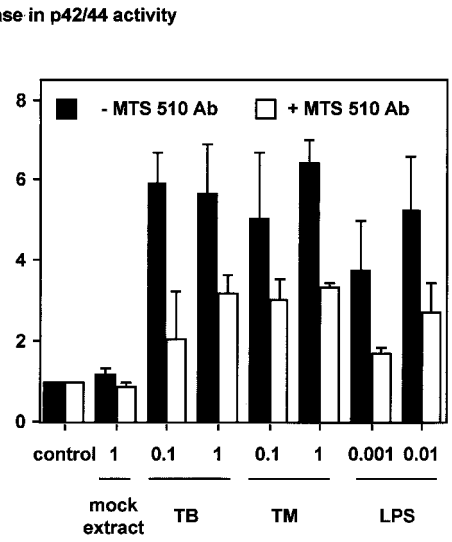

FIG. 9. **Decrease of activity of MAPKs p42/44 in the presence of a blocking TLR-4 antibody.** RAW 264.7 cells were incubated with the blocking anti-murine TLR-4 antibody MTS 510 for 30 min at 37 °C, followed by stimulation with *Treponema* LTA, mock extract, or LPS (*E. coli* 0111:B4) for 20 min in the presence of rmLBP (1 μg/ml). Cell lysates were harvested and investigated regarding activation of p42/44. Experiments were performed in triplicate. Shown is one representative of two experiments.

from TLR-4-defective C3H/HeJ mice appeared to stimulate cells via TLR-4, TM apparently preferentially utilizes TLR-2, as indicated by the lack of NF-κB translocation in TLR-2-defective Chinese hamster ovary cells (22). The inhibition by the anti-TLR-4 antibody observed here was more pronounced for TB as compared with TM when using low concentrations, further supporting a preferential utilization of TLR-4 by TB. This effect, however, in our hands could not be demonstrated for cytokine induction, which may be explained by the fact that cytokine release had to be assessed at a later time point.

In contrast to these observations, TM revealed a stronger activation of p42/44 in TLR-2 negative U373 cells as compared with TB, while IL-6 secretion caused by these stimuli resembled NF-κB translocation with TB being more active as TM. Taken together with our observations that treponeme LTA, although showing a strong activation of MAPKs in RAW 264.7, only induced a weak TNF-α release, these data could indicate a dissociation between the NF-κB pathway with subsequent cytokine release, and the MAPK pathway. On the other hand, our results obtained with the MEK and p38 inhibitors clearly support the notion that MAPK activation leads to cytokine release. Thus, although both the MAPK and NF-κB pathway lead to cytokine induction by bacterial compounds, they may not be linked in a synergistic manner.

As we have shown previously, chemical analysis of both treponeme compounds revealed differences regarding length and composition of the carbohydrate chain. Although TB exhibited a large number of small repeating units of ~5 sugars in size, TM contained fewer repeating units, each being composed of 20–30 sugars. Additionally, fatty acid content of TB appeared to be much lower as compared with TM (22). Although both LTA preparations induced similar signaling events in mononuclear cells and interact with LPS-binding proteins such as LBP and CD14, they appear to utilize TLRs in a divergent manner. Still, in line with our previous study describing the presence of LTA-like lipoglycan in *T. denticola*, the general structure of all treponeme lipoglycans described by us may be similar (23). We assume that the presence of a diacylglycerol lipid anchor is responsible for similar biological activity by interaction with LPS-binding structures, while the length and

composition of the carbohydrate chain may affect the affinity to the different TLRs. However, additional studies have to be performed to further elucidate this hypothesis.

In summary we have shown that the MAPK and SAPK pathways are induced not only by LPS but also by structurally distinct bacterial compounds such as treponeme LTA. Furthermore, we were able to demonstrate a connection of TLR-4 and activation of MAPKs p42/p44. Finally, we obtained evidence for a dissociation of the MAPK and the NF-κB pathway in myelomonocytic cells. The compounds investigated by us appear to be valuable tools for investigating signaling pathways involving TLRs, as here structurally related compounds apparently utilize TLRs in a different manner.

Acknowledgments—We acknowledge the excellent technical assistance of Nicole Siegemund, Fränzi Creutzburg, Michaela Müller, Marco Kachler, and Cyndi Hefenbrock.

REFERENCES

1. Nocton, J. J., and Steere, A. C. (1995) *Adv. Intern. Med.* **40,** 69–117
2. Lukehart, S. A., and Holmes, K. K. (1994) in *Harrison's Principles of Internal Medicine* (Isselbacher, K. J., Braunwald, E., Wilson, J., Martin, J. B., Fauci, A. S., and Kasper, D. L., eds) pp. 726–737, McGraw-Hill, Inc., New York
3. Nathan, C. F. (1987) *J. Clin. Invest.* **97,** 319–329
4. Rietschel, E. T., Brade, H., Holst, O., Brade, L., Müller-Loennies, S., Mamat, U., Zähringer, U., Beckmann, F., Seydel, U., Brandenburg, K., Ulmer, A. J., Mattern, T., Heine, H., Schletter, J., Hauschildt, S., Loppnow, H., Flad, H.-D., Schade, U. F., DiPadova, F., and Schumann, R. R. (1996) *Curr. Top. Microbiol. Immunol.* **216,** 39–81
5. Keller, R., Fischer, W., Keist, R., and Bassetti, S. (1992) *Infect. Immun.* **60,** 3664–3672
6. Bhakdi, S., Klonisch, T., Nuber, P., and Fischer, W. (1991) *Infect. Immun.* **59,** 4614–4620
7. Weidemann, B., Brade, H., Rietschel, E. T., Dziarski, R., Bazil, V., Kusumoto, S., Flad, H.-D., and Ulmer, A. J. (1994) *Infect. Immun.* **62,** 4709–4715
8. Takayama, K., Rothenberg, R. J., and Barbour, A. G. (1987) *Infect. Immun.* **55,** 2311–2313
9. Hardy, P. J., and Levin, J. (1983) *Proc. Soc. Exp. Biol. Med.* **174,** 47–52
10. Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., Gwinn, M., Hickey, E. K., Clayton, R., Ketchum, K. A., Sodergren, E., Hardham, J. M., McLeod, M. P., Salzberg, S., Peterson, J., Khalak, H., Richardson, D., Howell, J. K., Chidambaram, M., Utterback, T., McDonald, L., Artiach, P., Bowman, C., Cotton, M. D., and Venter, J. C. (1998) *Science* **281,** 375–388
11. Sellati, T. J., Bouis, D. A., Kitchens, R. L., Darveau, R. P., Pugin, J., Ulevitch, R. J., Gangloff, S. C., Goyert, S. M., Norgard, M. V., and Radolf, J. D. (1998) *J. Immunol.* **160,** 5455–5464
12. Radolf, J. D., Norgard, M. V., Brandt, M. E., Isaacs, R. D., Thompson, P. A., and Beutler, B. (1991) *J. Immunol.* **147,** 1968–1974
13. Norgard, M. V., Arndt, L. L., Akins, D. R., Curetty, L. L., Harrich, D. A., and Radolf, J. (1996) *Infect. Immun.* **64,** 3845–3852
14. Riviere, G. R., Wagoner, M. A., Baker, Z. S., Weisz, K. S., Adams, D. F., Simonson, L., and Lukehart, S. A. (1991) *N. Engl. J. Med.* **325,** 539–543
15. Riviere, G. R., Weisz, K. S., Simonson, L. G., and Lukehart, S. A. (1991) *Infect. Immun.* **59,** 2653–2657
16. Choi, B. K., Paster, B. J., Dewhirst, F. E., and Göbel, U. B. (1994) *Infect. Immun.* **65,** 1889–1895
17. Choi, B. K., Nattermann, H., Grund, S., Haider, W., and Göbel, U. B. (1997) *Int. J. Syst. Bacteriol.* **47,** 175–181
18. Moter, A., Hoenig, C., Choi, B., Riep, B., and Göbel, U. (1998) *J. Clin. Microbiol.* **36,** 1399–1403
19. Wyss, C., Choi, B. K., Schupbach, P., Guggenheim, B., and Göbel, U. B. (1996) *Int. J. Syst. Bacteriol.* **46,** 745–752
20. Offenbacher, S., Heasman, P. A., and Collins, J. G. (1993) *J. Periodontol.* **64,** 432–444
21. Rosen, G., Sela, M. N., Naor, R., Halabi, A., Barak, V., and Shapira, L. (1999) *Infect. Immun.* **67,** 1180–1186
22. 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
23. Schultz, C. P., Wolf, V., Lange, R., Mertens, E., Wecke, J., Naumann, D., and Zähringer, U. (1998) *J. Biol. Chem.* **273,** 15661–15666
24. Schumann, R. R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch, R. J. (1990) *Science* **249,** 1429–1431
25. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) *Science* **249,** 1431–1433
26. Schumann, R. R., Rietschel, E. T., and Loppnow, H. (1994) *Med. Microbiol. Immunol.* **183,** 279–297
27. Ulevitch, R. J., and Tobias, P. S. (1995) *Annu. Rev. Immunol.* **13,** 437–457
28. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189,** 1777–1782
29. Beutler, B. (2000) *Curr. Opin. Microbiol.* **3,** 23–28
30. Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P., and Malo, D. (1999) *J. Exp. Med.* **189,** 615–625
31. Poltorak, A., He, X., Smirnova, I., Liu, M., Huffel, C., 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
32. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752
33. 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
34. 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
35. Means, T. K., Lien, E., Yoshimura, A., Wang, S., Golenbock, D. T., and Fenton, M. J. (1999) *J. Immunol.* **163,** 6748–6755
36. Means, T. K., Golenbock, D. T., and Fenton, M. J. (2000) *Cytokine Growth Factor Rev.* **11,** 219–232
37. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Takada, H., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 443–451
38. Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., and Kirschning, C. J. (1999) *J. Biol. Chem.* **274,** 17406–17409
39. Geng, Y., Gulbins, E., Altman, A., and Lotz, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8602–8606
40. Geppert, T. D., Whitehurst, C. E., Thompson, P., and Beutler, B. (1994) *Mol. Med.* **1,** 93–103
41. Gardner, A. M., Vaillancourt, R. R., Lange, C. C., and Johnson, G. L. (1994) *Mol. Biol. Cell* **5,** 193–201
42. Brunet, A., Pages, G., and Pouyssegur, J. (1994) *FEBS Lett.* **346,** 299–303
43. Freed, E., McCormick, F., and Ruggieri, R. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59,** 187–193
44. Fu, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narsimhan, R. P., Mamon, H., Collier, R. J., and Roberts, T. M. (1994) *Science* **266,** 126–129
45. Reimann, T., Buscher, D., Hipskind, R. A., Krautwald, S., Lohmann, M. M., and Baccarini, M. (1994) *J. Immunol.* **153,** 5740–5749
46. Seger, R., Ahn, N. G., Posada, J., Munar, E. S., Jensen, A. M., Cooper, J. A., Cobb, M. H., and Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 14373–14381
47. Weinstein, S. L., Sanghera, J. S., Lemke, K., DeFranco, A. L., and Pelech, S. L. (1992) *J. Biol. Chem.* **267,** 14955–14962
48. Ding, A., Sanchez, E., and Nathan, C. F. (1993) *J. Immunol.* **151,** 5596–5602
49. Liu, M. K., Herrera, V. P., Brownsey, R. W., and Reiner, N. E. (1994) *J. Immunol.* **153,** 2642–5262
50. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) *Science* **265,** 808–811
51. Han, J., Richter, B., Li, Z., Kravchenko, V., and Ulevitch, R. J. (1995) *Biochim. Biophys. Acta* **1265,** 224–227
52. Herskowitz, I. (1995) *Cell* **8,** 187–197
53. Kotlyarov, A., Neininger, A., Schubert, C., Eckert, R., Birchmeier, C., Volk, H. D., and Gaestel, M. (1999) *Nat. Cell Biol.* **1,** 94–97
54. Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *J. Biol. Chem.* **270,** 7420–7426
55. Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) *Science* **267,** 682–685
56. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) *Cell* **76,** 1025–1037
57. Sanghera, J. S., Weinstein, S. L., Aluwalia, M., Girn, J., and Pelech, S. L. (1996) *J. Immunol.* **156,** 4457–4465
58. Liu, F., and Green, M. R. (1990) *Cell* **61,** 1217–1224
59. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) *Genes Dev.* **7,** 2135–2148
60. Vossler, M. R., Yao, H., York, R. D., Pan, M. G., Rim, C. S., and Stork, P. J. (1997) *Cell* **89,** 73–82
61. Westphal, O., and Jann, K. (1965) in *Methods in Carbohydrate Chemistry* (Whistler, R. L., ed) 5th Ed., pp. 83–92, Academic Press, New York
62. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., and Miyake, K. (2000) *J. Immunol.* **164,** 3471–3475
63. Lamping, N., Dettmer, R., Schröder, N. W., Pfeil, D., Hallatschek, W., Burger, R., and Schumann, R. R. (1998) *J. Clin. Invest.* **101,** 2065–2071
64. Weinstein, S. L., Gold, M. R., and DeFranco, A. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4148–4152
65. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364,** 229–233
66. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 27489–27494
67. Shapira, L., Takashiba, S., Amar, S., and Van, D. T. (1994) *Oral Microbiol. Immunol.* **9,** 112–117
68. Kirschning, C., Wesche, H., Merrill, A., and Rothe, M. (1998) *J. Exp. Med.* **188,** 2091–2097
69. Yang, R., Mark, M., Gray, A., Huang, A., Xie, M., Zhang, M., Goddard, A., Wood, W., Gurney, A., and Godowski, P. (1998) *Nature* **395,** 284–288
70. Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998) *J. Exp. Med.* **187,** 2097–2101