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The role of lipopolysaccharide moieties in macrophage response to *Escherichia coli*

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ABSTRACT

Lipopolysaccharide (LPS) is the main component of Gram-negative bacteria that – upon infection – activates the host immune system and is crucial in fighting pathogens as well as in the induction of sepsis. In the present study we addressed the question whether the key structural components of LPS equally take part in the activation of different macrophage immune responses. By genomic modifications of *Escherichia coli* MG1655, we constructed a series of strains harboring complete and truncated forms of LPS in their cell wall. These strains were exposed to RAW 264.7 macrophages, after which phagocytosis, fast release of pre-synthesized TNF and activation of NF-κB signal transduction pathway were quantified. According to our results the core and lipid A moieties are involved in immune recognition. The most ancient part, lipid A is crucial in evoking immediate TNF release and activation of NF-κB. The O-antigen inhibits phagocytosis, leading to immune evasion.

Introduction

Macrophages are cells of the innate immune system and react rapidly to diverse microbes. To shorten the reaction time, they store mRNA of pro-inflammatory cytokines in their cytoplasm, furthermore, they release pre-synthesized TNF from secretory granules immediately in response to bacteria [1]. Macrophages ingest bacteria by phagocytosis, destroy them within phago-lysosomes, and present components of the bacteria to T cells. It was noted that phagocytic receptors like scavenger receptors and complement receptor 3 (CR3, CD11b/CD18) bind LPS on bacterial surfaces and also recognize microbes that are coated with serum opsonins [2–5]. An overlapping set of genes is activated in RAW 264.7 macrophage-like cells either stimulated with LPS or infected with high numbers of *Salmonella typhimurium* [6–7]. It has been well elucidated that bacteria or LPS activates transcription of genes encoding inducible enzymes and cytokines through the Toll-like receptor-4/MD2 complex [8–10]. Toll-like receptors can co-operate with each other and also with CR3 [11–13]. Increasing evidence indicates the role of lipid rafts in the innate immune response: recruitment of TLR-4 and other proteins into lipid rafts has been observed upon LPS stimulation in RAW 264.7 macrophages [14–17].

We decided to study the efficiency of bacterium-internalization, the release of pre-synthesized TNF and production of pro-inflammatory cytokines as responses to *Escherichia coli* bacteria with different LPS composition.

LPS is a component of the outer membrane in the cell wall of Gram-negative bacteria and is composed of three distinct regions: the O-antigen polysaccharide, a short core oligosaccharide, and lipid A, also known as endotoxin. We used the *MG CL* (MG1655 laboratory strain) bearing core region and lipid A, the *MG OCL* genetically modified strain expressing complete LPS, and two deletion mutants: *MG L*, that expressed solely lipid A, and *MG CΔL* strain synthesizing normal core region but penta-acylated, rather than hexa-acylated lipid A (Fig. 1.). The role of eukaryotic membrane microdomains in the host–microbe interactions was also investigated.

Materials and methods

Cell lines. E. coli K-12 laboratory strain MG1655 – called *MG CL* in our experiments – produces complete lipid A and core region but lacks O-antigen due to a common mutation (*rfb-50*) of all K-12 strains [18] (Fig. 1.). *MG OCL* strain was made in our lab using a previously published method rescuing *rfb-50* mutation and leading to the production of complete LPS molecules [19–21]: *MG OCL* bacteria express the full length *wbbL* gene from a pUC19 plasmid (New England Biolabs, Inc. Beverly, MA, USA) in *MG CL*. *MG L* and *MG CΔL* strains are derivatives of *MG CL* made in our lab as well, using suicide (conditionally replicative) plasmid-based allele replacement. Detailed description of the method can be found in Ref. [22] and [23]. *MG L* strain did not produce the core region

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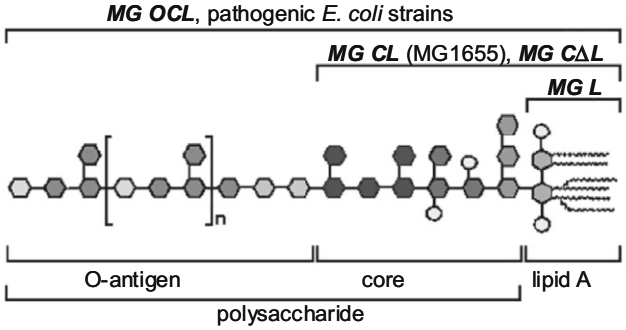

Fig. 1. Structure of the LPS molecules of different bacterial strains. *MG OCL* strain, producing complete LPS molecules, was made by completing rfb-50 mutation of laboratory strain *MG CL* (MG1655). The deletion strain *MG L* could synthesize only lipid A fragments. *MG CΔL* strain produced truncated, penta-acylated lipid A, although it made core regions, like MG1655 or DH5α.

due to deletion of a set of genes (Table S1), except for the lipid A moiety of LPS. *MG CΔL* strain contained the core region but its lipid A moieties were penta-acylated rather than hexa-acylated due to the deletion of the *msbB* gene coding for the myristoyl acyltransferase enzyme [24]. Successful deletion of genes was proved by polymerase chain reactions.

The RAW 264.7 mouse macrophage cell line (ATCC TIB 71) was grown in DMEM, 5% FCS (heat inactivated). Activation of the NF–κB pathway in macrophages was detected with a RAW 264.7 subline carrying the pNF-κB-luc/Neo plasmid. The pNF-κB-luc/Neo plasmid was made in our laboratory by cloning the Neo cassette of the pRc/CMV vector (Invitrogen) into the pNF-κB-luc plasmid (Stratagene, PathDetect cis-Reporting System). L929 mouse fibroblast cells (ATCC CCL-1) – transformed with the pNF-κB-luc reporter plasmid – were used for following the NF-κB pathway activation after exposure of cells to TNF produced by macrophages. L929 cells were grown in DMEM 10% FCS.

Following phagocytosis by cytoflurometry. Diluted, overnight cultures of bacteria (10^8 cells/ml) were labeled with the intracellular fluorescent dye Mitotracker Green FM (Molecular Probes) at 0.75 μg/ml for 1 h at 37 °C, then the labeled bacteria were pelleted and washed with PBS, resuspended in DMEM and added to the macrophages in different concentrations. Macrophages and the fluorescently labeled *E. coli* cells were co-incubated for 1, 2, or 3 h at macrophage: bacterium ratios of 1:1, 1:10, or 1:100. At the end of the co-incubation, the fluorescence of the eukaryotic cells was detected by a flow cytofluorometer. Data were acquired on a FACS CaliburTM and analyzed with CellQuestTM software (Becton Dickinson, USA).

Measuring luciferase activity in NF-κB-luc reporter cell lines. Macrophage activation was followed with RAW/NF-κB-luc cells, based on the fact that bacteria and LPS trigger the NF-κB pathway in macrophages. RAW/NF-κB-luc cells were plated onto 24 well plates in a density of 10^5 cells per well. The next day different bacteria (in concentrations of 10^6 or 10^7 cells/ml) or LPS (*E. coli* LPS, strain 055:B5; Sigma Aldrich) solutions (0.1; 1, or 10 μg/ml) were added. Cells were lysed after 2, 4, or 6 h-incubation periods and the kinetics of NF-κB activation was detected by the Promega Luciferase Assay Kit (Promega, Madison, USA).

Bioassay of TNF. TNF can also activate the NF-κB signaling pathway in L929 TNF-sensitive mouse fibroblast cells. This bioassay was used to detect TNF produced by macrophages. L929/NF-κB-luc cells were plated onto 24 well plates in a density of 10^5 cells per well. The next day, 150 times diluted supernatants of RAW 264.7 cells (previously co-incubated with different bacteria) were added to the reporter cells. After a 6 h-incubation, L929/NF-κB-luc cells were lysed and the luciferase activity of the extracts was measured using the Promega Luciferase Assay Kit. Quantity of TNF and activation of NF-κB were linear within a wide range of TNF concentrations. Anti-TNF antibodies completely neutralized the NF-κB-activating effect of RAW 264.7 supernatants (data not shown).

TNF ELISA. TNF-content of macrophage supernatants was detected by ELISA as well, according to instructions provided by the manufacturer (Mouse TNF-α/TNFSF1A DuoSet, R&D Systems).

IL-6 titration from RAW 264.7 supernatants. IL-6 content of RAW 264.7 supernatants was detected in a proliferation assay using B9 hybridoma cells [25]. B9 cells grew in RPMI 1640 medium, in the presence of IL-6 (hrIL-6 at 1000 u/ml a kind gift of Prof. A. Falus). Cultures were fed with IL-6-containing medium every 3 days. When performing the assay, serially diluted samples were loaded onto microtiter plates (100–100 μl/well) and then washed hybridoma cells were added (5000 cells/100 μl/well). 500 u/ml of hrIL-6 standard was used as positive control. After a 3 day-incubation, cell proliferation was detected by MTT assay [26]. Optical density of the samples was measured at 570 nm by a Multiscan ELISA reader (Labsystems Oy, Finland).

NO-determination. NO produced by cells was assayed as nitrite end product as described by Misko et al. [27]. Briefly, 30 μl of the RAW 264.7 supernatants were brought to 100 μl with double deionized water. Then, 10 μl of 2,3-diaminonaphtalene (DAN, Sigma) solution (0.05 mg/ml in 0.62 M HCl) was added and incubated at room temperature for 10 min. The reaction was terminated by the addition of 10 μl 1.4 M NaOH and the formation of 2,3-diamino-naphthotriazole was measured using a Labsystems Ascent Fluoroscan fluorescent plate reader with excitation at 355 nm and emission read at 460 nm. The amount of nitrite in the samples was calculated using a calibration curve. Known amounts of sodium nitrite (Merck) were added to the culture media and handled in the same way as samples were.

Membrane cholesterol depletion with methyl-β-cyclodextrin (Mβ-CD)-treatment. Macrophages were washed twice with PBS followed by the addition of 10 mM Mβ-CD solution (SIGMA) 1 h before the experiments. In preliminary experiments, we excluded toxicity of Mβ-CD on RAW 264.7 cells in a 2 h-incubation by propidium-iodide (1.5 μg/ml final concentration) labeling. The morphology of the Mβ-CD-treated cells did not change either, ruling out early apoptosis undetectable by PI incorporation (data not shown).

Cholesterol detection with thin layer chromatography. We compared the cholesterol content of control and Mβ-CD-treated cells by thin layer chromatography (Fig. 2C). Cholesterol was extracted from washed cells using the method of Folch et al. [28] and separated on thin layer chromatography plates (MERK) in hexane:ether:acetate solution (60:40:2). The plates were treated with H_2SO_4 and incubated at 180 °C to visualize cholesterol.

Results

Quantifying the uptake of different E. coli strains

We investigated the effect of bacterial LPS composition on the phagocytic activity of RAW 264.7 mouse macrophages. Mammalian cells were exposed to fluorescently labeled bacteria with complete or truncated LPS molecules on their surface. Fluorescent labeling of the bacteria and kinetics of the phagocytosis were followed by flow cytofluorometry. We compared the internalization of different bacteria in the presence and absence of serum. In serum free medium (Fig. 2A), the *MG OCL* and *MG CL* strains were phagocytosed with equal efficiency, while the absence of the core region in the case of the *MG L* or the absence of the myristoyl chain in the case of *MG CΔL* (i.e., penta-acylated lipid A instead of the wild type hexa-acylated form) strains decreased the phagocytosis by RAW 264.7 cells, indicating that in the absence of serum

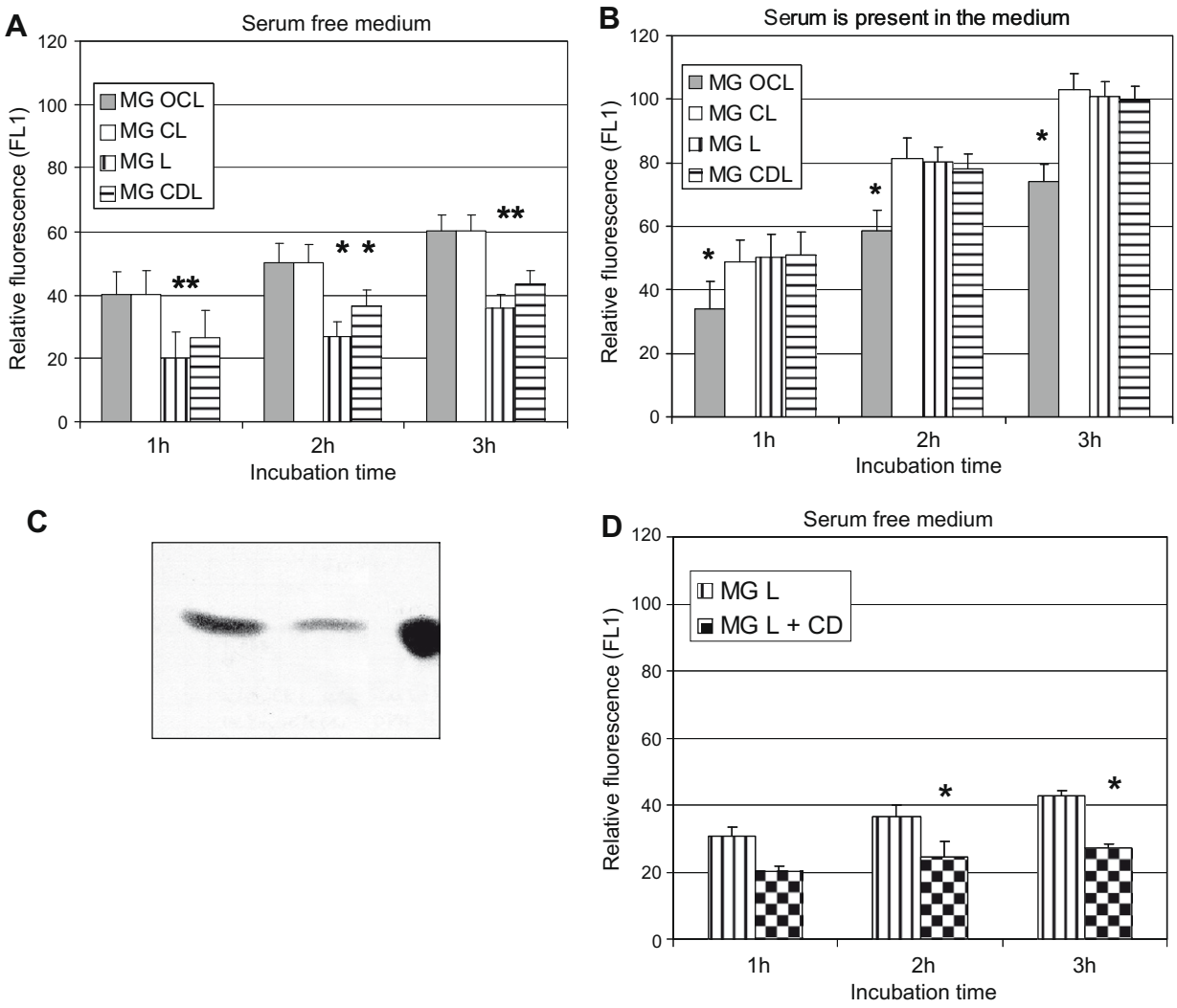

Fig. 2. Phagocytosis assay. Phagocytosis of fluorescently labeled bacteria by RAW 264.7 mouse macrophages was followed by flow cytometry: (A) The absence of core region (*MG L*) and penta-acylated lipid A moieties (*MG CΔL*) significantly decreased the efficiency of phagocytosis in serum free medium. (B) In the presence of serum, *MG OCL* strain inhibited phagocytosis, while *MG L*, *MG*, *CΔL*, and *MG CL* strains were internalized equally. (C) In further experiments, cholesterol was depleted from RAW 264.7 cells by Mβ-CD pretreatment. The effect was verified by thin layer chromatography. First lane: untreated RAW 264.7 cells. Second lane: Mβ-CD-treated RAW 264.7. Third lane: control cholesterol. (D) Mβ-CD treatment significantly decreased the uptake of *MG L* bacteria. ($p < 0.05$, paired, nonparametric two-tailed test).

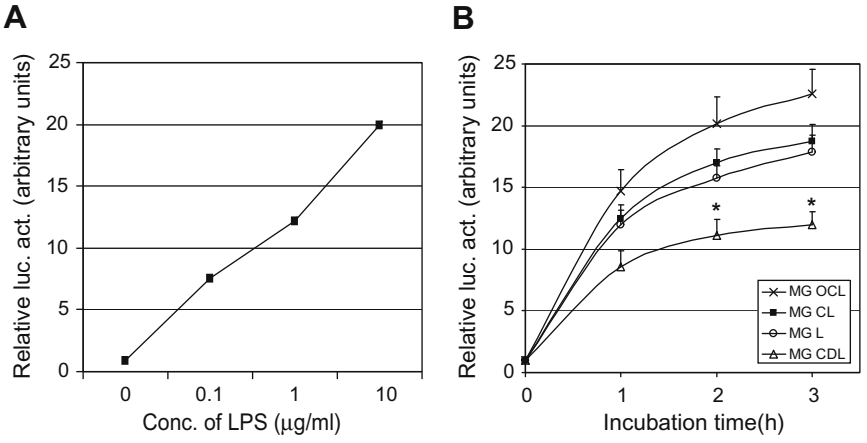

Fig. 3. Early TNF release of macrophages depends on the LPS structure of *E. coli* cells. TNF released by macrophages was detected by L929/NF-κB-luc reporter cells as described in Materials and methods. (A) RAW 264.7 cells released TNF in response to LPS in a concentration dependent manner. (B) In response to *MG CL* (MG1655) and *MG L* strains, macrophages released about the same amount of TNF into the supernatant. Introduction of *MG OCL* bacteria caused higher tumor necrosis factor production, but this difference was not significant. *MG CΔL* bacteria caused the production of significantly less TNF compared with the TNF produced in response to *MG CL*. ($p < 0.05$; paired Mann–Whitney test).

opsonins, core and lipid A regions are both targets of phagocytic receptors. In the presence of serum (Fig. 2B), *MG OCL* strain was internalized significantly less effectively then the other three strains, all lacking the O-antigen.

Mβ-CD treatment of RAW 264.7 cells reduced the level of cellular cholesterol by 65% compared to levels found in untreated control cells (Fig. 2C). The membrane modification did not alter the uptake of *MG OCL*, *MG CL*, and *MG CΔL* strains (data not shown). Surprisingly, the phagocytosis of *MG L* bacteria turned out to be sensitive to Mβ-CD-treatment (Fig. 2D).

"Early" TNF release

TNF release from secretory granules occurs in parallel with phagocytosis of the microbes. It is a rapid event as it does not need de novo mRNA and protein synthesis. TNF is detectable within 15 min of LPS exposure in the supernatant of stimulated immune cells. Newly synthesized TNF appears in the supernatant of macrophages later on. We measured the early TNF secretion in a bioassay using L929 cells expressing the luciferase gene controlled by NF-κB binding elements and with ELISA as well. Fig. 3A shows the NF-κB-activating effect of the supernatant of RAW 264.7 cells activated with LPS.

We found no significant differences in TNF production of RAW 264.7 cells in response to the *MG OCL*, the *MG CL*, and *MG L* strains, while *MG CΔL* bacteria caused the production of significantly less TNF (Fig. 3B). We confirmed the results with ELISA, i.e., the NF-κB activation was indeed due to TNF, and furthermore, the presence of potential contaminating bacterial products did not interfere with the bioassay. The differences in TNF production did not depend on the presence or absence of serum in the medium (data not shown).

Control and Mβ-CD pre-treated RAW 264.7 cells were exposed to different bacteria and early TNF release in supernatants was

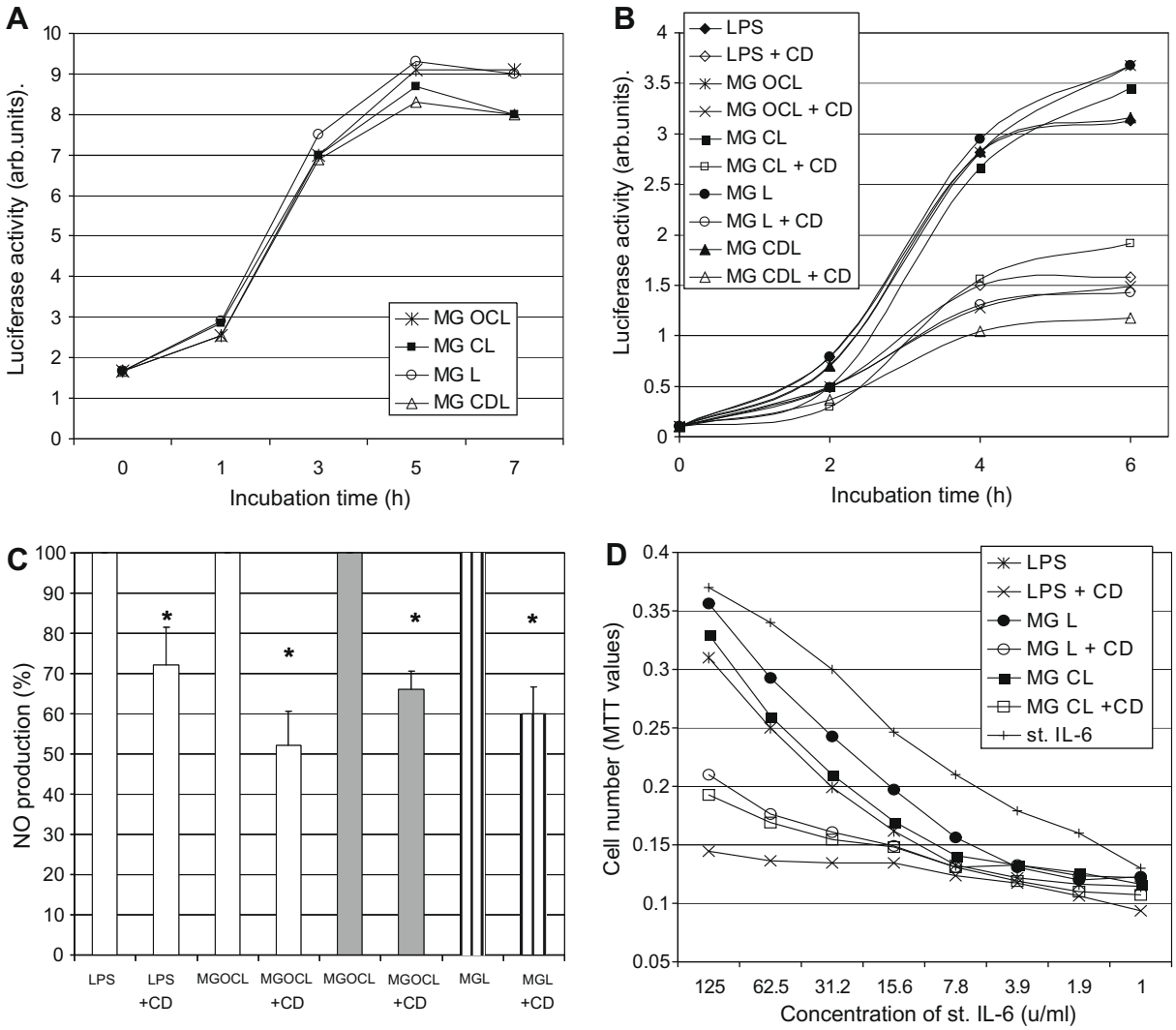

Fig. 4. Administration of *E. coli* cells bearing complete or truncated LPS molecules does not affect full activation of the NF-κB-pathway in RAW/NF-κB-luc cells (A). Bacteria and RAW 264.7 cells were co-incubated for 1, 3, 5, or 7 h. (B) Effect of Mβ-CD-treatment on NF-κB-activation in RAW/NF-κB-luc cells: macrophages were pre-treated with Mβ-CD then exposed to 10 μg/ml of LPS, *MG OCL*, *MG CL*, *MG L*, or *MG CΔL* bacteria, and luciferase activity was measured. NF-κB activation by different bacterial strains was practically identical and membrane modification severely inhibited the activation of the NF-κB pathway in each case. (Experiments were repeated 5 times with identical results.) (C) Macrophages were exposed to different bacterial strains and NO production was measured spectrophotometrically. Cholesterol depletion of macrophage membranes with Mβ-CD significantly decreased the NO-production of RAW 264.7 cells. NO production was expressed as a percentage of that of nontreated control macrophages (the LPS concentration was 10 μg/ml; macrophage: bacterium ratio was 1:100) ($p < 0.05$). (D) Macrophages were exposed to different *E. coli* strains for 3 h at a macrophage: bacterium ratio of 1–100. IL-6 level of the supernatants was measured in a bioassay using IL-6 dependent B9 hybridoma cells. Cell proliferation was followed using MTT assay. Cholesterol depletion of macrophage membranes with Mβ-CD decreased IL-6 production of RAW 264.7 cells in response to LPS and bacteria as well (experiments were repeated 3 times with similar results).

monitored. Fast TNF release seemed to be governed by a receptor(s) that was (were) not raft associated, as the amount of secreted TNF did not change upon Mβ-CD-treatment in the case of any of the bacterium strains (data not shown).

Induction of pro-inflammatory cytokine genes via the NF-κB pathway

According to present knowledge, LPS activation of TLR-4/MD2 is mainly responsible for the activation of the NF-κB pathway when macrophages are exposed to Gram-negative bacteria. NF-κB activation consecutively leads to transcription of genes of inducible NO-synthase and pro-inflammatory cytokines, like IL-1β, TNF-α, IL-6, IL-8, and IL-12. Indeed, we were able to detect activation of the NF-κB signal transduction pathway by using macrophages transformed with the pNF-κB-luc reporter plasmid. When different *E. coli* bacteria were co-incubated with RAW/NF-κB-luc cells, we measured no difference between the activation of NF-κB pathway (Fig. 4A). The presence or absence of serum did not change NF-κB-activation either (data not shown).

We also measured NO and IL-6 production of RAW 264.7 cells in response to the original *MG CL* strain and the modified LPS producing bacteria. NO- and IL-6-production of macrophages was equal in the case of the investigated strains (data not shown).

It is well documented that TLR-4 becomes raft-associated upon activation with LPS or bacteria. With this knowledge, it was not a surprise that disruption of membrane microdomains by cholesterol depletion caused a decrease in downstream signaling events like NF-κB-activation (Fig. 4B) and NO-(Fig. 4C) or cytokine (IL-6) production (Fig. 4D) in the case of all the investigated strains.

Discussion

We investigated macrophage functions in response to genetically modified *E. coli* bacteria that differed only in their LPS structure. According to our results, different parts of the LPS molecules react with different macrophage receptors/receptor complexes to initiate phagocytosis, release of pre-synthesized TNF, or induction of NF-κB signaling. When LPS did not contain carbohydrate moiety or the lipid A part was truncated, it attenuated the efficiency of phagocytosis (in serum free medium) and fast TNF release, but it did not impair these processes completely. It might mean that other kinds of receptors can recognize the truncated LPS molecules (or other parts of the bacterial cell wall) but only with lower efficiency, and initiate ingestion of bacteria (Fig. 2A) and immediate TNF release (Fig. 3B).

The anti-phagocytic effect of complete LPS has already been known: O-antigens can enhance the pathogenicity of *E. coli*, *Shigella* spp., and *Salmonella* [29] (Fig. 2B).

We did not find difference in the NF-κB activation in responses to different LPS-modified strains (Fig. 4A). Moreover, macrophages showed the same strong NF-κB-activation in case of pathogenic *E. coli* (enterohaemorrhagic *E. coli* O157:H7) and heat inactivated *MG CL* and O157:H7 bacteria as well (K. Eder and Z. Gyorfy, unpublished data). These data suggest that the same – full – activation of TLR-4 can be achieved by recognition of the essential part of LPS, lipid A (even if it is penta-acylated), and the process is not influenced by the carbohydrate moiety.

LPS induces raft association of TLR-4 leading to the nuclear translocation of several transcription factors (AP-1, NF-κB, Elk-1) and activation of genes of pro-inflammatory cytokines and inducible NO-synthase. Activation of TLR-4 proved to be Mβ-CD-sensitive according to data in the literature and our experiments: efficiency of downstream signaling events – NF-κB activation, IL-6- and NO-production – decreased upon disruption of membrane microdomains (Fig. 4B–D).

Fast TNF release and phagocytosis were found to be Mβ-CD-insensitive, only the internalization of *MG L* bacteria was impaired by membrane modification (Fig. 2D), a fact that is not easy to explain according to our current knowledge. Perhaps in the absence of carbohydrate moieties the remaining lipid A part of LPS is recognized by TLR-4 – a raft associated receptor – that is able to induce the uptake of these bacteria.

Until recently, the innate immune response was believed to be rather simple when compared to the acquired immune response. On the contrary, our results and recent studies by others have demonstrated that the innate immune response is as complex as the acquired, involving multiple receptors and a plethora of protein–protein interactions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.08.082.

References

[1] Y. Kobayashi, C. Miyaji, H. Watanabe, H. Umezu, G. Hasegawa, T. Abo, M. Arakawa, N. Kamata, H. Suzuki, T. Kodama, M. Naito, Role of macrophage scavenger receptor in endotoxin shock, J. Pathol. 192 (2000) 263–272.
[2] O. Elomaa, M. Kangas, C. Sahlberg, J. Tuukkanen, R. Sormunen, A. Liakka, I. Thesleff, G. Kraal, K. Tryggvason, Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages, Cell 80 (1995) 603–609.
[3] A.M. Pearson, Scavenger receptors in innate immunity, Curr. Opin. Immunol. 8 (1996) 20–28.
[4] C.A. Janeway, R. Medzhitov Jr., Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216.
[5] V. Le Cabec, S. Carreno, A. Moisand, C. Bordier, I. Maridonneau-Parini, Complement receptor 3 (CD11b/CD18) mediates type I and type II phagocytosis during nonopsonic and opsonic phagocytosis, respectively, J. Immunol. 169 (2002) 2003–2009.
[6] C.M. Rosenberger, M.G. Scott, M.R. Gold, R.E. Hancock, B.B. Finlay, *Salmonella typhimurium* infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression, J. Immunol. 164 (2000) 5894–5904.
[7] M.C. Royle, S. Totemeyer, L.C. Alldridge, D.J. Maskell, C.E. Bryant, Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live *Salmonella typhimurium* is a critical but not exclusive event leading to macrophage responses, J. Immunol. 170 (2003) 5445–5454.
[8] E. Andreakos, S.M. Sacre, C. Smith, A. Lundberg, S. Kiriakidis, T. Stonehouse, C. Monaco, M. Feldmann, B.M. Foxwell, Distinct pathways of LPS-induced NF-κB activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP, Blood 103 (2004) 2229–2237.
[9] C.M. Rosenberger, B.B. Finlay, Phagocyte sabotage: disruption of macrophage signaling by bacterial pathogens, Nat. Rev. Mol. Cell. Biol. 4 (2003) 385–396.
[10] C.R. Raetz, C.M. Reynolds, M.S. Trent, R.E. Bishop, Lipid A modification systems in Gram-negative bacteria, Annu. Rev. Biochem. 76 (2007) 295–329.
[11] A. Ozinsky, K.D. Smith, D. Hume, D.M. Underhill, Co-operative induction of pro-inflammatory signaling by Toll-like receptors, J. Endotoxin Res. 6 (2000) 393–396.
[12] D.H. Wyllie, E. Kiss-Toth, A. Visintin, S.C. Smith, S. Boussouf, D.M. Segal, G.W. Duff, S.K. Dower, Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses, J. Immunol. 165 (2000) 7125–7132.
[13] P.Y. Perera, T.N. Mayadas, O. Takeuchi, S. Akira, M. Zaks-Zilberman, S.M. Goyert, S.N. Vogel, CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene expression, J. Immunol. 166 (2001) 574–581.

[14] M. Triantafilou, K. Miyake, D.T. Golenbock, K. Triantafilou, Mediators of innate immune recognition of bacteria concentrate in lipid rafts and facilitate lipopolysaccharide-induced cell activation, J. Cell Sci. 115 (2002) 2603–2611.
[15] M.P. Haynes, M.C. Phillips, G.H. Rothblat, Efflux of cholesterol from different cellular pools, Biochemistry 39 (2000) 4508–4517.
[16] M. Triantafilou, K. Triantafilou, Receptor cluster formation during activation by bacterial products, J. Endotoxin Res. 9 (2003) 331–335.
[17] S. Olsson, R. Sundler, The role of lipid rafts in LPS-induced signaling in a macrophage cell line, Mol. Immunol. 43 (2006) 607–612.
[18] J.D. Perkins, J.D. Heath, B.R. Sharma, G.M. Weinstock, XbaI and BlnI genomic cleavage maps of *Escherichia coli* K-12 strain MG1655 and comparative analysis of other strains, J. Mol. Biol. 232 (1993) 419–445.
[19] D. Liu, P.R. Reeves, *Escherichia coli* K12 regains its O antigen, Microbiology 140 (1994) 49–57.
[20] Z. Yao, M.A. Valvano, Genetic analysis of the O-specific lipopolysaccharide biosynthesis region (rfb) of *Escherichia coli* K-12 W3110: identification of genes that confer group 6 specificity to *Shigella flexneri* serotypes Y and 4a, J. Bacteriol. 176 (1994) 4133–4143.
[21] G. Stevenson, B. Neal, D. Liu, M. Hobbs, N.H. Packer, M. Batley, J.W. Redmond, L. Lindquist, P. Reeves, Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its rfb gene cluster, J. Bacteriol. 176 (1994) 4144–4156.
[22] T. Feher, I. Karcagi, Z. Gyorfy, K. Umenhoffer, B. Csorgo, G. Posfai, Scarless engineering of the *Escherichia coli* genome, Methods Mol. Biol. 416 (2008) 251–259 (Humana Press).
[23] G. Posfai, G. Plunkett 3rd, T. Feher, D. Frisch, G.M. Keil, K. Umenhoffer, V. Kolisnychenko, B. Stahl, S.S. Sharma, M. de Arruda, Emergent properties of reduced-genome *Escherichia coli*, Science 312 (2006) 1044–1046.
[24] S.A. Khan, P. Everest, S. Servos, N. Foxwell, U. Zahringer, H. Brade, E.T. Rietschel, G. Dougan, I.G. Charles, D.J. Maskell, A lethal role for lipid A in *Salmonella* infections, Mol. Microbiol. 29 (1998) 571–579.
[25] L.A. Aarden, E.R. De Groot, O.L. Schaap, P.M. Lansdorp, Production of hybridoma growth factor by human monocytes, Eur. J. Immunol. 17 (1987) 1411–1416.
[26] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing, Cancer Res. 47 (1987) 936–942.
[27] T.P. Misko, R.J. Schilling, D. Salvemini, W.M. Moore, M.G. Currie, A fluorometric assay for the measurement of nitrite in biological samples, Anal. Biochem. 214 (1993) 11–16.
[28] J. Folch, M. Lees, S.G.H. Sloane, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[29] R. Morona, C. Daniels, L. Van Den Bosch, Genetic modulation of *Shigella flexneri* 2a lipopolysaccharide O antigen modal chain length reveals that it has been optimized for virulence, Microbiology 149 (2003) 925–939.