

Lipopolysaccharides from *Legionella* and *Rhizobium* stimulate mouse bone marrow granulocytes via Toll-like receptor 2

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Summary

Lipopolysaccharide (LPS) derived from enterobacteria elicit in several cell types cellular responses that are restricted in the use of Toll-like receptor 4 (TLR4) as the principal signal-transducing molecule. A tendency to consider enterobacterial LPS as a prototypic LPS led some authors to present this mechanism as a paradigm accounting for all LPSs in all cell types. However, the structural diversity of LPS does not allow such a general statement. By using LPSs from bacteria that do not belong to the *Enterobacteriaceae*, we show that in bone marrow cells (BMCs) the LPS of *Rhizobium species* Sin-1 and of three strains of *Legionella pneumophila* require TLR2

rather than TLR4 to elicit the expression of CD14. In addition, exposure of BMCs from TLR4-deficient (C3H/HeJ) mice to the lipid A fragment of the *Bordetella pertussis* LPS inhibits their activation by the *Legionella* lipid A. The data show selective action of different LPSs via different TLRs, and suggest that TLR2 can interact with many lipid A structures, leading to either agonistic or specific antagonistic effects.

Key words: Lipopolysaccharide, Toll-like receptors, Bone marrow, CD14, Innate immunity

Introduction

The outermost leaflet of the outer membrane of Gram-negative bacterial cell wall consists of lipopolysaccharide (LPS), a complex molecule which induces a dysregulated innate immune response. Because of the pathophysiological effects of LPSs and their action on a number of different cell types, it is important to get a picture as complete as possible of the early events involved in these responses. This picture may not be as simple as was thought a few years ago. The structural differences of LPSs produced by distinct microorganisms, and the distinct patterns of molecular sensors for LPSs on membranes of different cell types, seems to justify the expectation that the initial interactions and early events involved in the cellular effects of LPSs depend on both the particular LPS considered, and the cell type that is exposed to it.

It has been established that the activation of monocytes/macrophages by enterobacterial LPS requires at least four molecules: the serum LPS-binding protein LBP (Tobias et al., 1995), the GPI-anchored cell-surface protein CD14 (Wright et al., 1990; Golenbock et al., 1993), the type I transmembrane Toll-like receptor 4 (TLR4) (Poltorak et al., 1998), and the extracellular molecule MD2 that physically associates with TLR4 on the cell surface (Shimazu et al., 1999). Some investigators have presented this as the paradigm

accounting for cellular responses to LPS. However, other cell types, and particularly granulocytes, also play a crucial role in host protection. Furthermore, the considerable diversity of bacteria should incite caution in granting the status of representative model to a small group of bacteria such as enterobacteria. Therefore, it is important at the present stage of research in this area to examine what can occur when other cell types are exposed to other types of LPSs.

Concerning the responses of other cell types, it has been reported that Kupffer cells, the resident macrophages of the liver and most abundant tissue macrophages in the body, do not constitutively express CD14 (Tracy et al., 1995). They produce TNF- α via an LBP-independent and CD14-independent pathway, when stimulated by nanogram amounts of enterobacterial LPS (Lichtman et al., 1998). Furthermore, when CD14-deficient mice are injected with low doses of LPS, their liver produces normal levels of acute phase proteins (Haziot et al., 1998). In addition, we reported in previous studies that another mouse cell type, the bone marrow granulocyte (BMG), is sensitive to very low concentrations of enterobacterial LPS, although it does not constitutively express CD14 (Girard et al., 1993). Exposure of BMGs to this type of LPS actually induces a CD14-independent and serum-independent stimulation of the cells, and leads to the expression of CD14 (Pedron et al., 1999; Girard et al., 1997),

and downregulation of L-selectin (Pedron et al., 2001) and TNF-receptor 2 (T.P., R.G. and R.C., unpublished).

Regarding some LPSs that are structurally and/or functionally different from those isolated from enterobacteria, it has been shown that they induce a response in macrophages and B lymphocytes from TLR4-deficient mice (C3H/HeJ mice), which are unresponsive to enterobacterial LPSs. This is particularly the case with the LPSs from *Pseudomonas aeruginosa* (Pier et al., 1981), *Porphyromonas gingivalis* (Tanamoto et al., 1997; Ogawa et al., 1996) and *Prevotella intermedia* (Kirikae et al., 1999). In a recent study (Pedron et al., 2000), we found that LPSs from *Rhizobiaceae* and their structurally atypical lipid A moieties can stimulate BMGs from TLR4-deficient mice (C3H/HeJ and C57BL/10 ScCr) and induce the expression of CD14 in these cells.

It was therefore important to determine whether some LPSs, because of particular structural features of their lipid A moiety, can stimulate cells via a toll-like receptor distinct from TLR4 or some other receptor unrelated to the toll family. Recent publications proposed that LPSs from *Porphyromonas gingivalis* (Hirschfeld et al., 2001) and *Leptospira interrogans* (Werts et al., 2001) may activate macrophages via a TLR2-dependent mechanism. TLR2 is classically considered as mainly involved in the recognition of Gram-positive bacteria and mycobacteria (Yoshimura et al., 1999; Flø et al., 2000). It is also required in innate host defense to *Borrelia burgdorferi*, an atypical Gram-negative bacterium that lacks LPS but abundantly produces lipoproteins (Wooten et al., 2002). In addition to lipoproteins (Hirschfeld et al., 1999), porins can also activate immune cells by engaging TLR2 (Massari et al., 2002).

In the present study, we examined the contributions of TLR4 and TLR2 for the stimulation of BMGs by structurally atypical LPSs. We focused on LPSs for which a reliable background of structural data is available. As a follow-up to our previous studies (Pedron et al., 2000), we first examined the stimulation induced by the rough-type LPS of a plant pathogen: *Rhizobium species* Sin-1. We also examined LPSs from different strains of *Legionella pneumophila*, a human pathogen commonly associated with water-based aerosols and one of the top three causes of nosocomial pneumonias.

Materials and Methods

Animals and cell culture

C57BL/10 ScSn and C57BL/6 mice were purchased from Harlan (Gannat, France). C57BL/10 ScCr mice were a gift from Marina Freudenberg (Freiburg, Germany). Homozygous mutant mice for TLR2 (TLR2^{-/-}) generated by gene targeting, as described previously (Takeuchi et al., 1999), were back-crossed eight times with C57BL/6 to ensure similar genetic backgrounds. C3H/HeOU and C3H/HeJ mice were bred at the Pasteur Institute (Paris, France). 8- to 10-week-old female mice were used in all experiments. Bone marrow cells (BMCs) were collected from mouse femurs. Culture media was RPMI-1640 (Sigma, St Louis, MO) containing 20 mM Hepes, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Fetal calf serum was from Gibco (Grand Island, NY).

Lipopolysaccharides and lipid A

The rough-type LPSs from *Salmonella minnesota* Re-595 and from *Escherichia coli* J5 were from Sigma. The LPS from *Bordetella*

pertussis (LPS-Bp) and the lipid A fraction of the latter (BpLA), were prepared as described previously (Tahri-Jouti et al., 1990). The LPS from *Rhizobium species* Sin-1 ('rough' chemotype) and its lipid A fragment, were provided by R. W. Carlson (University of Georgia, Athens, GA). The LPS was extracted using hot phenol/water (Westphal and Jan, 1965) and purified by gel-filtration chromatography in the presence of deoxycholate (Reuhs et al., 1994). LPSs from three strains of *Legionella pneumophila* serogroup 1 (Philadelphia strain CS338, wild-type OLDA strain RC1 and phase variant OLDA strain 811) were prepared as described previously (Lüneberg et al., 1998; Zou et al., 1999; Kooistra et al., 2002b). The lipid A fragments of the *Legionella* LPSs were prepared by hydrolysis of 10 mg of LPS in 1 ml of 0.1 M sodium acetate/acetic acid buffer (pH 4.4) for 4 hours at 100°C. The lipid A fraction was recovered by centrifugation (2500 g, 15°C, 30 minutes), followed by two washes of the pellet by resuspension in apyrogenic water and centrifugation as described above. The washed pellets were lyophilized.

For removal of lipoprotein contaminants, LPSs and lipid A were re-extracted twice by phenol in the presence of sodium deoxycholate as recommended (Hirschfeld et al., 2000). The absence of contaminants in the LPS and lipid A samples was assessed by SDS-PAGE analysis of the samples and staining the gels with silver nitrate (Rabilloud et al., 1994).

Reagents

MALP-2, prepared as described (Aliprantis et al., 1999), was from Alexis (San Diego, CA). Tripalmitoyl pentapeptide was from Bachem (Bubendorf, Switzerland). Rabbit LBP was kindly provided by Richard Ulevitch (Scripps Research Institute, La Jolla, CA). Mouse recombinant LBP was from Biometec (Greifswald, Germany). The rat anti-mouse CD14 monoclonal antibody (Rm-C5-3) was from PharMingen (San Diego, CA). In FACS experiments, FITC-labeled or biotin-labeled goat anti-rat Ig antibodies (Southern Biotechnology Associates, Birmingham, AL), and FITC-labeled goat anti-hamster Ig antibody (Caltag, Burlingame, CA), were used as secondary antibodies, and biotin-labeled antibodies were stained with FITC-labeled streptavidin (Amersham-Pharmacia Biotech, Little Chalfont, UK). In western blot experiments, the biotin-labeled antibody was stained with a streptavidin-peroxydase conjugate (Southern Biotechnology Associates, Birmingham, AL). Autoradiography Hyperfilm MP, and all electrophoresis reagents, including molecular weight standards (rainbow markers), were from Amersham.

FACS analysis

Bone marrow cells (5×10⁵ cells in 400 µl CM without FCS) were incubated at 37°C with (10 ng/ml) or without LPS. When used, inhibitors were added to cell cultures 30 to 60 minutes before LPS. For detection of membrane antigens, the cells were incubated first (30 minutes, 4°C) with the primary antibody, and stained by reincubation (30 minutes, 4°C) with a labeled secondary antibody. Stained cells were layered on a 50% FCS solution, centrifuged and the cell pellet was resuspended in 0.5 ml of staining buffer (PBS, 5% FCS and 0.02% sodium azide) containing propidium iodide (0.2 µg/ml) to stain dead cells. Fluorescent cells were detected by analysis (5000 cells per sample) on a FACS flow cytometer (FACScan, Becton-Dickinson Electronic Laboratories, Mountain View, CA) using Cell Quest Software. Dead cells, which incorporated propidium iodide, were gated out of analysis. Cells with a fluorescence intensity higher than the maximal level of auto-fluorescence were scored as fluorescent cells.

Preparation of peritoneal macrophages and TNF-α assay

Mice were injected intraperitoneally with 2 ml of 4% thioglycollate (Difco, Detroit, MI). Three days later, peritoneal exudate cells were

isolated from the peritoneal cavity. Then the cells were cultured for 2 hours and adherent cells were used as peritoneal macrophages. Peritoneal macrophages (5×10^4) were cultured in RPMI-1640 medium supplemented with 10% FCS and exposed to LPS (100 ng/ml) for 24 hours. Concentrations of TNF- α in culture supernatants were determined by ELISA (Genzyme-Technique, Minneapolis, MN).

Expression vectors

pFLAG-TLR2 and pFLAG-TLR6 were obtained as described previously (Takeuchi et al., 2001).

Luciferase assay

Human embryonic kidney (HEK) 293 cells were transiently transfected with the vectors indicated above, together with a pELAM luciferase reporter plasmid (Takeuchi et al., 2001) and a pRL-TK (Promega, Madison, WI) for normalization of transfection efficiency by lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the cells were stimulated with the LPS from *Rhizobium* species Sin1 (100 ng/ml) for 8 hours. Then, the cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

SDS-PAGE analysis of membrane CD14

Membrane proteins were extracted from the cell pellet with 1% CHAPS in 300 mM NaCl, 50 mM Tris, pH 7.5, supplemented with a cocktail of proteases inhibitors (aprotinin 10 μ g/ml, PMSF 1 mM, pepstatin and leupeptin at 2 μ g/ml and iodoacetamide 2 mM.). Solubilized proteins were analyzed by SDS-PAGE in 10% polyacrylamide slab gels according to the method of Laemmli. Molecular mass markers from 14.3 to 220 kDa were run in parallel. Gels were fixed in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol) and proteins were transferred onto PVDF membranes (Millipore, Bedford, MA) with a semidry blotting system at 30 volts for 90 minutes. Membranes were blocked (18 hours at 20°C) with 2% BSA in PBS, and incubated (1 hour, 20°C) with the rat anti-mouse antibody rmC5-3 (1:1000 in PBS containing 2% BSA). The blots were washed with 0.1% Tween-20 in PBS, and then incubated for 1 hour at 20°C with a biotin-labeled goat-anti-rat antibody (1:2500 in the same buffer). After extensive washing and incubation with peroxidase-labeled streptavidin (1:20,000 in 2% nonfat milk in PBS), sites with peroxidase activity were detected by chemiluminescence with the Super Signal system (Pierce, Rockford, IL) according to the guidelines of the manufacturer.

Results

TLR2-mediated expression of CD14 induced by the lipid A of *Rhizobium species* Sin-1

We established in a previous study (Pedron et al., 2000) that the lipid A region of several LPSs from Rhizobiaceae can stimulate BMGs of the TLR4-deficient mouse strains C3H/HeJ and C57BL/10ScCr, and induce the expression of CD14 in these cells. This suggests that LPSs from Rhizobiaceae are recognized by another member of the Toll family, or by another receptor. We first analyzed the influence of the LPS isolated from one of these Rhizobiaceae, *R. species* Sin1, on macrophages. This LPS was purified by gel-filtration chromatography in the presence of deoxycholate (Reuhs et al., 1994). Its protein content, measured using a bicinchoninic acid assay kit from Pierce (Rockford, IL) was lower than 0.5%. We found that, unlike BMG, mouse peritoneal macrophages were not activated by this LPS: 100 ng/ml of the *Rhizobium* LPS

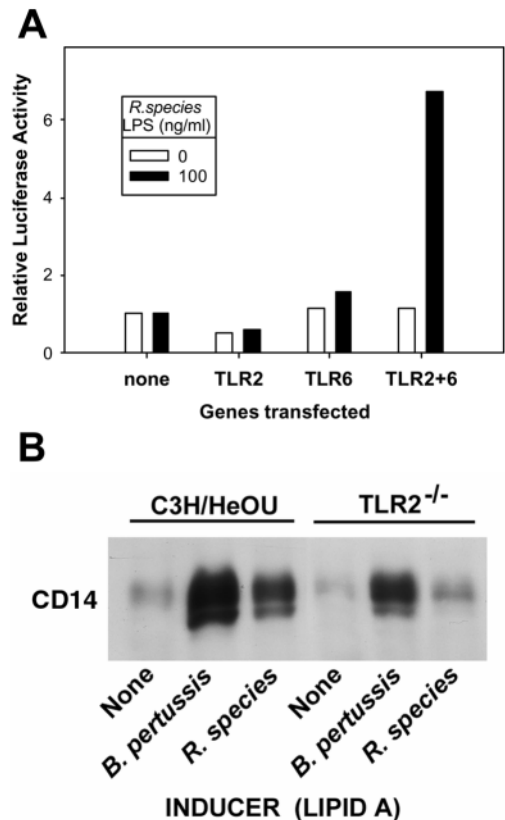


Fig. 1. TLR dependence of cellular responses to the LPS from *R. species*. (A) HEK293 cells transfected with the TLR2 and/or TLR6 expression vectors together with a pELAM luciferase reporter plasmid and pRL-TK were exposed 24 hours later to the *Rhizobium species* Sin1 LPS (100 ng/ml) for 8 hours. TLR-dependent activation was measured by the dual-luciferase reporter assay. (B) BMGs from C3H/HeOU and TLR2^{-/-} mice were incubated with lipid A fractions (1 μ g/ml) isolated from the LPSs of *Bordetella pertussis* and *Rhizobium species* Sin-1. Cell lysates (10^5 cells in 1% CHAPS, supplemented with a mixture of protease inhibitors) were analyzed for CD14 by SDS-PAGE and western blotting.

did not induce detectable levels of TNF- α , whereas the same amount of the *Salmonella minnesota* Re-595 LPS induced a production of 615 pg/ml of the cytokine. To determine whether the *Rhizobium* LPS can trigger cell activation via TLRs distinct from TLR4, HEK293 cells were transfected with TLR2- and/or TLR6-expression vectors along with an ELAM luciferase reporter plasmid, and exposed for 8 hours to 100 ng/ml of the *Rhizobium species* Sin1 LPS. The results in Fig. 1A indicate that, in this model, cell activation by the *Rhizobium* LPS requires the expression of both TLR2 and TLR6. As exemplified by this cooperation between two TLRs, another membrane component present in granulocytes and absent from macrophages may also be required for cell activation by some atypical LPSs, and thus explain why the *Rhizobium* LPS stimulates BMGs but does not activate macrophages at low concentrations.

To analyze the effect of the *Rhizobium* LPS in a more physiological cellular system than a transfected cell line, we re-examined the response of BMGs, and particularly the role of TLR2 in this response. We compared the expression of CD14 in BMGs from normal (C3H/HeOU) and TLR2-

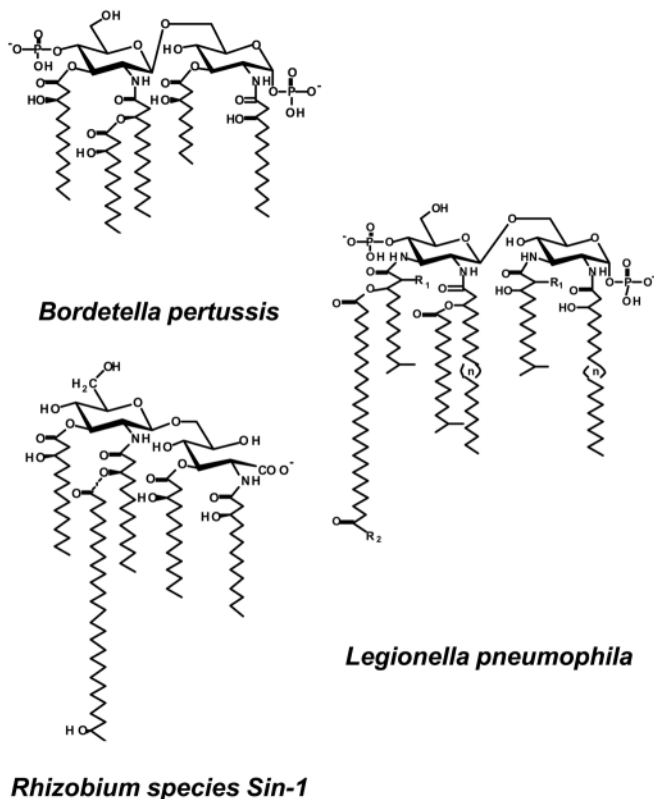


Fig. 2. Structures of the lipid A regions of LPSs used in this study. The structure of the lipid A region of *R. species Sin-1* is not completely determined but, as that from some other Rhizobiaceae (Bhat et al., 1994), it is devoid of phosphate, has 2-aminogluconate in place of glucosamine-1-phosphate, and contains the very long chain of 27-hydroxyoctacosanoic acid (27-OHC28:0) that may be ester-linked in the N-acyloxyacyl residue of the distal glucosamine unit (Basu et al., 1999). The lipid A regions of the LPSs from three strains of *Legionella pneumophila* (Zähringer et al., 1995; Kooistra et al., 2002a) are built upon the same structural model (two residues of 2,3-diaminoglucose substituted with one very long and five shorter fatty acids), with variations limited to the length or substituents of the fatty acid chains in positions 2 and 2': R₁=H or OH; R₂=OH or CH₃; *n*=18-22 (in strains CS338 and RC1), or *n*=16-18 (in strain 811).

deficient (TLR2^{-/-}) mice. The lipid A fragments of LPSs from *Bordetella pertussis* and *Rhizobium species Sin-1* were used as inducers because they represent the biologically active region of the LPSs, and also because eventual lipoprotein contaminants of LPSs are removed during the preparation of the lipid A fragments (as observed by SDS-PAGE analysis of a number of such lipid A preparations). Therefore, BMGs were exposed to lipid A for 24 hours at 37°C, in the absence of serum. The results of the analysis of CD14 expression by western blot (Fig. 1B) indicated that, in normal cells, the two lipid A fractions induced the expression of large amounts of CD14. The *B. pertussis* lipid A was also active in TLR2^{-/-} cells. In contrast, a much lower expression of CD14 was induced by the lipid A from *R. species Sin-1* in TLR2^{-/-} cells. This result explains why this atypical LPS was active in TLR4-deficient mice (Pedron et al., 2000), and shows that this effect is mediated by TLR2.

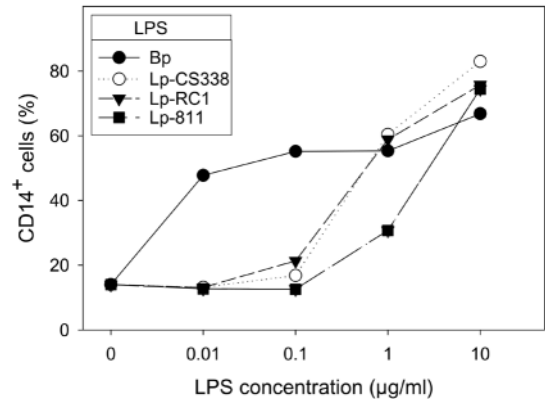


Fig. 3. Influence of *Legionella* LPSs on BMGs of LPS-responsive mice. BMGs (5×10^5 cells) from C3H/HeOU mice were incubated for 24 hours at 37°C with various concentrations of LPSs from *B. pertussis* (●), and from the *L. pneumophila* strains CS338 (○), RC1 (▼), and 811 (■). CD14 was detected by incubation with the anti-CD14 antibody rmC5-3 (2.5 µg/ml, 30 minutes, 0°C), and further incubation (30 minutes, 0°C) with a FITC-labeled goat anti-rat Ig mAb. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population.

LPSs from *Legionella* induced CD14 expression in BMGs from normal mice

The structure of the *Rhizobium* lipid A (Fig. 2) is atypical. When compared with the *Bordetella pertussis* lipid A, which acts via TLR4, the *Rhizobium* lipid A exhibits three major differences: the absence of phosphate groups, a modified sugar backbone (a glucosamine unit is replaced by 2-aminogluconic acid), and the presence of a very long fatty acid chain [28:0(27-hydroxy) residue] (Bhat et al., 1994; Basu et al., 1999). In searching for other LPSs with at least one of these atypical structural features, we chose the *Legionella* LPSs because they also contain a long fatty acid chain [27:0(1,27-dioic) or 28:0(27-oxo) residue]. The three strains of *Legionella pneumophila* used (CS338, RC1 and 811) have similar lipid A structures, that differ only in their heterogeneity in the proportion and length of the different fatty acid chains (Fig. 2) (Zähringer et al., 1995; Kooistra et al., 2002a). We exposed BMGs from C3H/HeOU mice to various concentrations of the three *Legionella* LPSs, and analyzed by FACS the expression of CD14. The results (Fig. 3) show that the three LPSs from *Legionella* can induce cell surface expression of CD14, although the concentrations of these LPSs required for this response (1 µg/ml) were much higher than that at which the *B. pertussis* LPS induced a similar effect (0.01 µg/ml).

Stimulation of BMGs from TLR4-deficient mice by the *Legionella* LPSs

To examine the role of TLR4 in the activation of BMGs by *Legionella* LPSs, we compared the expression of CD14 by BMGs from C3H/HeOU, C3H/HeJ and C57BL/10ScCr mice. In a first set of experiments, the cells were analyzed by FACS after exposure for 24 hours to 1 or 10 µg/ml of the LPSs. Several experiments gave similar results. As shown in one representative experiment (Fig. 4A), the three *Legionella* LPSs induced CD14 expression in C3H/HeJ and C57BL/10ScCr

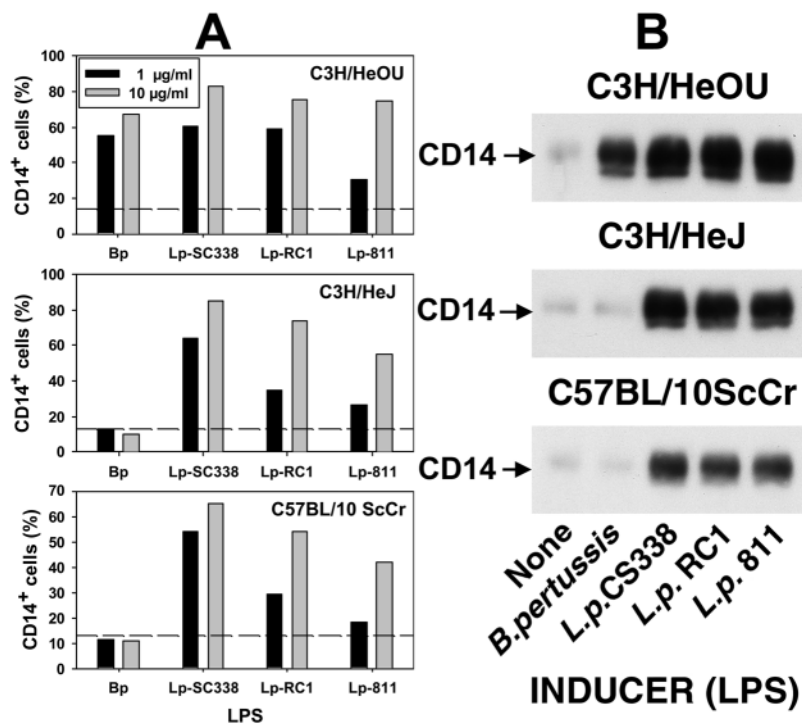


Fig. 4. CD14 expression in BMGs from normal and TLR4-deficient mice. BMGs from C3H/HeOU, C3H/HeJ and C57BL/10ScCr mice were incubated for 24 hours at 37°C with the *B. pertussis* LPS, or with LPSs from the different strains of *L. pneumophila*. (A) CD14 expression in BMGs incubated with 1 or 10 µg/ml of the LPSs was detected by FACS analysis of the gated granulocyte population, as described in the legend to Fig. 3. The dashed line represents the level of CD14 in non-stimulated cells. (B) CD14 expression in BMGs incubated with 10 µg/ml of the LPSs was analyzed by western blot in cell lysates, as described in the legend to Fig. 1.

mice, whereas the *B. pertussis* LPS did not. The most efficient activator was the LPS from the *L. pneumophila* CS338 strain. In a second set of experiments, expression of CD14 was analyzed by western blot. The results (Fig. 4B) were consistent with those obtained by FACS, and confirmed that the three LPSs of *Legionella* are efficient activators of BMGs from mice with a TLR4 defect.

Influence of the *Legionella* LPSs on TLR2-deficient macrophages and BMGs

The capacity of *Legionella* LPS to activate BMGs from TLR4-defective mice suggests that another receptor is involved. By analogy with the results obtained above with the *Rhizobium* LPS, we used TLR2-deficient (TLR2^{-/-}) mice to examine the role of TLR2. We analyzed first the responses of macrophages. We found (Table 1) that thioglycollate-elicited peritoneal macrophages from TLR2^{-/-} mice did not respond to the TLR2-dependent mycobacterial lipopeptide MALP-2, and produced normal levels of TNF-α in response to *Salmonella* and *Bordetella* LPSs. In contrast, these cells were not activated by the *Legionella* LPSs.

The stimulation of BMGs by the *Legionella* LPSs was then examined. The expression of CD14 in BMGs from TLR2^{-/-} mice was analyzed by FACS, and compared with that of BMGs from normal mice of the same genetic background (TLR2^{+/+}). Several experiments gave similar results. As shown in one representative experiment (Fig. 5A), the LPS isolated from the strain CS338 of *L. pneumophila* was completely unable to induce CD14 expression in TLR2^{-/-} cells. CD14 expression was detectable in TLR2^{-/-} cells exposed to the two other *Legionella* LPSs, but the responses were lower than those of TLR2^{+/+} cells, and were only induced by high concentrations of these LPSs (10 µg/ml). These residual responses may indicate that a receptor distinct from TLR2 reacts with these

LPSs or with a contaminant present in the LPS preparations. To ensure that contaminations cannot be put forward, the three *Legionella* LPSs were re-extracted twice by phenol in the presence of sodium deoxycholate (Hirschfeld et al., 2000). Because of their hydrophobic nature, the *Legionella* LPSs are fairly soluble in phenol, so that only 10% of the LPSs were recovered in the aqueous phase. However, the ability of these purified (lipoprotein-depleted) samples to induce CD14 in BMGs of different mouse strains was not different from that of the non-extracted samples. Analysis of CD14

expression by western blot (Fig. 5B) confirms that this cell response to *Legionella* LPSs is markedly reduced, but not completely abolished, in TLR2^{-/-} BMGs.

Effects of lipid A fragments in TLR4- and TLR2-deficient cells

Some cellular activities of LPS preparations are due to their polysaccharide region, or are modulated by that region. Other bacterial components (peptides, lipoproteins) present as contaminants in LPS preparations can also take part in cell responses (Hirschfeld et al., 2000). However, it is generally accepted that LPS effects are those mediated by the lipid A region of that molecule. To check that this was indeed the case for the effects of the *Legionella* LPSs mentioned above, we prepared the lipid A fragments of these molecules by hydrolysis for 4 hours at 100°C in a pH 4.4 buffer and washing

Table 1. LPS-induced TNF-α production by macrophages of wild-type and TLR2^{-/-} mice

Inducer	Concentration (ng/ml)	TNF-α produced (pg/ml)	
		Wild-type mice	TLR2 ^{-/-} mice
None	–	0±0	0±0
MALP-2	3	558±23	0±0
LPS Sm-595	100	615±64	800±200
LPS Bp-414	100	388±40	346±78
LPS Rs-Sin1	100	0±0	0±0
LPS Lp-338	100	386±28	0±0
LPS Lp-811	100	225±34	0±0
LPS Lp-RC1	100	1061±174	0±0

Thioglycollate-elicited peritoneal macrophages (10⁵ cells/well) from wild-type, and TLR2^{-/-} mice, were incubated for 24 hours with MALP-2 (3 ng/ml) or with different LPSs (100 ng/ml). The production of TNF-α was determined by ELISA. Data are the means±s.d. of three experiments.

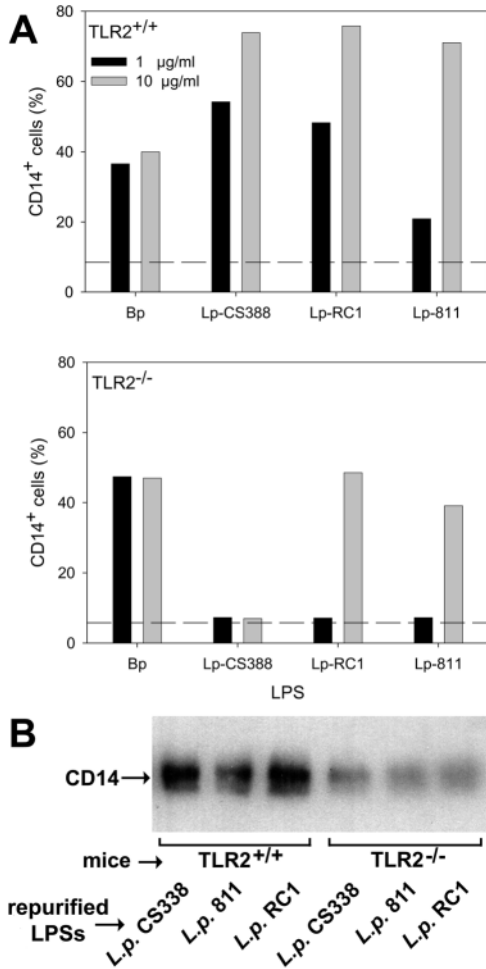


Fig. 5. Analysis of CD14 expression in BMGs from TLR2^{+/+} and TLR2^{-/-} mice. BMGs from TLR2^{+/+} and TLR2^{-/-} mice were incubated for 24 hours at 37°C with the *B. pertussis* LPS, or with LPSs from the different strains of *L. pneumophila*. (A) CD14 expression in BMGs incubated with 1 or 10 µg/ml of the LPSs was detected by FACS analysis of the gated granulocyte population, as described in the legend to Fig. 3. The dashed line represents the level of CD14 in non-stimulated cells. (B) CD14 expression in BMGs incubated with 10 µg/ml of re-purified LPSs (re-extracted with phenol/deoxycholate) was analyzed by western blot in cell lysates, as described in the legend to Fig. 1.

the insoluble material with water. Analysis of these lipid A preparations by SDS-PAGE stained with silver nitrate indicated an almost complete absence of protein contamination (protein content lower than 0.01%, as determined by comparison of band intensities of protein standards). BMGs from C3H/HeJ, TLR2^{+/+}, and TLR2^{-/-} mice were then exposed to these lipid A preparations, and CD14 expression was analyzed by western blot. The results (Fig. 6) were similar to those induced by the unfragmented LPS preparations: the three lipids were active in C3H/HeJ cells, and the activities were markedly reduced in TLR2^{-/-} cells.

Because *Legionella* LPSs are fairly soluble in phenol (see above), whereas their lipid A fraction is not, the lipid A isolated from *Legionella pneumophila* RC1 was re-extracted twice by phenol in the presence of sodium deoxycholate, to remove

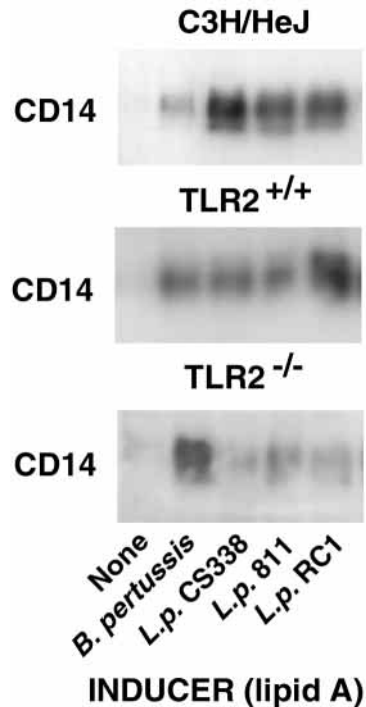


Fig. 6. CD14 expression induced by lipid A fractions in BMGs from TLR4- and TLR2-deficient mice. BMGs from C3H/HeJ, TLR2^{+/+} and TLR2^{-/-} mice were incubated for 24 hours at 37°C with lipid A from *B. pertussis* (10 µg/ml), or from the different strains of *L. pneumophila* (50 µg/ml). CD14 expression was analyzed by western blot in cell lysates, as described in the legend to Fig. 1.

residual amounts of unhydrolyzed LPS. This purified lipid A, in which the level of protein contamination was considerably lower than that of a standard *E. coli* LPS (Fig. 7A), was considerably more active in BMCs from TLR4-defective mice (Fig. 7B). This confirms that pure lipid A from *Legionella pneumophila* RC1 induces a TLR4-independent effect. We also found that the residual activation of TLR2^{-/-} BMGs induced by the re-purified LPS is markedly reduced with the re-purified lipid A (Fig. 7C). This observation suggests that the lipid A of *Legionella pneumophila* RC1 stimulates BMGs exclusively via TLR2, whereas the unfragmented LPS, which contains an additional polysaccharide region, stimulates BMGs via TLR2 and another receptor.

Dose-response experiments indicated that even low concentrations (100 ng/ml) of *L. pneumophila* lipid A can induce detectable levels of CD14 in BMGs from TLR4-deficient (C3H/HeJ) mice. The presence of fetal calf serum (10%), or of rabbit or recombinant mouse LBP (10 µg/ml) did not increase this response (data not shown).

Antagonist effect of *Bordetella pertussis* lipid A

We established in a previous study (Pedron et al., 2000) that the lipid A fragment of the *B. pertussis* LPS inhibits the expression of CD14 induced by the lipid A of *Rhizobium species* Sin-1 in BMGs from C3H/HeJ mice. As we had previously demonstrated that *Rhizobium* and *Legionella* LPSs are both TLR2-dependent stimulators of BMG, it was important to determine whether *B. pertussis* lipid A can also inhibit the TLR2-dependent response induced by the *Legionella* LPSs. In a first experiment, BMGs from C3H/HeJ mice, preincubated for 90 minutes with various concentrations of *B. pertussis* lipid A, were exposed for 20 hours to the LPS of *L. pneumophila* CS338. CD14 expression was detected by FACS. The results (Fig. 8A) show that 1 µg/ml of lipid A

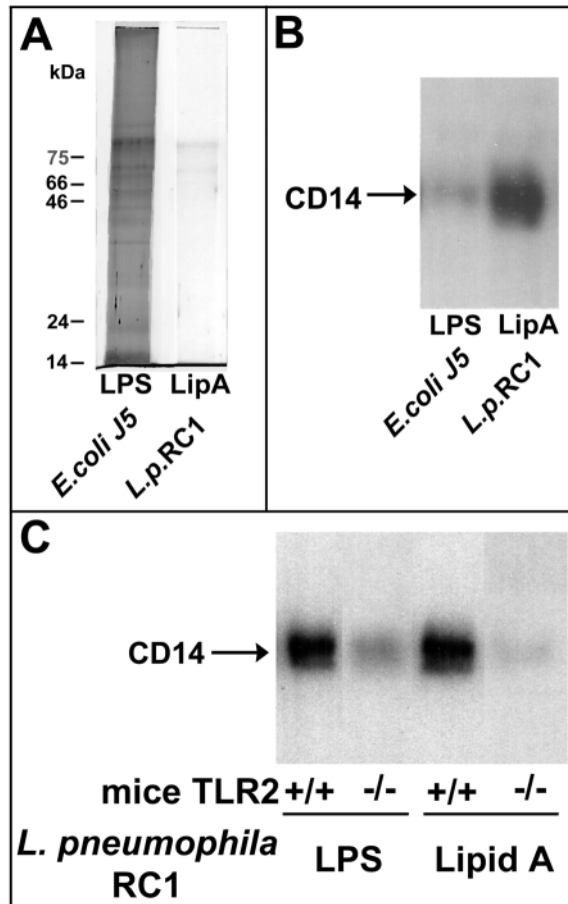


Fig. 7. Analysis of the re-purified lipid A fractions of *L. pneumophila* RC1. A re-purified lipid A fraction (re-extracted with phenol/deoxycholate) from *L. pneumophila* RC1 analyzed for the presence of contaminants (A) and for its ability to induce CD14 expression in BMGs from C57BL/10ScCr (B) and TLR2^{-/-} (C) mice. (A) Samples (40 µg) of LPS from *E. coli* J5 and of re-purified lipid A from *L. pneumophila* RC1 were analyzed by SDS-PAGE stained with silver nitrate. (B) Western blot analysis of CD14 expression induced in BMGs from C57BL/10ScCr mice by 10 µg/ml of the same samples of crude LPS from *E. coli* and re-purified Lipid A from *L. pneumophila* RC1. (C) Western blot analysis of CD14 expression induced in BMGs from TLR2^{+/+} and TLR2^{-/-} mice by 10 µg/ml of re-purified LPS and Lipid A from *L. pneumophila* RC1.

inhibited the stimulation induced by the LPS of *Legionella*. In a second experiment, BMGs from C3H/HeJ mice, preincubated for 2 hours with 10 µg/ml of *B. pertussis* lipid A, were exposed for 20 hours to the lipid A of *L. pneumophila* CS338. CD14 expression was detected by western blot. We found again (Fig. 8B) that *B. pertussis* lipid A induced a partial inhibition of the stimulation triggered by the *Legionella* lipid A. Similar results were obtained with the lipid A fragments of the two other strains of *Legionella* (data not shown). In contrast, pre-treatment of the cells with *B. pertussis* lipid A (10 µg/ml) did not inhibit CD14 expression induced in BMGs from C3H/HeJ mice by other TLR2-dependent ligands such as MALP-2 (0.1 to 5 ng/ml) and tripalmitoyl pentapeptide (0.5 to 2.5 µg/ml) (data not shown). This result suggests that *B. pertussis* lipid A, which is an agonist of TLR4, acts also as a

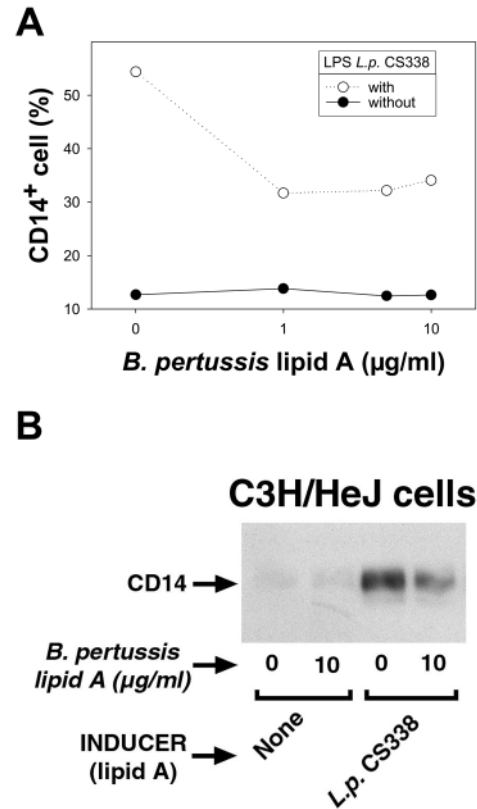


Fig. 8. Inhibitory effect of *B. pertussis* lipid A in C3H/HeJ cells. BMGs from C3H/HeJ mice were preincubated (90 minutes, 37°C) in the presence of various concentrations of *B. pertussis* lipid A. (A) The cells were reincubated (20 hours, 37°C) with (○) or without (●) LPS (1 µg/ml) from *L. pneumophila* CS338, and CD14 was detected by FACS analysis of the gated granulocyte population, as described in the legend to Fig. 3. (B) The cells were reincubated (20 hours, 37°C) with or without the lipid A fraction of *L. pneumophila* (0.1 µg/ml), and CD14 expression was analysed by western blot as described in the legend to Fig. 1.

specific antagonist of the interaction between TLR2 and *Legionella* lipid A.

Discussion

In a follow-up of our previous observation that *Rhizobium* LPSs induce CD14 expression in BMCs by a TLR4-independent mechanism (Pedron et al., 2000), we found that this effect is mediated by TLR2. Because this effect is due to the lipid A region of *Rhizobium* LPSs, and because one of the common structural features of these atypical lipid As is the presence of a very long fatty acid chain, we examined whether other LPSs containing long chain fatty acids can activate BMCs by the same mechanism. We found that this is indeed the case with *Legionella* LPSs. It should be noted that the presence of long chain fatty acids not only induces a switch from TLR4- to TLR2-mediated responses but also reduces the global reactivity of the LPS, as assessed by the higher concentrations of such atypical LPSs required to activate BMCs (1-5 µg/ml of *Rhizobium* and *Legionella* LPSs, vs. 10 ng/ml of enterobacterial or *B. pertussis* LPS). Long chains of

fatty acids in lipid A can even almost completely abolish the ability of an LPS to activate BMCs. For example, we found that with the lipid A from *Rhodopseudomonas viridis*, which contains the longest fatty acid ever seen in a lipid A [C30:0(27,29-di-hydroxy)], at least 10 µg/ml were required to induce CD14 in BMCs of normal mice, and that the LPS from *Brucella abortus*, which carries significant amounts of 27-hydroxy-C28:0 fatty acid (Moreno et al., 1990), was unable to induce CD14 expression, even at high concentrations (data not shown). This was also the case with the LPS from *Francisella tularensis* (Ancuta et al., 1996)

The observation that particular LPSs can activate cells of the immune system via TLR2 has already been reported by two groups, working on *Porphyromonas gingivalis* (Hirschfeld et al., 2001) and *Leptospira interrogans* (Werts et al., 2001). The chemical structure of the latter is presently under investigation, but that of the former has been fully elucidated. Therefore, comparisons are now possible between the structures of three LPSs with TLR2-dependent activities: those from *Porphyromonas*, *Legionella* and *Rhizobium*. Four features can be considered: the number of phosphate groups, the number of fatty acids, the length of the fatty acids, and the presence of branched fatty acid chains. The number of phosphate groups does not seem to be an important parameter for TLR2-dependence since 0, 1 and 2 phosphate groups are present in the lipid A regions of *R. species*, *P. gingivalis* and *L. pneumophila*, respectively. The number of fatty acid chains in their lipid A (5, 4 and 6, respectively) does not seem to be critical either. The length of the fatty acid chains may play some role since two of these LPSs have one long chain (C₂₈ in *R. species* and *L. pneumophila*) and one LPS has two fatty acids of medium size (C₁₇ in *P. gingivalis*). But the most critical feature seems to be the presence of a substituent or a branch on the penultimate carbon of a fatty acid chain: a hydroxyl group at C₂₇ in *Rhizobium* LPS, a ketone group at C₂₇ in *Legionella* LPSs, and a methyl branch at C_{n-1} of different fatty acids in *Legionella* and *Porphyromonas* LPSs. These structural features may also be important for the 3D supramolecular structures of lipid A (conical, cylindrical or lamellar) (Brandenburg et al., 1993), which have been postulated to determine differential interactions with TLRs (Netea et al., 2002). On the structural basis mentioned above, we can expect that other TLR4-independent LPSs such as those of *Flavobacterium meningosepticum* (Tanamoto et al., 2001), *Pseudomonas aeruginosa* (Pier et al., 1981), and *Prevotella intermedia* (Kirikae et al., 1999) could also activate cells via TLR2. This would then mean that the presently accepted paradigm of 'LPS activation via TLR4' is no more valid since an increasing number of examples indicate that structurally different LPSs can activate cells via different TLRs, and there is no objective reason to consider that one among the different LPS structures should be privileged, particularly when we are reminded that *Legionella* is a frequent cause of human nosocomial infection.

Our data on the activation of macrophages by *Rhizobium* LPSs (Fig. 1A) provide additional insight into the mechanism of action of this type of LPS. The requirement of both TLR2 and TLR6 is reminiscent of results obtained with some other microbial components such as the yeast cell-wall zymosan (Ozinsky et al., 2000), the mycoplasmal lipopeptide MALP-2 (Takeuchi et al., 2001), the *Staphylococcus epidermidis* modulin

(Hajjar et al., 2001), and the *Borrelia burgdorferi* outer surface protein A lipoprotein (Bulut et al., 2001), which all require both TLR2 and TLR6 for optimal activation of macrophages. This means that mechanisms triggered by microbial lipopeptides can be extended to some structurally atypical LPSs, and may suggest that heteromeric associations between TLR2 and TLR6 can recognize particular LPS structures that are unable to signal via TLR4.

Another interesting observation of the present study is that *Bordetella pertussis* lipid A (BpLA) partially inhibits the activation induced by *Legionella* lipid A (LLA) in BMCs from C3H/HeJ mice. One possible explanation for this observation is that BpLA induces a cross-inhibition via its interaction with TLR4. Indeed, reduction of surface expression of the TLR4-MD2 complex has been reported after LPS treatment, even in C3H/HeJ mice that are hyporesponsive to LPS (Nomura et al., 2000). Therefore, we can speculate that in BMCs from C3H/HeJ mice, the downregulation of the TLR4-MD2 complex triggered by BpLA induces a partial cross-inactivation of TLR2 that lowers the response to the TLR2-dependent LLA. This would mean that this partial cross-inhibition of TLR2 via TLR4 occurs via a MAL/TIRAP-independent signaling mechanism. Such inter-TLR cross-talks leading either to synergy (Beutler et al., 2001; Gao et al., 1999) or to inhibition/anergy (Sato et al., 2000) have already been reported.

However, this hypothesis of cross-inhibition of TLR2 via TLR4 must probably be rejected because we found that BpLA did not inhibit the action of other TLR2-dependent inducers such as MALP-2 and tripalmitoyl pentapeptide. Therefore, another explanation must be proposed for our observation. The most likely is that BpLA behaves as a specific antagonist of LLA on TLR2. This can occur if a partial occupancy of the LPS-binding site of TLR2 by BpLA is not sufficient for triggering the signaling cascade, but makes TLR2 inaccessible for LLA. If this hypothesis is correct, it would mean that TLR2 can interact with many lipid A structures, but only some of these interactions can lead to signaling and cell activation. This hypothesis, and the specificity of the antagonism induced by BpLA, also implies that the TLR2 activator (LLA) and the TLR2 antagonist (BpLA) interact with the same binding site of TLR2, whereas other TLR2 activators (lipopeptides) interact with another binding site on TLR2. Additional investigations are required to confirm this hypothesis.

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