



Lipopolysaccharide induces distinct alterations in the microtubule cytoskeleton of monocytes

S. Rußwurm¹, K.J. Böhm³, P. Mühlig³, M. Wiederhold¹, K. König² and K. Reinhart¹

¹University of Jena, Department of Anaesthesiology and Critical Care Medicine; ²Institute of Anatomy II, ³Institute of Molecular Biotechnology, Jena, Germany

Received 25 April 2000; accepted 17 October 2000

Keywords: lipopolysaccharide, lipid A, microtubule, cytoskeleton, monocytes

Abstract

Microtubules are obligate functional elements of almost all eukaryotic cells. They are involved in a broad range of essential cellular functions and structural changes of this system may trigger cell death. Recently, we have reported that lipopolysaccharides inhibit *in vitro* microtubule formation due to exclusion of microtubule-associated proteins. The distinct epitopes of lipopolysaccharides responsible for these effects and the *in vivo* relevance of these data are unknown. Therefore, this study was conducted to elucidate the effects of lipid A, the biologically active motif of lipopolysaccharides, on microtubule formation *in vitro* and to prove whether lipopolysaccharides affect the microtubule architecture of cultured human monocytes *in vivo*. Despite a dose- and pH-dependent inhibition of microtubule formation by lipopolysaccharides, inhibition of microtubule assembly could be mimicked by lipid A. Near-infrared two-photon microscopy revealed that human peripheral blood monocytes accumulate lipopolysaccharides. A vesicular distribution pattern of lipopolysaccharides within the monocytes was observed. Confocal laser scanning microscopy demonstrated alterations in the microtubule architecture of monocytes after incubation with lipopolysaccharides. Lipid A seems to be responsible for the observed crosstalk between lipopolysaccharides and microtubule proteins. Furthermore, our data indicate that lipopolysaccharides may affect the microtubule architecture in human monocytes after intracellular accumulation directly. Therefore, we conclude, that the microtubule cytoskeleton is an essential intracellular target for sepsis-relevant bacterial components such as lipopolysaccharides.

Abbreviations: DAPI, diamidinophenylindole; DTAF, dichlorotriazinylaminofluorescein; EGTA, ethylene glycol-bis(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid; FITC, fluorescein isothiocyanate; GTP, guanosine triphosphate; LPS, lipopolysaccharide; MAP, microtubule-associated protein; NIR, near-infrared two-photon microscopy; Pipes, piperazine-*N,N'*-bis[2-ethanesulphonic acid]

Introduction

The microtubule cytoskeleton is known to be involved in a wide range of specific cellular events such as mitosis, cytoplasmic transport, cell movement, and cell shape. Since attacks to this system may trigger cell death (reviewed by Jordan and Wilson, 1998), these components continue to attract enormous attention from both basic researchers and clinicians (MacRae, 1992; Mandelkow and Mandelkow, 1995).

Microtubules are proteinaceous elements occurring in almost all eukaryotic cells. They consist mainly of tubulin dimers which, starting from microtubule-organizing centers, assemble into a three-dimensional filamentous intracellular network. Further functionally essential proteins are linked to microtubules, the so-called microtubule-associated proteins (MAPs). Prominent members of the MAP family are the high-molecular-weight MAP-1 and MAP-2 and the tau proteins (MacRae, 1992; Mandelkow and Mandelkow, 1995). These proteins stimulate microtubule formation and stabilize microtubules against disassembling factors. In addition, MAPs are involved in determination of microtubule substructure, for example, the number of protofilaments forming the microtubule wall (Kuznetsov et al., 1981; Böhm et al., 1984; McKeithan et al., 1984).

Lipopolysaccharide (LPS) is the major toxic product from the cell wall of gram-negative bacteria. Application of LPS to animals induces manifestations of systemic inflammation, septic shock, and multiorgan failure (Alexander et al., 1991; von der Mohlen et al., 1996; Sevransky et al., 1997). It is known that *in vitro* LPS binds to microtubules (Ding et al., 1992), inhibits microtubule formation (Risco et al., 1993, 1995), and disassembles microtubules by both endwise tubulin dimer release and fragmentation (Böhm et al., 1998). The effects of LPS on the microtubule cytoskeleton were concluded to be a consequence of LPS-

MAP interaction (Ding et al., 1992; Risco et al., 1993; Böhm et al., 1999).

To evaluate the biological importance of previously obtained *in vitro* observations (Böhm et al., 1998), human peripheral blood monocytes were investigated by confocal laser scanning microscopy and near infrared two-photon microscopy (NIR). The present study contributes to discovery of the intracellular distribution pattern of LPS and elucidates possible alterations within the microtubule cytoskeleton induced by LPS. We have previously reported that LPS affects microtubule formation *in vitro* by interacting with MAP-2 as well as tau proteins (Böhm et al., 1998, 1999). The epitopes of LPS responsible for the observed inhibition of microtubule formation are so far unknown. Our study focuses on the direct interaction of lipid A with the microtubule cytoskeleton.

Material and methods

Microtubule protein

Microtubule protein (tubulin plus MAP-1, MAP-2, and tau proteins) was isolated from porcine brain homogenates by three cycles of temperature-dependent disassembly/reassembly (Shelanski et al., 1973). Together with the high-molecular-weight MAPs, the tau proteins were removed from the phosphocellulose column with 1 mol/L NaCl (Weingarten et al., 1975). MAP-1 was eliminated by heat treatment and MAP-2 by gel filtration, according to the protocol of Drubin et al. (1986).

Microtubule formation

Microtubule formation was induced by incubation of microtubule proteins with 0.35 mmol/L GTP in assembly buffer at 37°C and monitored by time-dependent turbidity measurements at 360 nm with a Cary 1E

spectrophotometer (Varian Instruments GmbH, Darmstadt, Germany). Assembly buffer consists of 20 mmol/L Pipes, 80 mmol/L NaCl, 1 mmol/L EGTA, 0.5 mmol/L MgCl₂. If not indicated otherwise, the experiments were performed at pH 6.8. The LPS, purified from *E. coli* by gel filtration (Sigma, Deisenhofen, Germany) and lipid A (a kind gift of Professor E. Rietschel, Borstel, Germany) was added from fresh stock solutions of 2 mg/ml in assembly buffer. To reduce aggregations (Risco and Pinto da Silva, 1995), the LPS solution was treated immediately before use for 15 min in a Sonopuls HD70 ultrasound homogenizer equipped with a MS73 Sonotrode (Bandelin Electronic, Berlin, Germany) at 30 W and 4°C.

Human peripheral blood monocytes

Human peripheral blood monocytes were obtained as previously described (Rußwurm et al., 1994). Mononuclear cells were plated on 4- or 8-well Permanox chamber slides (Nunc A/S, Roskilde, Denmark) for 1 h in RPMI 1640 medium without phenol red (Gibco BRL, Life Technologies Ltd, Paisley, UK). After differential adhesion, nonadherent cells were removed by excessive washing and adherent monocytes were cultured for 16 h in the presence of 10% human AB serum.

Confocal laser scanning microscopy

Cells were rinsed with PBS and fixed at 37°C for 1 h with 2% v/v formaldehyde, 0.5% v/v glutaraldehyde, and 2% Triton X-100 in PBS. After incubation with 0.02% (w/v) NaBH₄ to reduce autofluorescence, blocking of unspecific binding was performed in antibody dilution buffer (10% (v/v) goat serum (Sigma, Deisenhofen, Germany), and 0.1% (v/v) Tween 20 (Carl Roth, Karlsruhe, Germany) in PBS) for 1 h at room temperature. Thereafter, the cells were exposed overnight to primary anti- α -tubulin antibodies (all 1:500, Sigma) at 4°C.

Cells were rinsed three times with PBS, incubated with rhodamine- or dichlorotriazinylaminofluorescein (DTAF)-coupled secondary antibody (1:400; Jackson/Dianova, Hamburg, Germany) for 1 h at room temperature in antibody dilution buffer in the dark. Postfixing with prechilled methanol was performed after rinsing with PBS at 20°C for 30 min and the specimens were mounted in Vectashield containing diamidinophenylindole (DAPI) for staining nuclei (Vector Laboratories, Burlingame, CA) or Prolong (Molecular Probes, Eugene, OR) antifade kit and covered with glass coverslips. Micrographs were taken with Leica confocal laser scanning microscope (Leitz, Wetzlar, Germany) using a $\times 60$ objective (488 nm, Objective, NA 1.2).

Near-infrared two-photon microscopy

Cultured human monocytes were incubated with FITC-labeled LPS from *E. coli* B4 with concentrations up to 100 μ g for up to 24 h. Unbound LPS was removed by triple extensive washing with medium. Living cells were mounted in PBS and investigated with a near-infrared two-photon microscope (König et al., 1996) using $\times 100$ objective (800 nm, Objective, NA 1.30).

Results

The influence of LPS on microtubule assembly was investigated by recording time-dependent turbidity. Using this technique, it was confirmed that LPS is able to inhibit the formation of microtubules from microtubule proteins (tubulin plus MAPs) in a pH- and dose-dependent manner (Figure 1). LPS (50 μ g/ml) inhibited the assembly to an extent of 25%, whereas 200 μ g/ml LPS led to negligible microtubule assembly. The extent of assembly inhibition depended also on the LPS/microtubule protein ratio: if the concentration of microtubule protein was lowered to 0.5 mg/

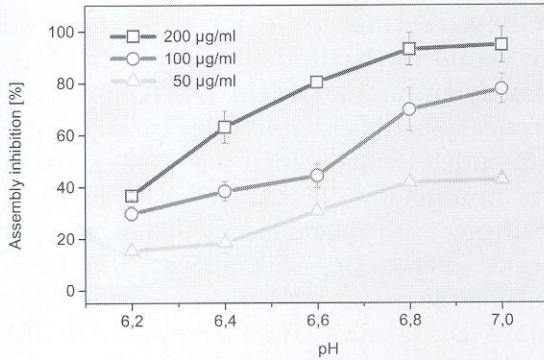


Figure 1. Inhibition of microtubule protein assembly by LPS. Microtubule protein (1.0 mg/ml), containing about 80% tubulin and 20% MAPs, was mixed with LPS (50, 100, 200 µg/ml from *E. coli* B4) at different pH. Assembly was initiated by raising the temperature to 37°C; the course of assembly was followed by turbidity measurement at 360 nm.

ml, total inhibition could be achieved with 50 µg/ml LPS (data not shown).

Incubation of cultured human monocytes with FITC-labeled LPS revealed a weak fluorescence (data not shown). To check whether this FITC-LPS derived fluorescence was intracellularly distributed, the monocytes were incubated for 12 h with FITC-labeled LPS (100 µg/ml) and thereafter investigated by NIR microscopy (Figure 2). Using this method, a clear fluorescence signal, significantly exceeding endogenous fluorescence level of control preparations, was measured inside the cells, confirming the uptake of LPS.

Figure 3 demonstrates LPS-caused alterations in the microtubule architecture of human

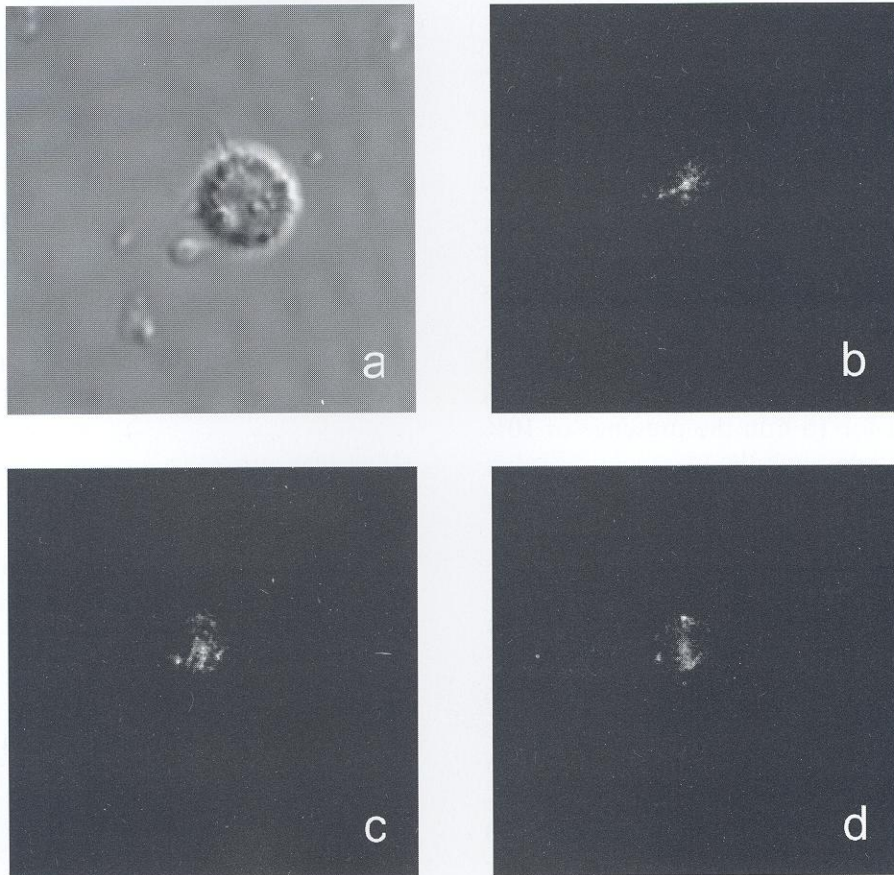


Figure 2. LPS uptake of cultured human monocytes. Cells were incubated for 12 h with FITC-labeled LPS (100 µg/ml medium), triple rinsed and mounted in PBS. Fluorescence was directly visualized with near-infrared two-photon microscopy. A phase-contrast micrograph is shown in (a) and optical sections from the middle of the cell in (b to d).

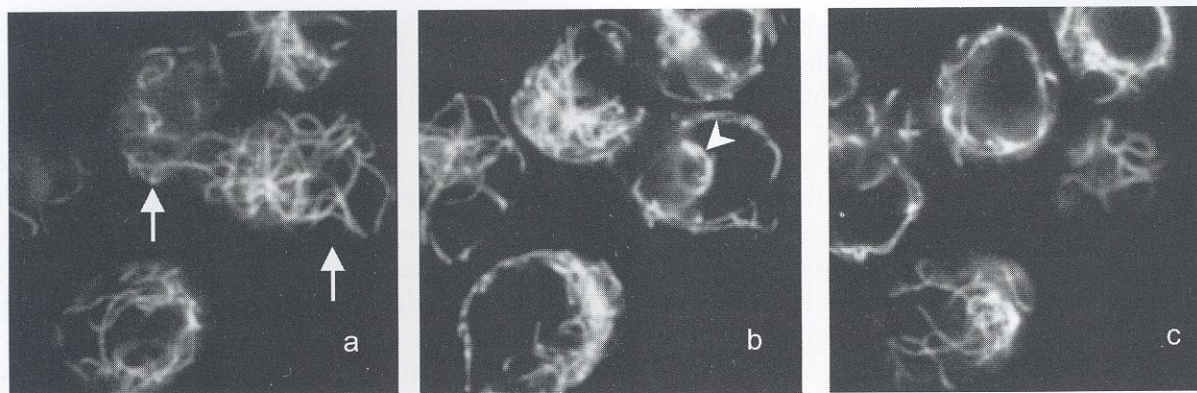


Figure 3. Basal (a), median (b), and apical (c) optical sections from monocytes incubated with LPS. Cells were incubated with 100 $\mu\text{g}/\text{ml}$ medium LPS from *E. coli* for 24 h, fixed, and incubated with anti- α -tubulin antibody followed by rhodamine-linked anti-mouse antibody. Slides were postfixed, mounted in Vectorshield and viewed with a Leica confocal laser scanning microscope using a $\times 60$ objective (arrows indicate handle-like protrusions in (a); arrowhead points to a transnuclear canal in (b)).

monocytes of the peripheral blood. Human monocytes incubated with LPS showed basal protrusions (Figure 3a, arrows). Alterations within the nuclear region were also detected: nuclei were perturbed by canals derived from microtubules (Figure 3b, arrowhead). Interestingly, the number, the distribution and the integrity of peripheral microtubules of these cells were not affected by LPS (Figure 3).

To clarify the distinct LPS epitopes responsible for the inhibition of microtubule assembly (Figure 1), the influence of lipid A on microtubule formation was investigated by recording time-dependent turbidity. The results indicate a marked inhibition of microtubule formation by lipid A (Figure 4). Incubation of 1.5 mg/ml microtubule protein with 100 $\mu\text{g}/\text{ml}$ lipid A resulted in a 60% assembly inhibition, which is comparable with the inhibition rate of 100 $\mu\text{g}/\text{ml}$ LPS.

Discussion

There is increasing evidence for a pathogenetically relevant interaction between toxic bacterial components, such as LPS, and the microtubule cytoskeleton. No data exist at

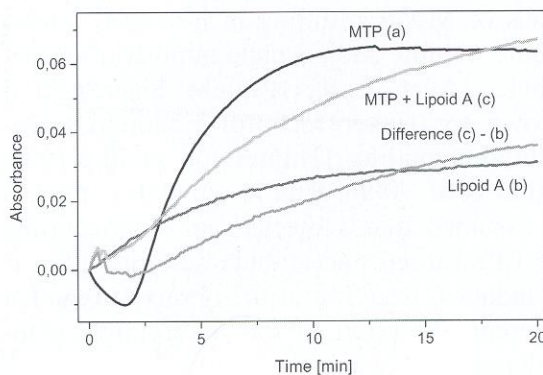


Figure 4. Inhibition of microtubule protein assembly by lipid A. Microtubule protein (1.0 mg/ml), containing about 80% tubulin and 20% MAPs, was mixed with lipid A (100 $\mu\text{g}/\text{ml}$, purified by Professor Dr E. Rietschel, Borstel). Assembly was initiated by raising the temperature to 37°C; the course of assembly was followed by turbidity measurement at 360 nm.

present on alterations of the microtubule cytoskeleton in monocytes challenged with LPS. We report here that LPS is able to cause the formation of peripheral protrusions and transnuclear channels. Since the microtubule cytoskeleton is known to be involved in a wide range of specific cellular events (including mitosis, cytoplasmic transport, cell movement, cell shaping), it could be speculated that these

morphological changes may reflect diversified physiological functions following LPS activation. Another explanation might be that these alterations may represent or induce proapoptotic diversification of LPS-treated cells. This is encouraged by the fact that alterations of cytoskeletal elements play a crucial role during the process of apoptosis and typical apoptotic characteristics result from cytoskeletal alterations (Blagosklonny et al., 1997; Vertessy et al., 1997).

Addressing the microtubule cytoskeleton, LPS seems to induce not only morphological alterations but also to enhance gene expression of microtubule proteins. It has been demonstrated that LPS stimulation of mononuclear phagocytes caused an increase in expression levels of MAPs resulting in increased microtubule stability and absolute number of microtubules (Allen et al., 1997a,b). Since LPS is known to trigger receptor-mediated intracellular signaling (Hambleton et al., 1995; Nahas et al., 1996; Paul et al., 1999), it could be assumed that a mechanism compensating the LPS-caused microtubule destabilization is co-induced, resulting in the observed regional different alteration of the microtubule cytoskeleton.

Our results, obtained by recording the time course of microtubule assembly, confirm earlier observations on an LPS-caused inhibition of microtubule formation, drawn from electron-microscopic and electrophoretic studies at pH 6.4 (Risco et al., 1993). Previous studies addressed the exclusion of MAPs, especially MAP-2 and tau proteins, as crucial for the observed microtubule fragmentation and inhibition of microtubule assembly (Böhm et al., 1998, 1999; Risco et al., 1993). In addition, we demonstrated that inhibition became stronger with increasing pH. The highest efficiency was observed at pH 6.8 and 7.0, that is, at values near to those measured within various LPS-target cells, such as macrophages (Swallow et al., 1990), monocytes

(Orlinska et al., 1995), hepatocytes (Portoles et al., 1991), and lymphocytes (Gerson and Kiefer, 1983). One explanation of the pH dependence of LPS-caused inhibition of microtubule assembly might be an ionic interaction between LPS and microtubule protein. The pH dependence of assembly inhibition might also be due to conformational alterations of tubulin and/or LPS: tubulin is known to be affected by pH, resulting in assembly products with special structural features (Matsumura and Hayashi, 1976; Burton and Himes, 1978; Rozycki et al., 1988). Shifts in pH were found to change LPS-LPS interaction (Coughlin et al., 1985) or LPS solubility (Din et al., 1993).

Since the distinct LPS-epitopes responsible for the crosstalk with the microtubule cytoskeleton remained unknown, this study focused on the direct influence of lipid A. Our present results obtained with LPS confirm recent data on the LPS-caused inhibition of microtubule formation (Böhm et al., 1999; Risco et al., 1993).

In addition, it was shown for the first time that inhibition of microtubule assembly could be achieved by lipid A (Figure 4). Comparable amounts of lipid A and LPS led to similar inhibitions rates, suggesting that the lipid A epitope is important for the crosstalk between LPS and microtubules. There are no data on the role of lipid A or other components of LPS explaining the LPS-microtubule interactions. However, findings of other groups support the hypothesis that lipid A represents the endotoxic principle of LPS (reviewed by Schromm et al., 1998).

Our results show that lipid A markedly inhibits the microtubule assembly *in vitro*. The intracellular uptake and a vesicular distribution pattern of LPS in monocytes was demonstrated by near-infrared two-photon microscopy. Morphological investigations of monocytes following LPS treatment revealed distinct alterations in the microtubule cyto-

skeleton. We therefore conclude that the microtubule cytoskeleton is an additional intracellular target for lipopolysaccharides. Since the intracellular interaction between LPS and microtubule proteins seems to be very complex, further studies are necessary addressing the direct morphological effects and regulation of mRNA and protein levels.

Acknowledgments

The authors thank Professor E. Rietschel, Borstel, Germany for kindly providing lipid A and Mrs Sabine Häfner for her skilful technical assistance. The present publication was supported in part by grants from the Deutsche Forschungsgemeinschaft (Re 653/51, Un 82/2-1) and the Thuringian Ministry of Science, Research and Culture (B30195026).

References

- Alexander HR, Doherty GM, Buress CM, Venzon DJ, Norton JA. A recombinant human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice. *J Exp Med.* 1991;173:1029-32.
- Allen JN, Moore SA, Liao Z, Wewers MD. Changes in mononuclear phagocyte microtubules after endotoxin stimulation. 1. Changes in microtubule stability. *Am J Resp Cell Mol Biol.* 1997a;16:119-26.
- Allen JN, Moore SA, Liao Z, Wewers MD. Changes in mononuclear phagocyte microtubules after endotoxin stimulation. 2. Changes in microtubule composition. *Am J Resp Cell Mol Biol.* 1997b;16:127-32.
- Blagosklonny MV, Giannakakou P, el Deiry WS, Kingston DG, Higgs PL, Neckers L, Fojo T. Raf-1/bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res.* 1997;57:130-35.
- Böhm KJ, Vater W, Fenske H, Unger E. Effect of microtubule-associated proteins on the protofilament number of microtubules assembled in vitro. *Biochim Biophys Acta.* 1984; 800:119-26.
- Böhm KJ, Vater W, Russwurm S, Reinhart K, Unger E. Lipopolysaccharide-caused fragmentation of individual microtubules in vitro observed by video-enhanced differential interference contrast microscopy. *FEBS Lett.* 1998;425 (1):134-6.
- Böhm KJ, Russwurm S, Ghaleb N, Reinhart K, Unger E. Microtubule formation and kinesin-driven microtubule gliding in vitro in the presence of lipopolysaccharide. *Cell Biol Int.* 1999;23:431-7.
- Burton PR, Himes RH. Electron microscope studies of pH effects on assembly of tubulin free of associated proteins. Delineation of substructure by tannic acid staining. *J Cell Biol.* 1978;77:120-33.
- Coughlin RT, Peterson AA, Haug A, Pownall HJ, McGroarty EJ. A pH titration study on the ionic bridging within lipopolysaccharide aggregates. *Biochim Biophys Acta.* 1985;821:404-12.
- Din ZZ, Mukerjee P, Kastowski M, Takayama K. Effect of pH on solubility and ionic state of lipopolysaccharide obtained from the deep rough mutant of *Escherichia coli*. *Biochemistry.* 1993;32:4579-86.
- Ding A, Sanchez E, Tancinco M, Nathan C. Interactions of bacterial lipopolysaccharide with microtubule proteins. *J Immunol.* 1992;148:2853-8.
- Drubin D, Kirschner M. Purification of tau protein from brain. *Methods Enzymol.* 1986;134:156-60.
- Gerson DF, Kiefer H. Intracellular pH and the cell cycle of mitogen-stimulated murine lymphocytes. *J Cell Physiol.* 1983;114:132-6.
- Haldar S, Basu A, Croce CM. Bcl2 is the guardian of microtubule integrity. *Cancer Res.* 1997;57:229-33
- Hambleton J, McMahon M, DeFranco AL. Activation of Raf-1 and mitogen-activated protein kinase in murine macrophages partially mimics lipopolysaccharide-induced signaling events. *J Exp Med.* 1995;182:147-54
- Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. *Curr Opin Cell Biol.* 1998;10:123-30
- König, U. Simon, K-J. Halhuber: 3D resolved two-photon fluorescence microscopy of living cells using a modified confocal laser scanning microscope. *Cell Mol Biol.* 1996; 42:1181-94
- Kuznetsov SA, Rodionov VI, Gelfand VI, Rosenblat VA. Microtubule-associated protein MAP1 promotes microtubule assembly in vitro. *FEBS Lett.* 1981;135:241-4.
- MacRae, TH. Towards an understanding of microtubule function and cell organization: an overview. *Biochem Cell Biol.* 1992;70:835-41.
- Mandelkow E, Mandelkow EM. Microtubules and microtubule-associated proteins. *Curr Opin Cell Biol.* 1995;7:72-81.
- Matsumura F, Hayashi M. Polymorphism of tubulin assembly. In vitro formation of sheet, twisted ribbon and microtubule. *Biochim Biophys Acta.* 1976;453:162-75.
- McKeithan TW, Rosenbaum JL. The biochemistry of microtubules. In: Shay JW, ed. *Cell and muscle motility*. New York: Plenum Publishing; 1984:255-88.
- Nahas N, Molski TF, Fernandez GA, Sha'afi RI. Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists. *Biochem J.* 1996;318:247-53
- Orlinska U, Newton RC. Modification of tumor necrosis factor-alpha (TNF-alpha) production by the Na(+)-dependent HCO₃-cotransport in lipopolysaccharide-activated human monocytes. *Immunopharmacology.* 1995;30:41-50.

- Paul A, Cuenda A, Bryant CE, Murray J, Chilvers ER, Cohen P, Gould GW, Plevin R. Involvement of mitogen-activated protein kinase homologues in the regulation of lipopolysaccharide-mediated induction of cyclo-oxygenase-2 but not nitric oxide synthase in RAW 264.7 macrophages. *Cell Signal*. 1999;11:491-7.
- Portoles MT, Ainaga MJ, Municio AM, Pagani R. Intracellular calcium and pH alterations induced by *Escherichia coli* endotoxin in rat hepatocytes. *Biochim Biophys Acta*. 1991;1092:1-6.
- Risco C, Dominguez JE, Bosch MA, Carrascosa JL. Biochemical and electron microscopy analysis of the endotoxin binding to microtubules in vitro. *Mol Cell Biochem*. 1993;121:67-74.
- Risco C, Pinto da Silva P. Cellular functions during activation and damage by pathogens: immunogold studies of the interaction of bacterial endotoxins with target cells. *Microsc Res Tech*. 1995;31:141-58.
- Rozycki MD, Stetzkowski-Marden F, Edelstein SJ. Conservation of tubulin alpha-beta differences in zinc-induced sheets with variations in pH and microtubule-associated protein content. *J Ultrastruct Mol Struct Res*. 1988;98:48-59.
- Rußwurm S, Krause S, Finkelberg L, Schauer U, Lösche W. Generation of reactive oxygen species and activity of platelet-activating factor acetylhydrolase in human monocyte-derived macrophages. *Thromb Res*. 1994;74:505-14.
- Schromm AB, Brandenburg K, Loppnow H, Zahringer U, Rietschel ET, Carroll SF, Koch MH, Kusumoto S, Seydel U. The charge of endotoxin molecules influences their conformation and IL-6-inducing capacity. *J Immunol*. 1998;161(10):5464-71.
- Sevransky JE, Shaked G, Novogrodsky A, et al. Tyrphostin AG 556 improves survival and reduces multiorgan failure in canine *Escherichia coli* peritonitis. *J Clin Invest*. 1997;99:1966-73.
- Shelanski ML, Gaskin F, Cantor CR. Microtubule assembly in the absence of added nucleotides. *Proc Natl Acad Sci USA*. 1973;70:765-8.
- Swallow CJ, Grinstein S, Rotstein OD. Regulation of cytoplasmic pH in resident and activated peritoneal macrophages. *Biochim Biophys Acta*. 1990;1022:203-10.
- Vertessy BG, Orosz F, Kovacs J, Ovadi J. Alternative binding of two sequential glycolytic enzymes to microtubules. Molecular studies in the phosphofructokinase/aldolase/microtubule system. *J Biol Chem*. 1997;272:25542-6.
- von der Mohlen MA, van der Poll T, Jansen J, Levi M, van Deventer SJ. Release of bactericidal/permeability-increasing protein in experimental endotoxemia and clinical sepsis. *J Immunol*. 1996;156:4969-73.
- Weingarten MP, Lockwood AH, Hwo SY, Kirschner MW. A protein factor essential for microtubule assembly. *Proc Natl Acad Sci USA*. 1975;72:1858-62.

Address for correspondence: Dr. Stefan Rußwurm, Department of Anaesthesiology and Critical Care Medicine, Friedrich-Schiller-University of Jena, Bachstr. 18, D-07740 Jena, Germany.

E-mail: russwurm@med.uni-jena.de