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Synthesis of lipid A and its novel analogues for investigation of the structural and conformational bases for their bioactivity

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To elucidate the structural requirements for the endotoxic and antagonistic activity of lipid A derivatives, we have focused on the effects of acyl moieties and also the acidic groups at the 1- and 4′-positions. *Rubrivivax gelatinosus* lipid A, that has shorter (C_{10}, C_{12}) acyl groups than *Escherichia coli* lipid A and is characteristic with their symmetrical distribution on two glucosamine residues, was synthesized. We also designed, based on molecular mechanics calculation, and synthesized lipid A analogues possessing acidic amino acid residues, with different combinations of acyl groups, in place of the non-reducing end glucosamine. The synthetic *R. gelatinosus* lipid A (C_{10}, C_{12}) showed potent antagonistic activity, although the natural counterpart (C_{10}, C_{12}/C_{14}) was reported to be endotoxic: the result may suggest only two methylene groups have a decisive influence on the biological activity. Bioactivities of the synthetic analogues will be discussed in comparison with the results of molecular modeling.

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Synthesis of labeled lipid A for biofunctional analysis

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Previously, we synthesized several fluorescence-labeled lipid A analogues but they were found to show only very weak fluorescence and some even exhibited no endotoxic activity probably owing to strong aggregation induced by the hydrophobic BODIPY® group employed as a fluorescent moiety. In the present study, we established a new method for synthesis of labeled bioactive lipid A by using glucose as a hydrophilic linker for labeling. The method includes the following steps: (i) preparation of lipid A derivatives possessing a reducing glucose tag; and (ii) introduction of a labeling group to the glucose residue via the formation of a hydrazone under mild conditions. Several fluorescence-labeled and biotin-labeled lipid A derivatives were efficiently synthesized without any damage to the phosphoryl and acyl groups. Alexa Fluor 568™-labeled *Escherichia coli* lipid A derivatives thus prepared showed strong fluorescence and bioactivity.

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Synthesis of chlamydial tetra- and penta-acyl lipid A

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Chlamydiae are obligatory intracellular Gram-negative pathogens which are responsible for a variety of acute and chronic diseases in animals and humans, such as urogenital infections and trachoma.[1] Although chlamydial LPS is less active than enterobacterial endotoxins, its role in local chronic infections and inflammatory processes needs to be clarified.[2] Chlamydial LPS is only available in minute quantities and the structural variability with respect to number, type and position of fatty acyl groups makes it impossible to isolate homogeneous chlamydial lipid A. Based on the structural data on

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Chlamydia trachomatis serotype L2 LPS,[3] chlamydial tetra-acyl lipid A and penta-acyl lipid A containing (*R*)-3-hydroxy-eicosanoic acid residues has been synthesized and fully characterized by NMR and MS data. Purity has been demonstrated by immunostaining with a lipid-A specific monoclonal antibody. Studies of their endotoxic activities and their function as an acceptor for chlamydial CMP-Kdo transferases are currently in progress.

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Biological activity of chlamydial lipopolysaccharide on mononuclear phagocytes

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Chronic *Chlamydia pneumoniae* infection is known to be a risk factor for atherosclerosis. Among *Chlamydia*-derived bioactive components, lipopolysaccharide (LPS) is one of the most important virulence factors. LPS from *C. pneumoniae* is structurally unique in that lipid A domain, a toxic center of LPS, has longer but less acyl chains than those of enterobacterial LPS. Thus, we investigated the biological activity of chlamydial LPS using monocytes/macrophages as target cells. FACS analysis showed that chlamydial LPS could bind to human peripheral monocytes and the murine macrophage-cell line RAW264.7 in CD14-dependent manner. In response to chlamydial LPS, human monocytes and RAW264.7 cells produced inflammatory cytokines (TNF-α and IL-6). Furthermore, both LPS binding and cytokine production were significantly enhanced by the presence of LPS-binding protein (LBP). Interestingly, although monocytes and RAW264.7 cells were much less responsive to chlamydial LPS than *Escherichia coli* LPS, binding study indicated that both chlamydial LPS and *E. coli* LPS could bind to recombinant LBP in a similar fashion. The difference in the affinity of chlamydial LPS and *E. coli* LPS to LBP and CD14 will be discussed.

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Biosynthesis of nucleotide-activated heptoses: new perspectives for structural biology and drug design

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Following the elucidation of the genetics and enzymology involved in the biosynthesis of ADP D- and L-*glycero*-β-D-*manno*- as well as GDP D-*glycero*-α-D-*manno*-heptose, increased evidence has been obtained for the wide-spread occurrence of these pathways, which are operative not only in the assembly of inner and outer core sugars, but also in O-antigen and capsular polysaccharide as well as bacterial glycoprotein biosynthesis.[1] Recently, the crystal structures of ADP heptose epimerase (GmhD) and of the *Escherichia coli* heptosyl transferase II have been solved which serve as defined targets for drug design.[2,3] C-Glycosyl-heptose phosphates, heptosyl phosphonates and the corresponding nucleotide analogs are being investigated as stable inhibitors of the biosynthetic pathways, comprising substrate and transition state analogs for the epimerase, nucleotide and heptosyl transfer reaction.

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Alteration of molecular conformation for artificial chiral receptors by embedding into lipid A membrane from *Erwinia carotovora*

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We have studied the membrane function of lipid A as an embedding material for the artificial chiral receptors (binaphthyl derivatives, BNA and BNAP). Lipid A from *Erwinia carotovora* FERM P-7576 differing in the number of phosphate groups was used in aqueous dispersions. For the study, synchrotron radiation X-ray diffraction was applied for the elucidation of the aggregate structures and Fourier-transform infrared spectroscopy for the gel-to-liquid crystalline phase transition of the acyl chains of lipid A. The artificial receptors by themselves formed ordered aggregate structures in buffer, with a high periodicity for BNAP but much lower for BNA. The mixed receptor:lipid A aggregates formed well-expressed three dimensional structures in particular for the dephospho-lipid A different from those of the pure compounds. In the case of receptor:monophosphoryl lipid A mixtures, neither the lipid A nor the receptor changed their structures. This holds true similarly for the BNA:bisphosphoryl lipid A mixture, whereas the BNAP:bisphosphoryl lipid A mixture again formed a well-defined aggregate structure, different from those of the pure compounds. In this way, the number of the phosphate group of lipid A affected the molecular conformation of the binaphthyl compounds as well as lipid A. Thus, we conclude that embedding into the lipid A membrane alters the space of the artificial receptors for a guest molecule.

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Physicochemical characterization and endotoxic activity of synthetic monophosphoryl analogues of lipid A

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It is known that variations in the lipid A moiety of endotoxically highly active LPS from enterobacteria may result in dramatic changes in bioactivity. For example, the removal of only one acyl chain in *Escherichia coli*-type hexa-acyl lipid A causes a reduction of cytokine induction in human mononuclear cells by 2–3 orders of magnitude, and the removal of one phosphate causes an activity decrease by one order of magnitude. In this way, such well-directed reduction of the toxic effects of LPS may lead to the development of lipid A-like compounds for clinical use as immunomodulators.

We have performed a physicochemical study of various synthetic diglucosamine phosphate or aminoalkyl glucosamine phosphates with six acyl chains and systematic variations in their lengths, and have correlated the data to results in biological test systems. The measurements comprise the determination of the gel-to-liquid crystalline phase transition of the acyl chains via Fourier-transform infrared spectroscopy and differential scanning calorimetry, the aggregate structure of the compounds as found by synchrotron radiation X-ray diffraction, their intercalation into target phospholipid liposomes induced by LBP, and their ability to induce tumor necrosis factor-α (TNF-α) in human mononuclear cells.

The results show characteristic variations of the aggregate structures parallel to the differences in biological activity. In contrast, the acyl chain melting behavior does not correlate directly with the biological data except for a modulating effect.

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Dephosphorylation of lipid A selectively reduces its activity to induce IL-1β production

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Lipopolysaccharide, a major component of the Gram-negative bacterial outer membrane, is known as endotoxin. Lipid A, a membrane anchor portion of lipopolysaccharide, is responsible for the endotoxin activity and shows many inflammatory

responses on macrophages, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) secretion. Monophosphoryl lipid A, which structurally lacks a phosphate residue of lipid A, is known as a non-toxic derivative of lipid A. Stimulation of mouse peritoneal macrophages with monophosphoryl lipid A induced the synthesis of IL-1β precursor, but not secretion of IL-1β from the macrophages. In the monophosphoryl lipid A-stimulated macrophages, IL-1β precursor was not processed to the mature form. On the other hand, TNF-α was secreted from the monophosphoryl lipid A-stimulated macrophages. Caspase-1, which catalyzes IL-1β precursor processing, was not activated in monophosphoryl lipid A-stimulated macrophages, but in lipid A-stimulated cells. These results indicated that lack of caspase-1 activation leads to selective reduction of IL-1β secretion in monophosphoryl lipid A-stimulated macrophages. Since caspase-1 has been shown to be essential for endotoxin shock, our results suggest that lack of caspase-1 activation is responsible for reduced toxicity of monophosphoryl lipid A.

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Cleavage and modification of the 1-phosphate group of *Helicobacter pylori* lipid A

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This study describes the two-step enzymatic incorporation of phosphoethanolamine (pEtN) directly to the 1-position of *Helicobacter pylori* lipid A. Using an *in vitro* assay system, we demonstrated the presence of lipid A 1-phosphatase activity in membranes of *H. pylori* and identified *hp0021* as the structural gene encoding the enzyme. In addition, we have determined the structural gene for the *H. pylori* lipid A pEtN transferase (Hp0022). Heterologous expression of Hp0021 in *Escherichia coli* K-12 strains resulted in the highly selective removal of the 1-phosphate group. While expression of Hp0022 alone in *E. coli* did not result in any lipid A modifications, dual expression of both Hp0021 and Hp0022 resulted in the addition of a single pEtN group at the 1-position indicating that modification of the 1-phosphate of *H. pylori* lipid A requires two enzymatic steps. Investigation of *H. pylori* strains lacking functional copies of *hp0021* and *hp0022* will be necessary to determine if pEtN addition to *H. pylori* lipid A plays an important role in pathogenesis.

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Physicochemical characterization of carboxymethyl lipid A derivatives in relation to biological activity

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The 'endotoxic principle' of LPS, lipid A, carries two negatively charged phosphate groups and six acyl chain residues in a defined asymmetric distribution (corresponding to synthetic compound 506). Tetra-acyl lipid A (precursor IVa or synthetic 406), which lacks the two hydroxylated acyl chains, is agonistically completely inactive, but is a strong antagonist to bioactive LPS when administered to the cells before LPS addition. The two negative charges of lipid A, represented by the two phosphate groups, are essential for agonistic as well as for antagonistic activity and no highly active lipid A are known with negative charges other than phosphate groups. We hypothesized that the phosphate groups could be substituted by other negatively charged groups without changing the endotoxic properties of lipid A. To test this hypothesis, we synthesized carboxymethyl derivatives of hexa-acyl lipid A (CM-506 and Bis-CM-506) and of tetra-acyl lipid A (Bis-CM-406) and correlated their physicochemical with their endotoxic properties. We found that, similarly to compounds 506 and 406 also for their carboxymethyl derivatives a particular molecular ('endotoxic') conformation and with that, a particular aggregate structure is a prerequisite for high cytokine-inducing capacity and antagonistic activity, respectively. In other parameters such as acyl chain melting behaviour, antibody binding, activity in the *Limulus* lysate assay, and partially the binding of Kdo transferase, strong deviations from the properties of the phosphorylated compounds were observed. These data allow a better understanding of endotoxic activity and its structural prerequisites.

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Physicochemical characterization and biological activity of lipo-oligosaccharides from *Neisseria meningitidis*

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The physicochemical characterization of *Neisseria meningitidis* lipopoly(oligo)saccharide (LOS) structures from the wild-type serogroup B strain NMB and from genetically-defined mutants (*orfA*: KDO_2-lipid A; and *kdtA*: lipid A) was assessed in relation to bioactivity. Meningococcal KDO_2-lipid A was the minimal structure required for optimal activation of the TNF-α release from macrophages. Fourier-transform infrared spectroscopy showed that meningococcal lipid A has a drastically reduced inclination angle of < 30° compared to meningococcal (KDO_2-lipid A) that has a value of 50–55°. This indicates an important role for the KDO moiety on the molecular conformation and bioactivity. The gel-to-liquid crystalline phase transition of the (β↔α) acyl chains in meningococcal lipid A structure was approximately 45°C in contrast to wild-type and KDO_2-lipid A meningococcal LOS that have a broad phase transition range of 15–30°C. X-ray diffraction experiments indicated that meningococcal lipid A has a unilamellar aggregate structure, which is indicative of less active or inactive endotoxin. Meningococcal lipid A was very weak agonist in stimulating human macrophages even at high doses. Finally, experiments with fluorescence resonance energy transfer (FRET) showed that meningococcal LOS structures as well as lipid A was able to incorporate into target cell membranes as prerequisite for agonistic as well as antagonistic activity.

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Differential induction of the TLR4-MyD88-dependent and independent signaling pathways by endotoxin

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The role of lipid A structure in the activation of the MyD88-dependent and independent signaling pathways in macrophage were investigated. Highly purified meningococcal lipo-oligosaccharide (LOS) was a potent inducer of the MyD88-dependent pathway molecules TNF-α, IL-1β, MCP-1, MIP-3α, and the MyD88-independent molecules IFN-β, nitric oxide and IP-10 at picomolar concentrations. *Escherichia coli* 55:B5 and *Vibrio cholerae* lipopolysaccharide (LPS) at the same concentrations induced TNF-α, IL-1β, MIP-3α, but did not induce significant amounts of IFN-β, nitric oxide and IP-10. In contrast, *Salmonella minnesota* and *Salmonella typhimurium* LPS released significant amounts of IFN-β, nitric oxide and IP-10 but significantly less TNF-α and MIP-3α in time-course and dose-response experiments when compared to meningococcal, *E. coli* and *V. cholerae* LPS. Blocking the MyD88-dependent pathway by DNMyD88 resulted in significant reduction of TNF-α release but did not influence nitric oxide release. IFN-β polyclonal antibody and IFN-α/β receptor 1 antibody significantly reduced nitric oxide release. At physiologically relevant picomolar concentrations, meningococcal endotoxin activated both the MyD88-dependent and independent signaling pathways in human macrophages. *E. coli* 55:B5 and *V. cholerae* LPS induced the MyD88-dependent pathway while *Salmonella* LPS selectively activated the MyD88-independent pathway.

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Expression of phosphorylcholine and *N*-acetylneuraminic acid on *Haemophilus somnus* lipo-oligosaccharide

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Haemophilus somnus is a Gram-negative, opportunistic pathogen of cattle. The lipo-oligosaccharide (LOS) of *H. somnus* contains a terminal lactosamine, in which the linkage undergoes phase variation between Gal(1-4)GlcNAc and

Gal(1-3)GlcNAc. The outer core oligosaccharide can be decorated with *N*-acetylneuraminic acid (NeuAc), which interferes with antibody binding, and phosphorylcholine (ChoP). The genes encoding for two sialyltransferases were identified from the *H. somnus* genome sequence. One sialyltransferase was shown to preferentially sialylate Gal(1-4)GlcNAc, while the other sialyltransferase preferentially sialylated Gal(1-3)GlcNAc. Induction of TLR4 signaling by sialylated LOS was reduced 28% compared to non-sialylated LOS. The genes for expression of ChoP contained homology to the *lic1* locus of *Haemophilus influenzae*. The *lic1*A homologue (choline kinase) contained a repeat tract of 5′-AACC-3′ indicating ChoP expression is phase variable. Following respiratory challenge of calves with *H. somnus* in which > 95% of the cells either expressed or did not express ChoP, the predominant population recovered from the upper respiratory tract of all calves expressed ChoP, indicating ChoP played a role in epithelial cell colonization. Furthermore, reactivity of ChoP with monoclonal antibody also varied through phase variation of the oligosaccharide extending beyond ChoP. Therefore, *H. somnus* has conserved as well as novel methods to avoid host defenses.

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Temperature dependence of the binding of endotoxins to the polycationic peptides polymyxin B and its nonapeptide

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The interaction between endotoxins – free lipid A and various lipopolysaccharide (LPS) chemotypes with different sugar chain lengths – and the polycationic peptides polymyxin B (PMB) and polymyxin nonapeptide (PMBN) has been investigated by isothermal titration calorimetry (ITC) between 20–50°C. The results show a strong dependence of the titration curves on the phase state of the endotoxins. In the gel phase (< 30°C for LPS and < 45°C for lipid A) an endothermic reaction is observed, for which the driving force is an entropically driven endotoxin–polymyxin interaction, due to disruption of the ordered water and cation assembly in the lipid A backbone and adjacent molecules. In the liquid crystalline phase (> 35°C for LPS and > 47°C for lipid A), an exothermic reaction takes place which is mainly due to the strong electrostatic interaction of the polymyxins with the negative charges of the endotoxins, *i.e.* the entropic change DS is much lower than in the gel phase. Whereas for endotoxins with short sugar chains (lipid A, LPS Re, LPS Rc), the stoichiometry of the polymyxin binding corresponds to pure charge neutralization, for the compounds with longer sugar chains (LPS Ra, LPS S-form) this is no longer valid, much higher peptide concentrations are required to cause charge neutralization. This indicates a more complex interaction under participation of the sugar moiety, due to steric changes and to the presence of further negative charges. This again can be related to the lower susceptibility of the corresponding bacterial strains to antibiotics.

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Francisella tularensis live vaccine strain lipopolysaccharide does not bind to lipopolysaccharide binding protein

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Francisella tularensis is a facultative intracellular Gram-negative bacterium that can cause overwhelming sepsis at a small inoculum. The lipopolysaccharide (LPS) of *F. tularensis* neither activates endotoxin-responsive human cells nor antagonizes cell activation by potent LPS species. LPS binding to lipopolysaccharide binding protein (LBP) is the first step in potent pro-inflammatory host responses to LPS. We hypothesized that the functionally inert properties of *F. tularensis* LPS could be due to an inability to bind LBP. We investigated the binding of *F. tularensis* live vaccine strain LPS to LBP using competition assays. In the initial experiments, LBP was adsorbed to a polystyrene plate, and we measured binding of [^{3}H]-lipo-oligosaccharide (LOS) from *Neisseria meningitidis*. Cold *N. meningitidis* LOS competed with the [^{3}H]-LOS, with a 10-fold excess of cold LOS inhibiting by 57% (SD ± 3%) [^{3}H]-LOS binding to LBP and a 100-fold excess inhibiting by 79% (± 6%). By contrast, *F. tularensis* LPS showed no competition at a 10-fold

excess and inhibited by only 23% (± 12%) and 30% (± 6%) at 100 and 1000-fold excesses, respectively. Because adsorbed LBP may not mimic LBP in its more physiological soluble form, we conducted an immune capture assay in which adsorbed polyclonal antibody to LBP pulled down LBP–[^{3}H]-LOS complexes. When cold LOS was co-incubated with LBP and [^{3}H]-LOS, a 10-fold excess of cold LOS fully abrogated formation of LBP–[^{3}H]-LOS complexes. By contrast, 10- and 100-fold excesses of *F. tularensis* LPS had no effect on formation of LBP-[^{3}H]-LOS complexes. Thus, *F. tularensis* LPS shows little or no binding to LBP. This inability to bind LBP may explain why *F. tularensis* LPS does not interact with host LPS-sensing machinery.

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Susceptibility of LPS mutants of *Actinobacillus pleuropneumoniae* to cationic antimicrobial peptides

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Actinobacillus pleuropneumoniae is an important pathogen of swine. We previously reported that lipopolysaccharides (LPSs) are involved in the adherence of *A. pleuropneumoniae* to host respiratory tract cells. Rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 were generated by using a mini-Tn10 transposon mutagenesis system, and the gene affected by the transposon was identified in each of these mutants. The purpose of the present study was to evaluate the susceptibility of various *A. pleuropneumoniae* LPS mutants to cationic antimicrobial peptides which are important components of the innate immune response. We determined the minimal inhibitory concentration of the peptides polymyxin B, protamine, cecropin P1, melittin, protegrin-1, and mastoparan. A rough LPS mutant of *A. pleuropneumoniae* exhibited the same susceptibility to these cationic peptides as that of the wild-type (WT) parent strain 4074 Nal^r. On the other hand, three core LPS mutants were more susceptible to cationic peptides than the WT strain. Structural analysis of the LPS from all mutants was performed. Our data indicate that an intact outer core is required for optimal protection of *A. pleuropneumoniae* against the antimicrobial activity of cationic peptides. It would be most interesting to infect pigs experimentally with the core LPS mutants and compare their virulence with that of the WT strain.

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Helicobacter varies LPS antigenic structure to evade surfactant protein D

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Helicobacter pylori is a common human gastric pathogen. Surfactant protein D (SP-D), a component of mucosal innate immunity, binds *H. pylori* causing aggregation of the organism. *H. pylori* establishes persistent infection raising the question of how the organism escapes SP-D. This study investigated the relationship between SP-D binding and LPS antigenic structure *in vitro* and *in vivo* to reveal potential mechanisms of bacterial persistence. Using laboratory strain J178, an SP-D 'escape variant' J178V was isolated through several rounds of SP-D agglutination, filtration and culture. Structural analysis of LPS extracted from J178 and J178V revealed changes in the degree of fucosylation in the O chain. Sequencing of the LPS biosynthesis genes identified a cytosine deletion in a poly-C tract of the fucosyltransferase A (*fucT 1*) gene leading to a premature stop codon in J178. Reversible switching of LPS antigenic structure was demonstrated indicating that SP-D evasion is a phase-variable phenomenon. SP-D-binding *H. pylori* strains *in vivo* are predominant in the mucus. We hypothesize that *H. pylori* has developed a strategy for evading the SP-D-mediated immune response through phase variation, which contributes to the persistence of this gastric pathogen.

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Construction of a deep-rough mutant in *Burkholderia cepacia* – characteristics of its LPS

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Burkholderia cepacia is a bacterium with increasing importance as a pathogen in patients with cystic fibrosis. The deep-rough mutant Ko2b was generated from *B. cepacia* type-strain ATCC 25416 by insertion of a kanamycin resistance cassette into the gene *waaC* encoding heptosyltransferase I. Mass spectrometric analysis of the de-O-acylated lipopolysaccharide (LPS) of the mutant showed that it consisted of a bisphosphorylated glucosamine backbone with two 3-hydroxyhexadecanoic acids in amide-linkage, 4-amino-4-deoxyarabinose residues on both phosphates and a core oligosaccharide of the sequence 4-amino-4-deoxyarabinose - D-*glycero*-D-*talo*-oct-2-ulosonic acid (Ko) - 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). The mutant allowed investigations on the biosynthesis of the LPS as well as on its role in human infections. Mutant Ko2b showed no difference in its ability to invade human macrophages as compared to the wild-type. Furthermore, isolated LPS of both strains induced the production of tumor necrosis factor α from macrophages to the same extent. Thus, the truncation of the LPS did not decrease the biological activity of the mutant or its LPS in these aspects.

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Structural analysis of KDO region of core oligosaccharides isolated from smooth Plesiomonas shigelloides and Hafnia alvei lipopolysaccharides

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Plesiomonas shigelloides and *Hafnia alvei* are Gram-negative bacteria and opportunistic pathogens in immunocompromised hosts and especially neonates. As the lipopolysaccharide (LPS) core modulates the biological and physical properties of endotoxin, the main virulence factor of these bacteria, it is of interest to determine the structure of this part of LPS isolated from smooth, pathogenic bacterial strains. The structure of the carbohydrate backbone of the core-lipid A region was determined in oligosaccharides obtained from de-acylated LPS. The products of alkaline de-acylation of LPS containing uronic acids are usually very complex and difficult to separate. The oligosaccharides suitable for structural analysis contain KDO that forms complexes with serotonin similarly to sialic acid. In this study, immobilized serotonin was used for one-step isolation of KDO oligosaccharides from the reaction mixture by affinity chromatography. The structural studies on pure oligosaccharide preparations were done by the use of NMR spectroscopy and MALDI-TOF mass spectrometry. It was found that analyzed *H. alvei* strains produce LPS with non-typical core structure. Besides KDO oligosaccharide linked directly to the lipid A part, an additional, substituted KDO molecule is situated on LPS core hexose region.

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The complete structure of *Plesiomonas shigelloides* O74 lipopolysaccharide and the immunodominant epitope within its O-antigen.

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Plesiomonas shigelloides is a Gram-negative bacterium, responsible for infections originating from the surface water contamination and an opportunistic pathogen in humans. The O-antigen defines the biological and physical properties of the

lipopolysaccharide (LPS) and is important in the pathogen–host interactions. As the structural data on the *P. shigelloides* O-antigens is scarce, the *P. shigelloides* O74 (CNCTC 144/92) LPS – the O-specific chain, the core, the lipid A and the linkages between them – was analyzed using NMR spectroscopy, MALDI-TOF mass spectrometry and chemical analysis. We conclude that the O-specific chain is built of a trisaccharide repeating unit, consisting of →3)-α-L-Rhap, →3)-α-D-FucpNAc and →2)-β-D-Quip3NHR, where R = 3-hydroxy-2,3-dimethyl-5-oxo-pyrrolidine-2-carboxylic acid, and the core is a novel octasaccharide devoid of phosphate residues. The structure of the O-antigen was confirmed directly on bacteria and isolated LPS, using the HR-MAS NMR technique. STD NMR experiments were used to identify the contribution of the structural elements in the O-antigen to the immunodominant epitope defined by anti-LPS antibodies.

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Structural analysis of *Plesiomonas shigelloides* lipid A molecules using MALDI-TOF and ESI mass spectrometry

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Biological activity of endotoxins depends mainly on lipid A structure and is modulated by the core oligosaccharide and O-specific chain. Chemical analysis of endotoxins, especially lipid A part, is an important step in the studies on the biological activity-structure relationship. Contrary to the detailed serotyping of *Plesiomonas shigelloides*,[1] lipopolysaccharide – main surface antigen and virulence factor – isolated from these bacteria seems to be poorly characterised. Preliminary results concerning lipid A structure of *P. shigelloides* 113/92 showed that it is built of glucosamine backbone substituted with two phosphate groups at the 1 and 4′ positions. The disaccharide backbone is substituted by 14:0(3-OH) fatty acids at positions 2, 2′ and 12:0(3-OH) at positions 3, 3′ and secondary ester bound 12:0 and 14:0.

As previously shown,[2–5] it is possible to determine the distribution of fatty acids in lipid A by combining data from negative and positive ion mode MS analysis and considering rules of fragmentation depending on the ionisation technique. The strategy based on ESI MSn and MALDI-TOF MS was used in comparative structural analysis of intact lipids A isolated from five strains of *P. shigelloides*, associated with human waterborn intestinal and extra-intestinal infections. Now, we report on the structures and microheterogeneity of lipid A among those strains.

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Structure of the O-polysaccharide and serological studies of the lipopolysaccharide of *Proteus penneri* 60 classified into a new *Proteus* serogroup O70

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LPS of *Proteus penneri* strain 60 was studied by chemical analyses and different variants of NMR spectroscopy, and the following structure of the linear pentasaccharide-phosphate repeating unit of the O-polysaccharide was established:

6)-α-D-Gal*p*-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Glc*p*NAc-(1→3)-β-D-Qui*p*4NAc-(1→6)-α-D-Glc*p*-1-*P*-(O→

Rabbit polyclonal O-antiserum against *P. penneri* 60 reacted with both core and O-polysaccharide moieties of the homologous LPS. Based on the unique O-polysaccharide structure and serological data, we propose to classify *P. pen-*

neri 60 into a new, separate *Proteus* serogroup O70. A weak cross-reactivity of *P. penneri* 60 O-antiserum with the LPS of *Proteus vulgaris* O8, O15 and O19 was observed and will be discussed in view of the chemical structures of the O-polysaccharides.

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Structure and serological studies of the O-polysaccharide of *Proteus penneri* 75: epitopes and subgroups of *Proteus* serogroup O73

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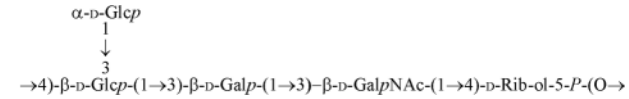
The O-specific polysaccharide of the lipopolysaccharide of *Proteus penneri* strain 75 consists of tetrasaccharide-ribitol phosphate repeating units and resembles ribitol teichoic acids of Gram-positive bacteria. The following structure of the polysaccharide was elucidated by chemical methods and ^{1}H and ^{13}C NMR spectroscopy:



The O-polysaccharide of *P. penneri* 128 has the same structure, and a similar structure has been established earlier for the O-polysaccharide of *P. penneri* strains 48, 90 and 103. Serological studies with rabbit polyclonal O-antisera showed that the two groups of strains could be classified into one *Proteus* serogroup, O73, as two subgroups 73a, 73b (for *P. penneri* 75 and 128) and 73a,73c (for *P. penneri* 48, 90 and 103). Epitopes responsible for the cross-reactivity of *P. penneri* O73 strains and a related strain of *Proteus mirabilis* O20 were tentatively defined.

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Characterization of LPS from the emerging human gastric pathogen, *Helicobacter bizzozeronii*

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Information on the LPS of non-*Helicobacter pylori* helicobacters is limited, hence this investigation was undertaken to characterize *Helicobacter bizzozeronii* LPS. Electrophoresis (SDS-PAGE) of LPS with silver staining showed production of predominantly low-molecular-mass LPS, although a less intensely staining, high-molecular-mass O-chain band was observed also. Unlike *H. pylori* LPS, no reactivity of anti-Lewis or anti-blood group antibodies was observed in immunoblotting with *H. bizzozeronii* LPS, indicating the absence of such molecular mimicry. Only 1 of 6 polyclonal antisera against the core of *H. pylori* LPS reacted with *H. bizzozeronii* LPS in serodot analysis, but not in Western blotting, indicating the occurrence of a shared epitope and the importance of antigen format for epitope recognition. Gel chromatography after mild acid hydrolysis of *H. bizzozeronii* LPS confirmed the predominance of a core oligosaccharide, rather than O-chain, consistent with the occurrence of low-molecular-mass LPS. The isolated core OS was subjected to detailed structural determination using NMR spectroscopy, mass spectrometry, and classical sugar methylation analysis. The established structure of the *H. bizzozeronii* core differs strikingly to that of *H. pylori*, only resembling that of *H. pylori* in the inner core. Epitopes unique to *H. bizzozeronii* LPS are likely to be present and thus could prove of diagnostic potential.

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Lewis expression on LPS of Japanese *Helicobacter pylori*-associated cancer strains and importance of serological test format for detection

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Expression of Lewis (Le) antigens, particularly Le^y, in the O-chains of *Helicobacter pylori* LPSs, has been implicated in generation of autoreactive antibodies contributing to gastric atrophy, a precursor of gastric cancer. Previously, both highly and weakly antigenic epitopes were detected in the O-chains of Japanese *H. pylori* isolates, but no correlation between these epitopes and Le presence was found in serological investigations. This study was undertaken to chemically determine whether Le and related antigens were present in two such Japanese cancer isolates (CA8 and CA9). Isolated O-chains and core oligosaccharides were subjected to sugar compositional and methylation-linkage analysis, and analysis by NMR and mass spectrometry. Both LPS produced O-chains of poly-*N*-acetyllactosamine that carried Le^x, Le^y and H-1 in CA8, but only Le^y and H-1 in CA9. The LPS core structure was identical in both isolates. Depending on antibody source and test format, extensive serological probing using dot blotting, Western blotting and ELISA with anti-Le and anti-blood group antibodies from various commercial sources detected the same determinants as seen in chemical analytical studies. Collectively, the data show the presence of Le^y and related antigens in Japanese cancer isolates and the dependence of immune recognition on antigen test format.

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Difference in the reactivity of *Helicobacter pylori*-lipopolysaccharides with patient sera in Poland and Japan

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Previously, we reported that LPS isolated from *Helicobacter pylori* Japanese strains were classified into 3 groups, highly (HAE) and weakly antigenic epitopes (WAE) having LPS and rough typed LPS on the basis of the reactivity with patient sera. In this study, we examined the reactivity of LPS isolated from 12 *H. pylori* Polish strains with Japanese and Polish patient sera positive to *H. pylori* by SDS-PAGE with immunoblotting. Three LPSs showed the semi-rough patterns by SDS-PAGE with silver stain and no reaction with any sera. The remaining 9 LPSs have ladder bands. Of these, 2 LPSs reacted with the HAE-positive Japanese sera, and one LPS reacted with the WAE-positive Japanese sera. Six LPSs did not react with any Japanese sera. These Polish LPSs, except 3 semi-rough typed LPSs, were reacted with *H. pylori*-positive Polish sera each with different pattern. On the other hand, in the reactivity with the anti-Lewis (Le) antibodies, 3 LPSs were reacted with anti-Le^x antibody, 5 LPSs with anti-Le^y antibody, 2 LPSs with anti-Le^b antibody, and 1 LPS with anti-H1 antibody. Collectively, Polish LPSs were reacted with Polish patient sera more than the Japanese sera, and it is necessary to classify Polish LPS based on the reactivity with Polish patient sera and with anti-Lewis antigen antibodies.

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Adaptation of Lewis antigen expression in LPS of *Helicobacter pylori* from experimentally infected rhesus monkeys

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Expression of Lewis antigens (Le^x and Le^y) in *Helicobacter pylori* LPS has been hypothesized to be related to human Lewis phenotype, consistent with selection of host-adapted bacterial populations. To understand the molecular complexity

of this adaptation, infection studies were performed using rhesus monkeys of non-secretor, Le(a^+,b^-), or secretor, Le(a^-,b^+), status. LPS of the inoculating strain (J166) and three isolates (98-149 and 98-169, non-secretor monkey; 98-181, secretor monkey), recovered 40-weeks post-colonization, were studied. First, LPS was probed with anti-Lewis and blood group antibodies. Second, acid-liberated saccharide chains of LPS were subjected to detailed structural determination using NMR spectroscopy, mass spectrometry, and sugar methylation analysis. All strains produced an O-chain of *N*-acetyllactosamine (LacNAc) units, some of which were fucosylated, yielding internal Le^x units (J166, 54%; 98-149, 69%; 98-169, 65%; 98-181, ~30%). Isolate 98-181 produced O-chains whose majority (~70%) of LacNAc units carried glucose (at C-4 od galactose). Terminal units of O-chains differed (J166, Le^y or H-1 antigen predominated over an unknown fucose-containing determinant (U) and Le^x; 98-149, Le^x and U; 98-169, only Le^x; 98-181, only Le^y). Therefore, greater expression of internal/terminal Le^x units occurred in non-secretor monkey isolates, but lower internal expression of Le^x with terminal Le^y in the secretor monkey isolate.

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Relevance of Lewis epitopes on outer membrane vesicles of *Helicobacter pylori* to autoantibody production

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Helicobacter pylori extrudes protein- and LPS-containing outer membrane vesicles (OMVs) from its cell surface which have been postulated to act in delivery of virulence factors to the host. The aim of this study was to characterize the expression of LPS on the surface of these OMVs and, in particular, expression of Lewis (Le) antigens by LPS, and their association with autoantibody production in the host. Using transmission electron microscopy of stained thin-sections, OMV production was observed in biopsies, and by *H. pylori* culture collection strains and clinical isolates grown in liquid and solid culture media. Immunoelectronmicroscopy with anti-Le antibodies revealed expression of Le^y epitopes both on the bacterial cell surface and OMVs. Immunoblotting of LPS from bacterial cells and OMVs confirmed the expression of Le^x and Le^y epitopes. Using ELISA, high antibody reactivity was detected in sera of 10 *H. pylori*-positive patients with gastric cancer but not in 10 *H. pylori*-negative controls. Following OMV absorption, this anti-Le reactivity decreased but not completely in some samples (70–100%). Likewise, cancer patient sera exhibited autoreactive anti-canicular antibodies to human gastric mucosa in immunohistochemistry, which were reduced, but not completed ablated, after absorption with OMVs. Thus, other protein-based host epitopes are likely involved in autoreactivity.

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Pseudomonas aeruginosa glycosyltransferases WbpLXYZ: molecular cloning, overexpression, purification and first characterization

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The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* synthesizes two different types of LPS O-antigen, A-band and B-band. A-band O-antigen is a homopolymer of D-rhamnose whereas B-band O-antigens are structurally diverse heteropolysaccharides determining 20 serotypes classified in the International Antigenic Typing Scheme. In the course of chronic lung infections, an antigen shift occurs: immunological active B-band LPS is replaced by less antigenic A-band LPS.[1]

In our aim to evaluate suitable targets for a new anti-infective treatment strategy, we investigated the biosynthesis of A-band specific D-rhamnan. Starting from GDP-D-mannose the activated building unit GDP-D-rhamnose is provided by the activity of Gmd and Rmd. Polymerization is initiated by transfer of one GlcNAc-moiety to the highly lipophilic membrane anchor undecaprenylphosphate catalyzed by WbpL. Subsequently, D-rhamnosyl units are transferred successive to this primary acceptor by the action of rhamnosyltransferases WbpX, WbpY and WbpZ in a highly co-ordinated mode.[2]

We cloned and overexpressed the appropriate genes *wbp*XYZ from clinical isolates of *P. aeruginosa* in *Escherichia coli*. All three enzymes remained in the insoluble fraction after cell disruption and were solubilized by addition of detergents. Due to a N-terminal His_6-tag, we were able to purify the putative rhamnosyltransferases by Ni-NTA affinity chromatography to near homogeneity.

In contrast, WbpL is a highly hydrophobic protein with presumably 11 membrane spanning domains. Various attempts to express His_6-tagged WbpL in *E. coli* failed. Solubility-enhancing protein fusions with MalE and NusA resulted in insufficient amounts for activity assays. Data will be presented about our efforts to synthesize WbpL in a cell-free expression system based on *E. coli* S30-extracts.

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Characterization of a galactosyltransferase gene, *lgtH*, in the biosynthesis of lipo-oligosaccharide (LOS) in *Neisseria meningitidis*

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The genetic locus with a cluster of glycosyltransferase genes, *lgtABE/H*, is responsible for the biosynthesis of lacto-*N*-neotetraosyl chain of LOS in *Neisseria meningitidis*. To characterize the function of *lgtH*, a recombinant plasmid of the gene was constructed by insertion of kanamycin resistance cassette into the coding region of *lgtH*. Group B *N. meningitidis* strain 6275 (*lgtABH*, L3 LOS) was transformed by electroporation for construction of the *lgtH* isogenic mutant. The mutant LOS had a reduction in molecular weight on SDS-PAGE gel and lost reactivity with an L3 antibody on immunoblot. Sugar analysis showed that galactose (Gal) was missing in the mutant LOS compared to the wild-type LOS. MALDI-TOF mass spectrometry showed that the O-deacylated mutant LOS had a major component with a mass of 2389 corresponding to $Glc.Hep_2.GlcNAc.PEA_2.Kdo_2$.-lipid A and that the wild-type one had two major components with mass of 3330 and 3039 corresponding to $Gal.GlcNAc.Gal.Glc.Hep_2.GlcNAc.PEA_3.Kdo_2$.lipid A ± NeuNAc. These results show that the *lgtH* gene encodes a galactosyltransferase that uses the LOS acceptor truncated at Glc of the lacto-*N*-neotetraosyl chain.

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Proteomic and functional analyses of membrane blebs isolated from *Neisseria meningitides* serogroup B

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We used an acetate auxotroph of *Neisseria meningitides* serogoup B to facilitate metabolic labeling of bacterial endotoxin (lipo-oligosaccharide, LOS) and compared interactions of purified LOS aggregates and of membrane-associated endotoxin with LBP, CD14 and endotoxin-responsive cells. Membrane blebs constitutively shed into the bacterial culture medium were purified by differential centrifugation and gel sieving chromatography. The endotoxin, phospholipid and protein composition of the recovered blebs indicate that the blebs derive from the bacterial outer membrane. Proteomic analysis revealed an unusual enrichment in highly cationic proteins (pI > 9) proteins. Both purified LOS aggregates and blebs activated monocytes and endothelial cells in a LBP, CD14 and TLR4/MD-2 dependent fashion, but the blebs were 3–10 times less potent when normalized for the amount of endotoxin added. Differences in potency correlated with differences in efficiency of LBP-dependent delivery to and extraction of endotoxin by CD14. Both membrane phospholipids and endotoxin were extracted by LBP/sCD14 treatment, but only LOS:sCD14 reacted with MD-2 and triggered TLR4-dependent cell activation. These findings indicate that the pro-inflammatory potency of endotoxin may be regulated by its association with neighboring outer membrane molecules as well as by its intrinsic structural properties.

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Antibiotic-induced release of Shiga toxin and endotoxin from *Shigella dysenteriae* type 1

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Twenty clinical isolates of *Shigella dysenteriae* type 1 were tested for release of endotoxin and Shiga toxin (STX) *in vitro* in response to ciprofloxacin, imipenem, ceftriaxone, ceftazidime, and mecillinam. STX in the medium was measured using a monoclonal antibody based indirect sandwich ELISA procedure, and endotoxin levels were measured by *Limulus* amebocyte lysate (LAL) assay. Morphological changes in the bacteria were examined under light microscopy after treatment with the respective antibiotics. The median levels of STX released in response to imipenem, ciprofloxacin, mecillinam, ceftazidime, ceftriaxone, and in control after 6 h of incubation were 110.68, 66.78, 62.87, 29.87, 18.8, and 0 ng/ml, respectively. Endotoxin release in response to ceftazidime, ceftriaxone, ciprofloxacin, imipenem, mecillinam and in the control were 2.35, 1.84, 1.75, 1.31, 0.78, 1.36 x 10^5 EU/ml, respectively. Endotoxin levels were higher than control ($P < 0.002$) with ceftriaxone and ceftazidime, lower than control with mecillinam ($P < 0.04$), and comparable with control in case of ciprofloxacin and imipenem. Microscopy revealed long filamentous changes in the bacteria when treated with ceftriaxone, ceftazidime, or ciprofloxacin; rod-shaped changes with nalidixic acid; and round-shaped transformation with imipenem or mecillinam. Further investigation is warranted to determine if similar effects of antibiotics are observed *in vivo*.

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New methods for the extraction, purification and quick characterization of bacterial endotoxins

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A quick and easy way to extract rough-type endotoxins at room temperature was developed using ammonium isobutyrate. Few steps of filtration and dialysis are required to obtain LPSs in good yields. Preparations to be used for biological tests were further purified by a new method. Each of the methods could be performed in 24 h and avoided the use of phenol. The resulting LPSs induced IL-1β, IL-6 and TNF while only activating macrophages from wild-type or $TLR2^{-/-}$ but not $TLR4^{-/-}$ mice. Furthermore, using the NF-κB luciferase reporter gene assay, these LPSs activated human MD2-TLR4 transfected HEK293 cells but not hTLR2 transfected HEK293 cells, confirming the degree of purity of the endotoxins. The purification method uses mineral acid added to a mixture of solvents to dissociate the non-covalent bonds existing between LPS and contaminants such as lipoproteins. This treatment can be followed by conventional enzymatic procedures when necessary. The method is broadly applicable to different Gram-negative bacteria. A mini extraction procedure was also devised for SDS-PAGE samples as well as a quick hydrolytic procedure for obtaining lipid A directly from bacterial cells and giving spectra in less than 24 h. These mini experiments are ideal for detecting structural modifications due to culture conditions or strain variability.

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A rapid and sensitive assay to detect circulating endotoxin in children with diarrhea

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The levels of circulating endotoxin in sera were determined with a kinetic assay using endotoxin-specific chromogenic *Limulus* reagent combined with alkaline pretreatment (Endospec ES test-MK) from children hospitalized due

to diarrhea in the International Center for Diarrhoeal Disease Research, Dhaka, Bangladesh. A significant level of endotoxin was determined in the sera from 23 of 42 subjects (54%), and the serum endotoxin levels with higher than 100 pg/ml were detected in 10 patients (23%). None of 32 healthy controls had any significant level of endotoxin. Three patients with malnutrition as well as diarrhea had extremely high levels of endotoxin exceeding 1000 pg/ml. However, comparison between the two groups on a statistical basis suggested that there was no significant difference in the serum endotoxin level between patients with and without severe malnutrition. Antibiotic therapy markedly reduced the level of endotoxin and improved the general condition of most patients. Non-survivors ($n = 5$) had higher levels of endotoxin with Gram-negative bacteremia before antibiotic therapy than survivors ($n = 37$), suggesting that a high level of circulating endotoxin might be a useful marker as a diagnostic aid to early treatment and management for diarrheal diseases.

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A quarter of a century for endotoxin assay: role of 'internal standard' revisited

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Twenty-five years have passed since the development of a quantitative endotoxin assay using a synthetic chromogenic substrate for the endotoxin-sensitive *Limulus* enzyme. The turbidometric endotoxin assay by *Limulus* lysate has also been improved to enable quantitative assay. Endotoxin-specific chromogenic substrate developed in Japan has added more accuracy to the assay. However, popular interest in endotoxemia has recently declined because of the poor detection rate of endotoxin in the clinical situation. The major problem is that an optimal method for eliminating plasma inhibitors has not yet been established. We have stressed the necessity of an internal standard for checking recovery in all endotoxin assay system. In order to improve the recovery of endotoxin in the dilution and heating method, we added a detergent, Tween 80, after heating the plasma. Large amounts of endotoxin lost in the precipitate fraction by the perchloric acid method have been successfully recovered by adding triethylamine. The internal standard is essential for checking optimal pH conditions by this acid and neutralizing system. We have reported the clinical significance of endotoxemia by several endotoxin-specific assay systems with this internal standard. In this paper, we would like to focus on this important point again in the newly-developed endotoxin assay system.

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A cell line assay system for predicting the response of human blood to endotoxin

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There are parenteral drugs that augment *in vivo* action of endotoxin. It is necessary to regulate overall toxic action of contaminated endotoxin by a clinically relevant test method for the safety of such drugs. Although responses of human peripheral blood cells (hPBC) to endotoxins to produce tumour necrosis factor-α (TNF-α) interleukin 6 (IL-6) and IL-1β showed considerable variations depending on endotoxin and also on source individuals of blood, the responses to each of the endotoxins evaluated relatively to that to Japanese Pharmacopeia endotoxin reference standard (RSE) were found to be highly reproducible irrespective of hPBC sources. The evaluation procedure according to the relative responsiveness to various endotoxins was shown to be highly effective to detect difference in responsiveness among the endotoxin test, the pyrogen test and the cytokine induction in hPBC. When eight human monocytoid cell lines were examined, only THP-1 and 28SC cells showed a significant dose-dependent IL-6 production. However, THP-1 failed to show consistency with hPBC in responses to the panel of endotoxins. 28SC cells showed appropriate consistency with hPBC not only in the responses to the endotoxins but also in detecting the effect of human interferons to augment endotoxin to induce IL-6.

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A quantitative *in vitro* assay for detecting biological activity of endotoxin using rabbit peripheral blood

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The pyrogen test and the bacterial endotoxin test (the LAL test) have been playing crucial roles in detecting endotoxin contamination in parenteral drugs. The current test methods, however, have disadvantages such as requiring a large number of animals or an inadequacy in direct evaluation of *in vivo* activity of endotoxin. We attempted to establish a new assay method that can overcome the shortcomings of the current assay methods. We standardized the *in vitro* assay method by the use of prostaglandin E_2 induction from peripheral blood of rabbits for detecting activity of endotoxin. A linear dose-response regression from approximately 0.15 to 5.0 EU/ml of Japanese Pharmacopoeia Endotoxin Reference Standard was attained by the *in vitro* assay. The assay showed a fine correlation with the pyrogen test but not with the LAL test, when endotoxins from various bacterial sources were tested. The *in vitro* prostaglandin E_2 induction test using rabbit blood was, therefore, suggested to be the appropriate test method for guaranteeing the same level of safety of parenteral drugs as by the pyrogen test.

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Pseudomonas LPS, a most prominent contamination of water samples is vastly overestimated by LAL with regard to pyrogenicity

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We have compared the immune stimulatory potency of 11 different LPSs using human whole blood incubations. LPS from *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella abortus equi*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella typhosa*, *Serratia marcescens* and *Shigella flexneri* equipotently induced the release of all investigated cytokines. In contrast, 1000-fold more LPS from *Pseudomonas aeruginosa* and *Vibrio cholerae* were still less potent in inducing TNF, IL-1β, IL-10 and IFN-γ but induced huge amounts of IL-8. All LPSs showed clear TLR4-dependence except for the LPS from *P. aeruginosa* which was TLR4 and TLR2 dependent. Since *P. aeruginosa* is an important source of LPS contamination able to grow in water at 4°C, its mechanisms of cytokine release were further investigated. Repurification of the commercial LPS preparation by phenol re-extraction led to a complete loss of the TLR2 dependency, indicating contamination with lipoproteins. The *Limulus* amebocyte assay revealed that LPS from *P. aeruginosa* is 5-fold less potent than an *E. coli* LPS. This leads to an enormous overestimation of pyrogen burden, since the biological activity of highly pure LPS from *P. aeruginosa* was about 1000-fold weaker than the one of the reference preparation, as confirmed by monocyte activation in human whole blood.

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An antimicrobial peptide amplifies LPS-induced exocytosis of horseshoe crab hemocytes

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LPS-induced exocytosis of granular hemocytes is a key component of the horseshoe crab's innate immunity to infectious micro-organisms; stimulation by LPS induces the secretion of various defense molecules from hemocytes. Using a newly established assay for exocytosis, we clearly show that a granular protein called factor C, an LPS-recognizing serine protease zymogen that initiates the hemolymph coagulation cascade, also exists on the hemocyte surface as a biosensor for LPS. Our data also demonstrate that the proteolytic activity of factor C is both necessary and sufficient to trigger exocytosis through a heterotrimeric G protein-mediating signaling pathway (Ariki *et al. Proc Natl Acad Sci USA* 2004; **101**: 953–958). Recently, we found an amplifying mechanism in LPS-induced exocytosis of hemocytes. In the absence of LPS, an antimicrobial peptide tachyplesin, a major component of exocytosed fluid, induced exocytosis of hemocytes. A phospholipase C inhibitor U-73122 and a G protein inhibitor pertussis toxin inhibited tachyplesin-induced exocytosis. Furthermore, surface plasmon resonance analysis showed that tachyplesin directly interacts with G protein, indicating that tachyplesin-induced exocytosis occurs through a G protein-mediating signaling pathway. Tachyplesin functions not only as an antimicrobial substance, but also as an amplifier of LPS-induced exocytosis at injured sites.

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Apolipoprotein CI augments the inflammatory response to LPS and correlates with improved infection-related survival in mice and humans

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We recently discovered that plasma apolipoprotein CI (apoCI) contains an important LPS-binding motif, and now evaluated whether apoCI can modulate the response to LPS in mice and humans. Pre-incubation of [^{125}I]-LPS with apoCI (1:1 molar ratio) greatly prolonged the plasma residence of LPS in mice by preventing the association of [^{125}I]-LPS with the liver (9.4 ± 0.9 vs 68.2 ± 2.3%), and increased the LPS (25 μg/kg)-induced TNF-α response 4-fold (10.8 ± 4.5 vs 2.8 ± 1.7 ng/ml, $P < 0.001$). Likewise, human apoCI-expressing (APOC1) mice had a 7-fold increased LPS-induced TNF-α response as compared to controls (28.2 ± 9.1 vs 4.1 ± 2.3 ng/ml, $P < 0.001$). Also, in patients undergoing cardiac surgery with cardiopulmonary bypass, a significant positive correlation was found between apoCI and endotoxemia-induced peri- and postoperative TNF-α levels ($P = 0.004$; $n = 58$). ApoCI protected against infection-related mortality, as APOC1 mice showed increased survival after pulmonary infection with *Klebsiella pneumoniae* (42%) as compared to wild-type (29%) and apoCI-deficient mice (8%). Moreover, survivors from infection-related febrile illness ($n = 270$) had 36% increased apoCI levels upon hospitalization, as compared to non-survivors ($n = 29$; 5.83 ± 0.17 vs 4.28 ± 0.42 mg/dl, $P < 0.01$). We conclude that apoCI may have a physiological role in facilitating an efficient attack towards bacterial infections in mice and humans by providing an increased pro-inflammatory response to LPS.

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Novel LPS-binding cyclic peptides and cytokine-binding peptides

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Although various LPS-binding proteins and peptides have been reported, no appropriate therapeutic agents for sepsis have been identified, so far. We attempted to make a new approach of isolating LPS-binding peptides and cytokine-binding peptides using phage-display method. We established libraries of phages displaying cyclic peptides with random sequences of 4–12 amino acids, whose cysteine residues were covalently bound to form S–S bonds. We screened the libraries to isolate phages with significant affinity to lipid A, and LPS to synthesize possible LPS-binding peptides according to the DNA sequence of the phages. We also attempted to isolate peptides that bind to cytokines. The procedure is expected to be effective in searching for novel LPS-binding peptide and cytokine-binding peptide sequences and structures. We have isolated several kinds of peptide of 14 amino acids with 4–12 circularized amino acids to identify peptides that have a significant level of affinity to LPS. We also have isolated several kinds of linear peptide of 7–12 amino acids to identify peptides that have a significant level of affinity to cytokines. We are further evaluating those peptides for their practical applicability for medical or therapeutic purposes.

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Serum factor other than LBP is necessary for soluble MD-2 to gain its function as a part of the LPS receptor

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To clarify the nature of soluble MD-2 (sMD-2), we purified sMD-2 from yeast transfected with human MD-2 and studied the effect of sMD-2 on the activation of NF-κB in response to LPS in HEK293 cells. When the cells expressing TLR4, MD-2

and CD14 were stimulated with LPS in DMEM containing 10% FCS, the activation of NF-κB was observed without the addition of sMD-2. Although the cells expressing TLR4 and CD14 did not respond to LPS stimulation in DMEM containing 10% FCS, those cells conferred the response when sMD-2 was added to the medium. In a serum-free medium containing LBP, LPS-induced activation of NF-κB was observed in cells expressing TLR4, MD-2 and CD14 either with or without the addition of sMD-2; however, the cells expressing only TLR4 and CD14 did not respond to LPS stimulation even with sMD-2. When cells expressing TLR4 and CD14 were pre-incubated with sMD-2 in DMEM containing 10% FCS, these cells responded to LPS stimulation in serum-free DMEM containing LBP. Taken together, these results suggest that sMD-2 is transferred to cell surface in the presence of a serum factor other than LBP.

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Soluble MD-2 activity in plasma from patients with severe sepsis and septic shock

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We have found that plasma from patients with severe sepsis and septic shock but not normal plasma supports LPS activation of epithelial cells expressing TLR4. Recombinant soluble MD-2 complemented normal plasma, and allowed LPS activation of epithelial cells to levels measured with 'septic' plasma, whereas soluble MD-2 depleted plasma lost its effects. The same 'MD-2 activity' was found in urine from a patient with septic shock and in lung edema fluids from ARDS patients. The addition of LBP and soluble CD14 to soluble MD-2 increased the LPS-dependent activation of cells bearing TLR4, but were not sufficient to support LPS activation of TLR4-expressing epithelial cells. An anti-MD-2 monoclonal antibody blocked LPS activation of TLR4-expressing cells only in the presence of septic plasma or septic urine. Septic plasma was also markedly more efficient in supporting LPS activation of human primary endothelial cells, an effect that was soluble MD-2 independent, but required LBP and soluble CD14. These results suggest that septic plasma containing soluble MD-2 leaking into the extravascular space supports LPS activation of TLR4-expressing epithelial cells. We therefore propose that soluble MD-2 is an important mediator of organ inflammation during sepsis.

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A region of human MD-2 required for the antagonistic activity of lipid IVa

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A synthetic lipid A precursor, lipid IVa, activates murine macrophages while it functions as a lipopolysaccharide (LPS)-antagonist in human macrophages, and MD-2 participates in this species-specific activity. We explored amino acid regions of human (h)MD-2 required for the antagonistic activity in HEK293 cells. Lipid IVa stimulated an NF-κB-dependent reporter activity in cells expressing mouse (m)CD14, mToll-like receptor and mMD-2 but was almost inactive and functioned as an LPS-antagonist when mMD-2 was replaced with hMD-2. The amino acid sequence of mMD-2 was divided into 6 regions and each region was replaced with the corresponding hMD-2 sequence. The activity of lipid IVa and a synthetic *Escherichia coli*-type lipid A (compound 506) as well as the antagonistic activity of lipid IVa were examined with these mutants and we found that an MD-2 mutant in which amino acids 57–73 were replaced with the human sequence exhibited the human phenotype. MD-2 mutants in which each amino acid in this region was individually replaced with the human sequence exhibited the mouse phenotype. These results indicate that amino acids 57–73 of human MD-2 is involved in the antagonistic activity of lipid IVa.

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TLR4 variant Asp299Gly induces hyporesponsiveness to LPS in human epithelial cells

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Toll-like receptor 4 (TLR4) has gained importance as a receptor for the recognition of lipopolysaccharide (LPS) in both blood cells and epithelial cells. It has been recently reported that two commonly occurring mutations in the human TLR4

gene (Asp299Gly and Thr399Ile) are associated with hyporesponsiveness to inhaled LPS as well as with increased risk of Gram-negative bacteraemia in sepsis patients. However, the importance of these variants during the LPS responses in epithelial cells is largely unknown. In this study, we investigated the nature of TLR4 variants (Asp299Gly, Thr399Ile and Asp299Gly/Thr399Ile) in human epithelial cells. Functional analysis of these TLR4 variants in HeLa cells was performed by RT-PCR using IL-8 specific primer and luciferase assay using IL-8 promoter construct. Here, we show that LPS treatment increased IL-8 mRNA expression and IL-8 promoter activity in wild-type and Thr399Ile-expressing cells but not in Asp299Gly- and Asp299Gly/Thr399Ile-expressing cells, suggesting that the Asp299Gly variant might be important for the responsiveness to LPS in human epithelial cells. To investigate whether expression of TLR4 variants itself can affect the endogenous TLR4 surface expression, we transfected the CHO cells, which stably express TLR4, MD-2 and CD14 (TLR4/MD-2/CD14-CHO cells), with TLR4 variants. Surprisingly, transfection of the cells with Asp299Gly and Asp299Gly/Thr399Ile but not wild-type and Thr399Ile reduced the endogenous expression of TLR4 in TLR4/MD-2/CD14-CHO cells. Taken together, TLR4 variants Asp299Gly and Asp299Gly/Thr399Ile might have a dominant-negative effect on endogenous expression of TLR4 in human epithelial cells and these effects might be important for hyporesponsiveness of these variants to LPS.

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The tetraspanins CD37 and CD81 are involved in LPS signaling

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Stimulation with lipopolysaccharide (LPS) induces an innate immune receptor complex consisting of several molecules including TLR-4, CD14, CD11/18 and CD81, which belongs to the tetraspanin superfamily (TM4SF). TM4SF proteins, including CD37, CD53 and CD151, have been shown to associate with a variety of membrane proteins and receptor complexes. The role of CD81 as a scaffolding protein which increases the stability of functional complexes by assembling specific proteins has been suggested and might be important for the stability of the LPS receptor complex, since CD81-deficient mice are less susceptible to LPS-induced shock. Because members of the TM4SF have been shown to form a network, we wanted to investigate if CD37 is involved in LPS signaling as well. Corresponding to CD81-deficient mice, LPS-induced macrophages from CD37-deficient mice showed a decreased TNF-α response which was almost reduced to background levels in CD37/CD81 double deficient mice. However, LPS-induced activation of MAP kinases such as p38 and ERK were comparable to the wild-type control. Thus, the absence of CD37 and CD81 does not lead to a general defect of LPS signaling. Taken together, our data suggest a specific role for TM4SF proteins in the LPS receptor complex and LPS-induced cytokine production.

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Expression of Toll-like receptor 4 and tumor necrosis factor-α in rat macrophages

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It has recently been shown that Toll-like receptor (TLR) 4 of macrophages is responsible for signal transduction leading to production of tumor necrosis factor (TNF)-α in response to lipopolysaccharide (LPS). As our previous study revealed differences of TNF-α production among various rat macrophages, we examined the expression of TNF-α mRNA and TLR4 mRNA in rat Kupffer cells, splenic macrophages and alveolar macrophages. Kupffer cells, splenic macrophages and alveolar macrophages separated from male Wistar rats were incubated with 0, 10, 100, 1000 ng/ml LPS for 3 h. Total RNA was extracted from these cells. TNF-α mRNA and TLR4 mRNA were determined through reverse transcription polymerase chain reaction (RT-PCR). The PCR products were size-fractionated by a 2% agarose gel electrophoresis, and DNA bands were visualized by staining the gel with ethidium bromide. TNF-α mRNA expression was greatest in alveolar macrophages, although TLR4 mRNA expression was not different among these cells. TNF-α mRNA tended to be increased and TLR4 mRNA expression tended to be decreased with higher LPS concentration in all macrophages. Although it has been believed that LPS-stimulated TNF-α production of macrophages was mostly TLR4 dependent, our results suggest that expression of TLR4 mRNA and TNF-α mRNA was not always parallel in these macrophages.

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Endotoxin-stimulated tumor necrosis factor-α production by various macrophages in rats with acute alcohol loading

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It is suggested that endotoxin, pro-inflammatory cytokines and lipopolysaccharide binding protein (LBP) play an important role in the development of alcoholic liver disease. In order to know the pathophysiological roles of various macrophages in alcoholic liver diseases, we examined the production of TNF-α by rat Kupffer cells, splenic macrophages and alveolar macrophages with acute alcohol loading in the presence or absence of LBP. Kupffer cells, splenic macrophages and alveolar macrophages were isolated from male Wistar rats given 5 mg/g body weight of ethanol intraperitoneally after an hour. The production of TNF-α by these cells incubated with 1000 ng/ml endotoxin in the presence or absence of LBP (1% rat serum) was determined. Acute alcohol loading did not affect the production of TNF-α by Kupffer cells. With acute alcohol loading, splenic macrophages tended to produce TNF-α more. Alveolar macrophages produced more TNF-α than Kupffer cells, and although the production of TNF-α by alveolar macrophages tended to be suppressed by acute alcohol loading, the production of TNF-α by alveolar macrophages still remained high in the presence of rat serum. There is a possibility that splenic macrophages and alveolar macrophages may be related to excessive production of TNF-α in acute alcoholics with endotoxemia.

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Effect of alcohol and serum on endotoxin-stimulated secretion of tumor necrosis factor (TNF)-α by macrophages

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Much interest has been focused on an association between endotoxin (LPS), pro-inflammatory cytokines, LPS binding proteins and the progression of alcoholic liver diseases. Until now the effect of LPS on macrophages has been investigated in the absence of serum factors, which may influence LPS action *in vivo*. In order to know the pathophysiological roles of macrophages in alcoholic liver diseases, we examined the effect of ethanol on LPS-stimulated TNF-α secretion of rat Kupffer cells, alveolar macrophages and peritoneal macrophages in the presence or absence of normal rat serum. In the absence of serum, addition of ethanol (10–100 mM) suppressed TNF-α secretion of alveolar macrophages. Kupffer cells and peritoneal macrophages were less affected. An addition of 1% rat serum led to marked enhancement (7–24-fold) of TNF-α secretion of all macrophages either with or without ethanol in the medium. Although ethanol tended to suppress TNF-α secretion of these cells, alveolar macrophages were less affected in the presence of serum. These results suggest that: (i) serum LPS binding proteins enhance TNF-α secretion by macrophages; and (ii) the alveolar macrophages are important for excessive secretion of TNF-α in alcoholics, which may contribute to the development of multiple organ failure.

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Synergistic transcriptional activation of inflammatory genes by NF-κB and IκB-ζ, a nuclear protein induced by LPS

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IκB-ζ, a nuclear protein with the ankyrin-repeats, is strongly induced by microbial components stimulating Toll-like receptors (TLRs), such as LPS, and IL-1β. Recent studies have demonstrated that IκB-ζ plays a crucial role in expres-

sion of inflammatory genes including IL-6 upon activation of TLR/IL-1R signaling pathways. Here, we show that co-expression of IκB-ζ and the NF-κB p50 subunit in HEK293 cells synergistically activates transcription of human β-defensin 2 gene, whereas it inhibits NF-κB-mediated transcription of the IL-8 gene. Reporter analyses of the β-defensin 2 promoter showed that a fragment spanning –205 to +50 of the gene was essential for the IκB-ζ-mediated activation. Mutations at an NF-κB site in the fragment abolished the activation. However, IκB-ζ acted as an NF-κB inhibitor on a promoter constructed with the NF-κB site alone, suggesting that IκB-ζ-mediated transcriptional activation requires another *cis*-element(s). A mutation at a C/EBP site adjacent to the NF-κB site in the promoter completely eliminated the stimulating activity of IκB-ζ. Furthermore, we found that genes that require IκB-ζ in their expression also harbor a C/EBP site(s) in their promoters. These results suggest that IκB-ζ acts as a transcriptional activator by forming a complex with NF-κB on promoters harboring NF-κB and C/EBP sites.

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Molecular dissection of IκB-ζ, a transcriptional regulator induced by activation of innate immunity

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IκB-ζ is an inducible nuclear protein, which we found in lipopolysaccharide (LPS)-stimulated macrophages. Overexpressed IκB-ζ inhibits NF-κB reporter activities by interacting with NF-κB via the C-terminal ankyrin-repeats. In the present study, we constructed a series of deletion mutants of IκB-ζ and analyzed their functions, focusing on the roles of the N-terminal region with no homology to other proteins. Indirect immunofluorescent studies demonstrated the presence of the nuclear localization signal in the region of amino acids 153–187. The GAL4-UAS reporter assay revealed that GAL4-fusion proteins containing amino acids 188–456 of IκB-ζ exhibited transcriptional activation activity. Interestingly, the activity was not detected in the full-length or a longer fragment of IκB-ζ with the C-terminal ankyrin repeats. On the other hand, we detected the GAL4-dependent transcriptional activity when a GAL4-fusion protein of the NF-κB p50 subunit was co-expressed with the full-length IκB-ζ, neither of which alone exhibited the activity. Wild-type IκB-ζ, but not a splicing variant lacking 236–429, augmented IL-6 production by LPS-stimulated NIH3T3 cells. These results indicate that IκB-ζ harbors latent transcriptional activation activity, and the activity is expressed upon interaction between IκB-ζ and the NF-κB p50 subunit, which would be crucial for the activation and regulation of innate immunity.

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Differential regulation of human and murine iNOS expression in response to lipopolysaccharide

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It is known that mouse macrophages produce nitric oxide (NO) in response to lipopolysaccharide (LPS) stimulation; however, LPS stimulation is not enough to stimulate the production in human macrophages. We explored this differential regulation in the RAW 264 murine macrophage cell line and the THP-1 human monocyte cell line. Since NO production in response to LPS is regulated by the transcriptional expression of inducible NO synthase (iNOS), 1.6–1.8 kb fragments of human (h) and mouse (m) iNOS promoter regions were inserted upstream of a luciferase reporter vector and reporter activity in response to LPS stimulation was examined. When each reporter was transfected into RAW 264 cells, LPS stimulation caused a remarkable induction of both hiNOS and miNOS promoter activity. However, when transfected into THP-1 cells, almost no induction was observed in both reporter constructs. These results suggest that human macrophages lack transcription factors that are induced in mouse macrophages in response to LPS stimulation. We are now attempting to identify the difference in transcription factors induced by LPS stimulation.

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The enhancing action of D-galactosamine on lipopolysaccharide-induced nitric oxide production in RAW 264.7 macrophage cells

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The effect of D-galactosamine (D-GalN) on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells was examined. D-GalN augmented the production of NO, but not tumor necrosis factor (TNF)-α in LPS-stimulated RAW 264.7 cells. Pretreatment of D-GalN augmented the NO production whereas its post-treatment did not. D-GalN augmented the NO production in RAW 264.7 cells stimulated with either TNF-α and interferon-γ. The augmentation of LPS-induced NO production by D-GalN was due to enhanced expressions of an inducible type of NO synthase mRNA and proteins. Intracellular reactive oxygen species (ROS) were exclusively generated in RAW 264.7 cells stimulated with D-GalN and LPS. Scavenging of intracellular ROS abrogated the augmentation of NO production. It was, therefore, suggested that D-GalN might augment LPS-induced NO production through the generation of intracellular ROS.

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Inhibition of mitogen and stress-activated protein kinase 1/2 induces mitochondria-mediated apoptotic cell death in lipopolysaccharide-stimulated RAW 264.7 macrophage cells

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The effect of inhibition of mitogen and stress-activated protein kinases 1/2 (MSK1/2) on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells was investigated. Pretreatment of Ro 31-8220, an inhibitor of MSK1/2, induced cell death in LPS-stimulated RAW 264.7 cells. On the other hand, calphostin C, another inhibitor of protein kinase C, did not cause cell death. The cell death was not mediated by the release of pro-inflammatory mediators from LPS-stimulated RAW 264.7 cells. The cell death was accompanied by DNA fragmentation and annexin V binding, suggesting apoptotic cell death. Further, several caspase inhibitors did not prevent LPS-induced cell death of Ro 31-8220-pretreated RAW 264.7 cells. The nuclear translocation of apoptosis-inducing factor (AIF) was detected in Ro 31-8220-pretreated cells after LPS stimulation. The cell death was due to mitochondrial damage. Ro 31-8220 exclusively inhibited the phosphorylation of cAMP-responsive element binding protein (CREB) as the substrate of MSK1/2. RAW 264.7 cells transfected with the dominant negative MSK1 clones underwent cell death in response to LPS. Therefore, it was suggested that MSK1/2 might play a critical role in the survival of LPS-stimulated RAW 264.7 cells.

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LPS inhibits doxorubicin-induced apoptosis through the inhibition of p53 activation

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The effect of LPS on doxorubicin-induced cell death was studied in RAW 264.7 cells. LPS at 10 ng/ml prevented the cell death and the inhibition was roughly dose-dependent. Doxorubicin-induced cell death was characterized by DNA fragmentation and activation of caspase 3, suggesting apoptotic cell death. LPS inhibited DNA fragmentation and caspase 3 activation. CpG DNA also inhibited doxorubicin-induced apoptosis whereas tumor necrosis factor-α and interferon-γ did not. The apoptosis was prevented by the addition of LPS at 1 h after treatment with doxorubicin. LPS inhibited doxorubicin-induced p53 accumulation and stabilization and the activation of caspase 3. On the other hand, the inhibition of doxorubicin-induced apoptosis by LPS was independent on mitogen-activated protein kinases, nuclear factor-κB and AKT kinase or the cytotoxic action. The detailed mechanism of the inhibition of doxorubicin-induced apoptosis by LPS will be discussed.

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Modified pattern of proteins reflecting the anti-apoptotic activity of lipopolysaccharides in primary mouse B lymphocytes

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Lipopolysaccharides (LPSs) are potent polyclonal activators of B cells. Recently, we have shown the capability of LPS to inhibit both spontaneous and drug-induced apoptosis in B lymphocytes. Cell death by apoptosis plays an essential role in the development and maintenance of homeostasis of the immune system, its dysfunction leading to various pathologies such as lymphomas or autoimmune diseases. In this report, to better understand the signaling pathways involved in the anti-apoptotic activity of LPSs, we investigated the mechanisms of LPS-induced inhibitory kappa B (IκB) expression and NF-κB translocation. As shown by immunoblotting analysis, LPS induced a time-dependent degradation of IκBβ and IκBε isoforms, preferentially to IκBα, and nuclear translocation of NF-κB p52 and c-Rel. In addition, LPS led to reduced levels of the active form of caspase 3, whereas an increased synthesis of the anti-apoptotic protein Bcl-2 was observed. These results suggest that LPS acts at a pre-mitochondrial stage of the intrinsic apoptotic pathway. Further studies, carried out with B cells from TLR4 defective mice, showed that LPS had no effect on apoptosis, on IκBβ degradation, and on Bcl-2 synthesis, whereas IL-4 maintains its anti-apoptotic activity, thus suggesting that a TLR4-dependent signaling is required for the anti-apoptotic activity of LPS.

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MARCKS-LPS interaction in epithelial cells and its effect on actin filament depolymerization

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Transcription of myristoylated alanine-rich C-kinase substrate (MARCKS) and its homologue, MARCKS-related protein (MRP), is significantly increased on stimulation by bacterial lipopolysaccharide (LPS). MARCKS contains the lysine-rich effector domain, which mediates the cross-talk between signal transduction pathways involving cellular membrane, protein kinase C, calmodulin and actin. We report that MARCKS from murine brain and human MRP binds to LPS with high affinity. The effector domain of MARCKS binds specifically to LPS and inhibits TNF-α secretion by mononuclear cells stimulated by LPS. Direct intracellular interaction between MARCKS and internalised LPS was detected by FRET effect in human epithelial cells. FRET was confirmed by the increased fluorescence of donor (MARCKS-GFP) after photobleaching of the acceptor (rhodamine-LPS). LPS-binding site of MARCKS was mapped to the heptapeptide KRFSFKK within the MARCKS effector domain using high resolution NMR. This LPS binding site of MARCKS overlaps with the actin-binding site. The consequence of this overlap is that the addition of LPS prevents polymerization and bundling of actin by MARCKS and depolymerizes F-actin. These findings suggest that direct interaction between MARCKS and LPS may be part of the cellular response to he internalised LPS, significant particularly in cells exposed to high levels of LPS, such as epithelial cells.

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LPS-induced IL-18 in mouse peritoneal cells; the induced amount is very small but it plays an important role as a mediator

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We have demonstrated that interferon (IFN)-γ is produced by mouse peritoneal cells upon LPS stimulation and contributes largely to nitric oxide (NO) production. In the production of IFN-γ, participation of IL-18 was suggested but

detection of LPS-induced IL-18 was not easy. To detect significant amount of IL-18 (> 100 pg/ml), LPS-stimulated culture supernatants were concentrated and detected 200–800 pg/ml in 10-fold concentrated samples. Compared to the amount of LPS-induced IL-12 and IFN-γ (in the order of ng/ml), that of IL-18 was revealed to be very small. IL-18 is secreted from the cells as an active form via processing by caspase-1. In the presence of an inhibitor of caspase-1, the LPS-induced IL-18 production was markedly reduced. The inhibitor also reduced the production of IFN-γ and NO, while scarcely reduced that of IL-12. Similar results were obtained by the use of anti-IL-18-receptor antibody. When the cells were cultured in the presence of IL-12 at 2.5 ng/ml, supplement of IL-18 at 20 or 100 pg/ml was enough to induce synergistic effect on production of substantial amounts of IFN-γ and NO. These results indicated that LPS induces only a small amount of IL-18; nevertheless, it plays an important role as a mediator.

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The role of lysophosphatidylcholine acyltransferase in leukocyte responses to lipopolysaccharide

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Mononuclear phagocytes play a pivotal role in the progression of sepsis by producing inflammatory mediators including TNF-α in response to bacterial lipopolysaccharide (LPS). Monocytes and macrophages can be made sensitive to LPS by treatment with IFN-γ (priming) and our previous studies showed this correlates with changes in monocyte membrane phospholipid composition, particularly in phosphatidylcholine, mediated by acyltransferases.

We report that IFN-γ can selectively activate lysophosphatidylcholine acyltransferase (LPCAT) but not lysophosphatidic acid acyltransferase in monocytic cells. Acyltransferase activity was measured by incorporation of radiolabelled fatty acyl CoA into lysophospholipid acceptors. Furthermore, specific inhibitors of LPCAT were found to inhibit (> 90%) the TNF-α and IL-6 production upon LPS challenge in both primed and unprimed cells. This inhibition of inflammatory cytokines with LPCAT inhibitors was also demonstrated in peripheral blood monocytes. The inhibition was not dependent on lysophosphatidylcholine (lysoPC) as blocking the lysoPC receptor (G2A) had no effect on the inhibition of cytokines. Our recent data indicate that LPCAT may influence LPS receptor aggregation in the membrane by controlling the accessibility to lipid raft domains.

These data suggest an important role for LPCAT in the response of leukocytes to LPS and that inhibition of LPCAT activity may have anti-inflammatory therapeutic potential.

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The effect of synthetic Kdo on human spermatozoa

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We know that LPS is spermicidal and our data suggest that this is primarily due to lipid A. The aim of this work was to evaluate if synthetic Kdo was spermicidal, had immune stimulatory activity, signalled via TLRs and induced apoptosis. The effects of lipid A and Kdo were assessed with and without anti-CD14 and polymyxin B. HeLa transfection assays were used for TLR activity. IFN-γ induced THP-1 cells were investigated for TNF-α activity. Finally, sperm apoptosis and caspase activity were assessed by flow cytometry and fluorimetry respectively, with and without inhibitors. Kdo was shown to be as spermicidal as lipid A but unlike lipid A was not affected by anti-CD14 or polymyxin B. Lipid A signalled via TLR4 whilst Kdo did not signal via TLR2 or TLR4. TNF-α production was activated by lipid A but not by Kdo. Both lipid A and Kdo induced sperm apoptosis which was primarily caspase-mediated. Kdo is spermicidal and produces death by caspase-mediated apoptosis as does lipid A. However, unlike lipid A, Kdo does not interact via CD14, signal via TLRs or activate TNF-α production. In conclusion, although Kdo induces apoptosis, it would appear that activation is via a different route to lipid A.

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Treponemal glycoconjugate inhibits LPS-induced cell activation by blocking LPS-binding protein and CD14 functions

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In this study, we found that a glycoconjugate preparation obtained from *Treponema medium* (Tm-Gp), an intermediate-sized oral *Treponema*, diminished the activation of a human monocytic cell line, Mono Mac 6, induced by LPS, but not that induced by TNF-α. NF-κB activation in Ba/F3 cells expressing mouse Toll-like receptor (TLR) 4 and MD-2 stimulated with LPS and its active center lipid A, but not with Taxol, was definitely inhibited by Tm-Gp, which was dependent on CD14/LPS-binding protein (LBP). Tm-Gp blocked the binding of LPS to immobilized CD14 and immobilized LBP, and also inhibited nitric oxide production by a mouse macrophage cell line, J774-A1, stimulated with peptidoglycan as well as LPS, but not with poly(I:C) RNA or CpG DNA, in the presence of FBS. Tm-Gp consisted of a glycoconjugate (Tm-GC) and a polysaccharide (Tm-PS), where Tm-GC was Tm-PS combined with phosphatidylglycerol (PG). Further, we demonstrated that PG was the major inhibitory molecule in Tm-Gp. These results indicate that *T. medium* contains a glycoconjugate possessing an inhibitory effect on TLR-mediated cell activation by interacting with LBP and CD14.

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A novel endotoxin antagonist – *Helicobacter pylori* lipopolysaccharide attenuates endotoxicity

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Endotoxin or lipopolysaccharide (LPS) is found on the outer membrane of Gram-negative bacteria. Its toxicophore, lipid A, is responsible for the pathophysiology associated with Gram-negative bacterial endotoxaemia. *Helicobacter pylori* LPS/lipid A, however, exhibits attenuated endotoxicity due to structural deviations from the endotoxic LPS. At nanomolar concentrations, the ENC_{50} of *H. pylori* LPS /lipid A, suppresses *Escherichia coli* endotoxin-induced hTNF-α production and inhibits LAL activation. Using two-dimensional gel electrophoresis, the proteome profiles of macrophages treated with various LPS regimens showed that *H. pylori* LPS/lipid A was able to neutralize *E. coli* LPS-induced endotoxic challenge. *H. pylori* LPS attenuated the up-regulation of several signal transduction proteins, implying that *H. pylori* LPS acts as an antagonist to suppress the induction of the pro-inflammatory signal transduction intermediates such that progress towards inflammation, apoptosis or sepsis is pre-empted. Since *H. pylori* LPS/lipid A is minimally cytotoxic, non-haemagglutinating and non-haemolytic, it is a good candidate for the development of anti-endotoxin agents, purportedly to treat Gram-negative bacterial endotoxaemia.

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Inhibition of cell activation: shedding new light on antagonism

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The tetra-acylated precursor of lipid A, lipid IVa, corresponding to synthetic compound 406, is an established antagonist for the LPS-induced immune cell activation in humans. The molecular mechanisms underlying this antagonistic

action of lipid IVa are not fully understood. It is generally accepted that the antagonist competes with LPS for the binding to soluble or membrane-bound proteins involved in LPS-induced cell activation such as LBP, CD14, and TLR4. Here, we present data showing that compound 406 is not only antagonistically active in molar excess, but even when less than 0.1% of the amount of biologically active LPS is administered to the cells prior or simultaneously to stimulation with LPS. Similar observations have been made with other LPS-antagonists such as synthetic compound E5531 or phospholipids. These findings are not consistent with a competitive model of antagonism. The surprising observation that antagonists not only antagonizes cell activation by LPS but also by the cytokine interleukin (IL)-1, suggests a more general principle of antagonism. We will present data supporting a so-called co-operative model, suggesting that the antagonist is intercalated into cell membranes by LPS-binding protein (LBP) where it may lead to a disturbance of the formation of the signaling complex, eventually leading to inhibition of the production of cytokines.

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Phage display method for isolating novel LPS-binding peptides

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Although various LPS-binding proteins and peptides have been reported, no appropriate therapeutic agents for sepsis have been identified, so far. We attempted to make a new approach of isolating LPS-binding peptides using phage-display method. We established libraries of phages displaying random sequences of linear peptides of 5, 7 and 12 amino acids or cyclic peptides of 4–12 amino acids. We screened the libraries to isolate phages with significant affinity to lipid A or LPS to synthesize possible LPS-binding peptides according to the DNA sequence of the phages. The procedure is expected to be effective in searching for novel LPS-binding peptide sequences and structures. We have isolated several kinds of linear peptide of seven amino acids to identify a peptide that have a significant level of affinity to LPS. The peptide neutralized, in a dose-dependent manner, LAL activity and the activity of LPS to induce IL-6 from 28SC cells that have the similar responsiveness to LPS to that of human peripheral blood cells. We are further attempting to isolate linear and cyclic peptides with much higher affinity to LPS.

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Limulus innate immune molecule for development of novel endotoxin diagnostics, endotoxin clearance and endotoxin antagonist

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After nearly three decades of conventional endotoxin testing methods using the *Limulus* amoebocyte lysate (LAL), the problems associated with LAL are finally resolved by a recent breakthrough in genetic engineering of factor C, an innate immune serine protease, which is activated by Gram-negative bacteria or nanograms of LPS to trigger the coagulation cascade in the horseshoe crab blood. Recombinant factor C (rFC) has a remarkable sensitivity to LPS (0.001 EU/ml). Thus, rFC serves as a novel, environmentally-friendly and standardised source of 'LAL' for sensitive and specific detection of LPS. Based on rFC, a novel microassay has been commercialized (PyroGene®, Cambrex Inc., USA), as a high throughput diagnostic kit for endotoxin in pharmaceuticals and parenterals. Furthermore, being capable of binding both free and bound LPS/lipid A with high affinity, sushi peptides derived from rFC are highly efficacious in the removal of LPS from contaminated fluids. Additionally, the sushi peptides effectively neutralize the endotoxicity, thus offering potentials for development into antimicrobial peptides.

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Efficacy of endotoxin-absorption therapy against septic shock

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Endotoxin plays a role in the development of septic shock caused by Gram-negative bacteria. In Japan, polymyxin B-attached fibers are used clinically to neutralize the biological activity of lipid A. Since hemodynamic improvement is not seen in all cases, it cannot be assumed that endotoxin absorption therapy will be effective against all cases of septic shock.
Methods: The blood endotoxin level was more than 1 pg/ml in 20 of these cases. These 20 cases were separated into two groups: the negative-postabsorption group in which the blood endotoxin level decreased to less than 1 pg/ml after endotoxin-absorption therapy; and the positive-postabsorption group in which the blood level stayed above 1 pg/ml after the therapy. Blood pressure, cardiac index and the dose of catecholamine, before and after therapy and the survival rate after 28 days were compared between the two groups.
Results: After endotoxin-absorption therapy, the mean blood pressure increased significantly to 86.4 ± 6.3 mmHg from 67.9 ± 11.4 mmHg in the negative-postabsorption group, whereas it showed no change in the positive-postabsorption group. After the therapy, the endotoxin level became undetectable in many cases, and patients in the negative-postabsorption group recovered earlier from shock, with a significantly higher rate of survival.

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Research on the adsorption of endotoxin by new polymer lignophenol

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Endotoxin is composed of lipopolysaccharide, the outer cell-wall component of Gram-negative bacteria. Endotoxin leads organism to fever, in serious case it can cause patient shock. Endotoxin is very stable to heat or organic solvents, and the usual sterile operation is difficult to remove endotoxin. Our research shows that lignophenol, which was separated from milled wood in phase-separative reaction system composed of phenol derivative and concentrated acid, can adsorb endotoxin. At room temperature, 50 mg lignophenol can adsorb 1000–2000 EU in aqueous solution. The adsorbed endotoxin was released when organic solvent was added to the system. Separated lignophenol from the lignophenol–endotoxin complex can adsorb endotoxin again. It is hoped that lignophenol will become a functional material for removing endotoxin in the medical industry.

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Enhancement of the bactericidal and LPS-neutralizing activities of guinea pig CAP11-derived antibacterial peptide by amino acid substitutions

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Mammalian myeloid and epithelial cell express several antibacterial peptides (defensins and cathelicidins) that contribute to the innate host defense by killing invading micro-organisms. CAP11 (cationic antibacterial polypeptide of 11 kDa) isolated from guinea pig neutrophils was shown to possess potent bactericidal and LPS-neutralizing activities. Recently, we have characterized the biologically active region of CAP11, and revealed that an 18mer peptide of CAP11 (1–18) is the most potent among various derivatives; however, its bactericidal and LPS-neutralizing activities were much lower than those of native CAP11. In this study, we tried to enhance the biological activity of CAP11 (1–18) by substituting amino acids. A novel peptide, CAP11 (1–18m2), whose hydrophobicity and positive charge were modified by the amino acid substitutions, exhibited more potent bactericidal activities against *Staphylococcus aureus* and *Escherichia coli*, and more strongly inhibited the LPS-binding to $CD14^+$ RAW264.7 cells, than CAP11. Notably, the biological activity of CAP11 (1–18m2) was about 10-fold greater than that of CAP11. Moreover, CAP11 (1–18m2) completely killed both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) even at 0.1 μg/ml. Thus, the bactericidal and LPS-neutralizing activities of CAP11-derived antibacterial peptides could be enhanced by the modification of their hydrophobicity and positive charge.

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Modulation of lipopolysaccharide-induced suppression of neutrophil apoptosis by antibacterial cathelicidin peptide CAP11

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Peptide antibiotics possess potent antimicrobial activities against invading micro-organisms and contribute to innate host defense. We previously revealed that antibacterial cathelicidin CAP11 exerts protective actions against endotoxin shock model. During Gram-negative bacterial sepsis, LPS activates neutrophils and their apoptosis is suppressed. Prolonged presence of activated neutrophils causes uncontrolled release of toxic metabolites, leading to the systemic tissue injury. To evaluate a therapeutic potential of CAP11 for Gram-negative sepsis, we here investigated the action of CAP11 on LPS-induced suppression of neutrophil apoptosis. LPS suppressed neutrophil apoptosis, accompanied with NF-κB activation, ERK phosphorylation, Bcl-XL expression and caspase 3 inhibition. Interestingly, CAP11 reversed the actions of LPS to trigger these changes, and induced neutrophil apoptosis. Furthermore, LPS activated monocytes to produce anti-apoptotic cytokines (IL-1β, TNF-α and IL-8) and suppressed neutrophil apoptosis. Importantly, CAP11 inhibited the cytokine production from monocytes, thereby inducing neutrophil apoptosis. Finally, CAP11 strongly suppressed LPS-binding to neutrophils and monocytes. Thus, CAP11 is able to block the LPS-induced prolongation of neutrophil survival via the suppression of anti-apoptotic signaling in neutrophils and cytokine production from monocytes by inhibiting LPS-binding to target cells. CAP11 is expected to have a therapeutic potential in Gram-negative sepsis to induce neutrophil apoptosis, thereby possibly attenuating tissue injury.

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Enhancement of endotoxin neutralization by coupling of a C_{12}-alkyl chain to a lactoferricin-derived peptide

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Peptide acylation, which mimicks the structure of the natural lipopeptide polymyxin B, increases antimicrobial and endotoxin neutralizing activities. The interaction of the lactoferricin-derived peptide LF11 and its N-terminally acylated analog, lauryl-LF11, with different chemotypes of lipopolysaccharide (LPS Re, Ra and S-form) was investigated biophysically and related to the peptide's biological activities. Lauryl-LF11 shows a much stronger inhibition of LPS-induced cytokine induction in human mononuclear cells than LF11. Although the peptide:LPS interaction is essentially of electrostatic nature, the lauryl-modified peptide displays a strong hydrophobic component. Such a feature might than explain the fact that saturation of the peptide binding takes place at a much lower peptide:LPS ratio for LF11 than for lauryl-LF11 and that an overcompensation of the negative LPS backbone charges is observed for lauryl-LF11. The influence of LF11 on the phase transition of LPS is negligible for LPS Re, but clearly fluidizing for LPS Ra. In contrast, lauryl-LF11 causes a cholesterol-like effect in the two chemotypes, fluidizing of the acyl chains in the gel and rigidifying in the liquid crystalline phase. Both peptides convert the unilamellar/cubic aggregate structure of lipid A into a multilamellar one. These data contribute to the understanding of the mechanisms of the peptide-mediated neutralization of endotoxin.

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Structure/function studies on endotoxin-neutralizing and antibacterial peptides derived from NK-lysin

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Increasing resistance of pathogenic bacteria against antibiotics is a severe problem in health care. In addition, classical antibiotics may release the most important pathogenicity factor of Gram-negative bacteria, lipopolysaccharide (LPS, endotoxin) during the process of killing bacteria thus promoting endotoxic shock. Natural antimicrobial peptides and derivatives thereof have emerged as promising candidates for 'new antibiotics' with novel mechanisms of action. We have designed antibiotic and anti-sepsis peptides based on NK-lysin, an antibacterial protein of mammalian immune cells. The most promising candidate, NK-2, a peptide of 27 amino acid residues, adopts an amphipathic, α-helical secondary structure and interacts specifically with membranes of negatively charged lipids. NK-2 is active against Gram-positive and Gram-negative bacteria, but is itself of low hemolytic activity and cytotoxicity. For structure/function analyses and further improvement, we synthesized shorter versions of the peptide NK-2 and screened their antibacterial, cytotoxic and LPS-neutralizing activities. These biological data correlate nicely with results obtained from biophysical studies (*i.e.* FTIR, SAXS, calorimetry, surface potential) with bacterial and eukaryotic membrane mimetics, including charged and uncharged phospholipids, and lipopolysaccharides of varying sugar lengths, and thus facilitate the deduction of a molecular model of the peptide/lipid interaction.

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Binding and neutralization of LPS/lipid A by argininosuccinate synthase, a rate-limiting enzyme in urea cycle and NO-citrulline cycle – analysis of its binding region

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Liver is thought to be a pivotal organ to eliminate LPS from the circulation. Pursuing the molecule(s) from liver that interact with LPS/lipid A, we found that a cytosolic urea cycle enzyme, argininosuccinate synthase (AS) binds LPS/lipid A and blocks its biological activity. Alternatively, LPS/lipid A blocked the enzyme activity dose-dependently. The binding site(s) for LPS/lipid A in AS was then investigated by analysis of proteolytic fragments of AS purified from mouse liver. Several fragments that bind LPS were first determined by 2D-PAGE analysis (native with LPS followed by SDS-treatment) of total peptide mixture. Sequence analysis revealed that LPS-interacting peptide bands commonly contain Thr210–Arg304. Another analysis carried out on peptide fragments separated by RP-HPLC revealed that fragments Tyr113–Lys140, Asn158–Lys176 represent LPS-blocking activities. Since these regions are reportedly to compose the substrate-binding site of the enzyme, the blocking of enzyme activity by LPS/lipid A supports that these regions interact with LPS/lipid A. While the sequences are rich in hydrophobic and basic amino acids, the patterns are different from LPS-binding sites of CAP18, BPI, and LBP.

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Protein-bound polysaccharide isolated from basidiomycetes inhibits LPS-induced cell activation and prevents endotoxin shock

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The protein-bound polysaccharide isolated from basidiomycetes (PSK) is a biological response modifier capable of exhibiting various biological activities, such as antitumor and antimicrobial effects. In the present study, we demonstrated

that PSK suppressed IL-6 production in murine peritoneal macrophages stimulated with LPS and its synthetic lipid A (compound 506). Nitric oxide production and p38 mitogen-associated protein kinase phosphorylation induced in the murine macrophage cell line J774-A1 by LPS and compound 506 were also inhibited by PSK. Further, PSK distinctly suppressed NF-κB activation in Ba/F3 cells expressing mouse Toll-like receptor 4 and MD-2, following stimulation with LPS and compound 506, however, not with Taxol. These PSK-induced inhibitory actions were caused by inhibition of the physical associations of LPS with LPS-binding protein (LBP) and CD14. PSK also protected mice from LPS-induced lethality, presumably by down-regulating IL-6 and TNF-α concentrations in serum. These findings indicate that PSK, which also has an ability to regulate LBP/CD14 functions, may be useful for clinical control of endotoxic sepsis.

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LPLUNC1 is a secreted product of airway epithelia with proposed innate immune functions

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We used cDNA library sequencing and expression profiling to identify novel transcripts in primary air-liquid interface cultures of human airway epithelia (HAE). We identified two transcripts, termed PLUNC (Palate, Lung, Nasal Epithelium Clone) and LPLUNC1 (long PLUNC1), as among the most abundant messages in these cells. Both are members of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family of proteins; in particular, they share homology with bactericidal/permeability increasing protein (BPI), which exhibits both antibacterial and endotoxin-binding properties. Current studies focus on expression and activity of LPLUNC1. We hypothesized that LPLUNC1 is a secreted protein involved in the innate immune responses of airway epithelia. RT-PCR and microarray screening confirmed that PLUNC and LPLUNC1 are highly expressed LT/LBP family members in HAE. Consistent with a possible role in host defense responses, *in situ* hybridization localized LPLUNC1 mRNA expression to the surface epithelium and submucosal glands of the conducting airway, and Western blotting with a polyclonal LPLUNC1 antibody detected LPLUNC1 protein in washings from regions of the conducting airways including HAE apical secretions and BAL fluid. Ongoing experiments are evaluating antimicrobial properties of LPLUNC1 as well as its ability to associate with endotoxin.

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Novel effects of scavenger receptor ligands on bactericidal/permeability-increasing protein (BPI)-mediated delivery of purified endotoxin to human CD14$^+$ monocytes

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Using fluorescent (BODIPY-FL)- and metabolically [^{14}C]-labeled meningococcal lipo-oligosaccharide (LOS), we demonstrate that BPI promotes association of purified endotoxin with human CD14$^+$ monocytes in a CD14-independent manner. BPI-dependent delivery of LOS to monocytes was greatest at low BPI/LOS molar ratios (1:20) and reduced at higher BPI/LOS ratios. Decreased BPI-dependent uptake of LOS at higher BPI/LOS molar ratios paralleled decreased net negative charge of the BPI:LOS aggregates suggesting that cell association of BPI:LOS aggregates depended on the polyanionic properties of the aggregates. Several polyanionic scavenger receptor (SR) ligands (*e.g.* polyI, polyG, dextran sulfate), but not other polyanions, potently increased BPI-dependent delivery of LOS to CD14$^+$ monocytes at low SR ligand concentrations (1–10 μg/ml) but not at higher concentrations (> 100 μg/ml). The enhancing effects of the SR ligands on BPI-dependent delivery of LOS to monocytes depended on high BPI/LOS molar ratios (up to 1:1) and the polycationic N-terminal domain of BPI. Increased BPI-dependent delivery of LOS to monocytes did not stimulate cell activation but promoted AOAH-like deacylation of LOS. In summary, BPI promotes clearance and partial detoxification of endotoxin by human CD14$^+$ monocytes in a manner that can be significantly regulated by the presence of scavenger receptor ligands.

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A complex of soluble TLR4 and MD-2 blocks LPS activity *in vitro*

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The evolutionary conserved Toll-like receptor (TLR) family as specific pathogen recognition molecules and signal transducers is critical for the activity of the innate immune system. Because TLR-induced signaling is involved in infectious diseases, chronic inflammatory diseases and sepsis, we aimed at modulating TLR-signaling via ligand-binding soluble receptors. TLR2 and TLR4 recognize Gram-positive and Gram-negative cell wall structures, respectively. Therefore, in our current study, we prepared the mouse recombinant fusion proteins mrsTLR2–IgGFc (T2Fc) and mrsTLR4–IgGFc (T4Fc) in *Drosophila melanogaster* Schneider 2 (S2) cells to modulate signaling through these membrane-bound TLRs. To determine the function of soluble TLRs, IL-6 concentrations were measured after stimulation of mouse macrophage cell line cells RAW 264.7 with the proper ligands. Surprisingly, T2Fc had a positive synergistic effect on (S)-[2,3-*bis*(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH] (Pam_3Cys)-induced IL-6 production, whereas T4Fc had no influence on IL-6 levels induced with purified lipopolysaccharide (LPS). Because MD-2 is required for efficient stimulation of membrane TLR4, we prepared tagged MD-2 to generate a T4Fc/MD-2 complex. This complex blocked LPS activity in vitro and might thus represent a new therapeutic option in sepsis.

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C-reactive protein: an acute-phase pathogen recognition receptor for bacterial clearance

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The horseshoe crab possesses a powerful innate immune system capable of clearing Gram-negative bacterial infections at dosages that would have been lethal to mice. This suggests the existence of extracellular frontline defense molecules that recognize LPS, resulting in rapid bacterial clearance. LPS-affinity chromatography of the cell-free hemolymph of the horseshoe crab isolated a repertoire of LPS-binding proteins, of which C-reactive protein (CRP) is the major component. The chromatogram suggests that CRP binds most proximally to LPS. Cloning of CRP revealed multiple CRP genes. This was confirmed by immunochemical characterization of the purified CRP isoforms from the cell-free hemolymph of naïve and infected horseshoe crabs. Two-dimensional electrophoresis revealed 6 predominant CRP isoforms. The relative ratios of these CRP isoforms remain unchanged during infection. *In vivo*, a pre-existing pool of CRP was rapidly consumed during the first hour of bacterial infection. However, the CRP gene expression was up-regulated and the protein was replenished within 6 h post-infection. We propose that CRP plays an important role in the frontline defense against Gram-negative bacteria and its consumption during infection must thus be rapidly replaced by up-regulation of both CRP-1 and CRP-2 genes.

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The acute phase protein haptoglobin prevents hemoglobin-induced dispersion of LPS aggregates and suppresses the hemoglobin-mediated enhancement of cellular activation by LPS

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Hemolytically released hemoglobin (Hb) represents a major source of iron required for systemic growth of Gram-negative and Gram-positive bacteria. The acute phase protein haptoglobin (Hp) is the central high affinity ligand for extracellular hemoglobin in serum and initiates the clearance of cell-free hemoglobin by macrophages via the Hb-Hp scavenger receptor CD163. Aside from its primary function in oxygen transport, hemoglobin has been shown to induce the dispersion of lipopolysaccharide (LPS) aggregates and changes in LPS conformation. In addition, Hb has been found to enhance LPS-induced cellular activation *in vitro* as well as the toxicity of LPS in a murine model of septic shock. This immunomodulatory function of extracellular hemoglobin has been discussed to provide an additional increase in sensitivity in the early immunodetection of LPS during Gram-negative bacteremia. Here, we report that the binding of haptoglobin to hemoglobin prevents the LPS disaggregation activity of Hb and suppresses the Hb-mediated enhancement of LPS-induced human monocyte activation *in vitro*. During systemic infections by Gram-negative bacteria haptoglobin may have an additional protective function in dampening an otherwise excessive and potentially harmful enhancement of LPS-induced intravascular immunoreactions by haemolytically released hemoglobin.

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An endotoxin:sCD14 complex is the preferred substrate for acyloxyacylhydrolase

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The removal of secondary fatty acyl chains contained in the lipid A component of endotoxin by treatment with the enzyme acyloxyacylhydrolase (AOAH), greatly modifies the pro-inflammatory potency of endotoxin from Gram-negative bacteria. While AOAH in intact host cells works efficiently against both isolated endotoxin and intact Gram-negative bacteria, purified enzyme requires low pH and detergent. To address the physiological form of the endotoxin substrate, we have compared the efficiency of AOAH to release metabolically labeled radioactive fatty acids from endotoxin isolated from *Neisseria meningitidis* (LOS) presented either as aggregates or as isolated, purified complexes with endotoxin-binding proteins (sCD14 or MD-2) that are necessary intermediates in endotoxin-dependent activation of TLR4. The experiments were carried out in balanced Hanks' buffer salt solution containing HEPES and 0.1% albumin. As judged by time and [AOAH] requirements, LOS:sCD14 was > 10 times more readily deacylated by AOAH than were LOS aggregates. In contrast, there is no apparent activity of AOAH on LOS presented as LOS:MD-2. These results support the suggestion that the lipid portion of endotoxin is deeply buried within a pocket of MD-2 and, thus, is rendered inaccessible to AOAH. The reactivity of LOS:sCD14 emphasizes the accessibility of the lipid A portion of LOS when bound to CD14, consistent with the need for albumin in its formation and stabilization. Our studies suggest the efficiency of deacylation of endotoxin by cellular AOAH may depend upon the transfer of endotoxin to mCD14, *i.e.* formation of an endotoxin:mCD14 complex.

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High-density lipoprotein (HDL) suppresses the inhibitory activity of LPS binding protein (LBP)

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LBP plays a major role in initiating cell responses to LPS by rapidly transferring LPS to CD14. LBP also plays a role in controlling responses to LPS, and it can inhibit responses to LPS in a concentration-dependent manner by at least three mechanisms: (i) LBP can transfer LPS to HDL; (ii) LBP can promote the formation of large LPS-LBP aggregates that have decreased ability to stimulate cells; and (iii) LBP can inhibit responses to LPS that has already bound to mCD14 by removing LPS from mCD14 (*J Biol Chem* 2003; **278**: 28367). We found that when THP-1 monocytes were incubated with a constant amount of LPS in serum-free medium, low concentrations of LBP promoted cytokine production, whereas high concentrations inhibited cytokine production as was previously reported in mouse macrophages. When these assays were performed in the presence of native HDL (nHDL), we found that the nHDL suppressed the inhibitory activity of LBP. In contrast, LDL did not suppress the inhibitory activity of LBP. nHDL was not contaminated with LPS. Thus, nHDL appears to suppress either mechanisms (ii) or (iii). We hypothesize that the ability of nHDL to modulate LBP activity is determined by an apolipoprotein that is uniquely present in nHDL.

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Involvement of lipid rafts in lipopolysaccharide-induced down-regulation of surface TLR4 expression in TLR4/MD-2/CD14-CHO cells

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Lipopolysaccharide (LPS) induces inflammatory activation through Toll-like receptor 4 (TLR4)/MD-2/CD14 complex. Although LPS is a potent cellular activator, repeated challenge may lead to temporary insensitivity of both blood cells and epithelial cells toward subsequent LPS stimulation (so-called LPS tolerance). Down-regulation of surface TLR4 expression has been considered as one of the reasons for LPS tolerance. To determine the molecular mechanisms responsible for LPS-induced down-regulation of surface TLR4 expression in epithelial cells, we established the stable transfectants that overexpress TLR4/MD-2 or TLR4/MD-2/CD14 in CHO cells. Expression level of surface TLR4 was determined by biotinylation experiment and flow cytometry using anti-TLR4 antibody. Here, we show that LPS treatment (1 μg/ml, 3 h) decreased surface TLR4 expression in TLR4/MD-2/CD14-CHO cells, but not in TLR4/MD-2-CHO cells, suggesting that CD14 expression is required for LPS-induced TLR4 down-regulation. To identify the pathway involved in LPS-induced TLR4 down-regulation, we treated the cells with or without inhibitors that impede the formation of lipid rafts (filipin, nystatin) and inhibit clathrin-mediated internalization (chlorpromazine, sucrose). Pretreatment of the cells with filipin and nystatin reduced LPS-induced TLR4 down-regulation. On the other hand, chlorpromazine and sucrose treatment did not affect TLR4 internalization. Taken together, the function of lipid rafts is required for LPS-induced TLR4 down-regulation and this down-regulation might be important for LPS tolerance in epithelial cells.

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A general role of cAMP-elevating agents in suppression of pro-inflammatory responses to LPS in human whole blood and Kupffer cells

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Circulating and tissue macrophages respond to bacteria by a pro-inflammatory cytokine burst in order to mobilize defence mechanisms. To avoid overwhelming inflammation that may lead to sepsis, several natural mechanisms of

counter-regulating inflammatory cytokine production by macrophages exist. Among them is the corresponding production of anti-inflammatory cytokines, as well as induction of intracellular cAMP signaling.

In order to map the effects of the cAMP signaling pathway in macrophages on cytokine responses to lipopolysaccharide (LPS), we have pre-treated whole human blood and primary cultures of human Kupffer cells with cAMP analogue (8-CPT-cAMP) or various cAMP elevating drugs (rolipram, forskolin, isoproterenol or PGE_2), and measured pro- and anti-inflammatory cytokine release by ELISA. We demonstrate that cAMP-elevating drugs apart from forskolin clearly limit the production of TNF-α ($P < 0.01$), but also the release of IL-6 ($P < 0.05$), with no reduction of IL-10 or G-CSF levels in either human whole blood or Kupffer cell cultures. Prolonged incubation with 8-CPT-cAMP slightly elevates G-CSF, IL-6 and IL-8 levels in unstimulated whole blood, but not in Kupffer cell cultures. The study clearly indicates that a variety of cAMP elevating agents suppresses pro-inflammatory responses to bacteria in the two main macrophage populations of the body.

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Negative regulation of LPS-induced IL-12 p40 production in RAW264.7 cells by activation of GA-12 repressor element through overactivation of ERK1/2

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We have been studying the LPS-induced pathway for IL-12 production by mouse macrophage cells. In response to LPS stimulation, murine peritoneal macrophages produced IL-12 p70, an active form of IL-12 and a heterodimer of p35 and p40 subunits, but murine macrophage cell lines, such as J774.1 and RAW264.7 cells did not. Both of these cell lines produced no IL-12 p35 subunit, and J774.1 cells produced high IL-12 p40 subunit, while RAW264.7 cells produced only slightly. These results suggested that some negative regulation mechanisms against LPS-induced IL-12 p40 subunit production are functioning in RAW264.7 cells. RAW264.7 cells activated ERK1/2 more strongly than J774.1 cells in response to LPS stimulation. Treatment with U0126, a synthetic inhibitor of MEK1/2 (upstream kinases of ERK1/2), enhanced LPS-induced IL-12 p40 subunit production in RAW264.7 cells. Strong binding of GAP-12 to a potent novel repressor element, GA-12, in the IL-12 p40 promoter, was detected in LPS-stimulated RAW264.7 cells and the effect was abolished by pretreatment with U0126. Taken together, these results indicated that strong activation of ERK1/2 in LPS-stimulated macrophages might inhibit IL-12 p40 subunit production through the increased binding of GAP-12 to GA-12 repressor element in the IL-12 p40 promoter.

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MEK inhibition (PD98059) does not block LPS tolerance inhibition of macrophage ERK

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Bacterial lipopolysaccharide (LPS) activates macrophage release of inflammatory cytokines that participate in development of SIRS/sepsis. Macrophages previously exposed to LPS develop endotoxin tolerance (impaired LPS-dependent signal transduction [decreased ERK] and cytokine release [TNF]). We hypothesized that MEK inhibition would prevent expression of impaired ERK activation in LPS-tolerant macrophages.

Methods: *In vitro* RAW 264.7 macrophages were pretreated (PreRx) for 24 h with medium, or 10 ng/ml *Escherichia coli* O111B4 LPS. Medium was discarded and macrophages were re-challenged with 0–100 ng/ml LPS ± 10 μM PD98059 to block phosphorylation of ERK (Western blot of phospho or total ERK in cell lysates 30 min after LPS). Supernatant TNF was determined at 3 h using ELISA (pg/ml ± SEM). Statistics by ANOVA and Student's *t*-test.

Results: Western blot showed impaired phospho ERK activation by macrophage PreRx with LPS vs. medium (naïve). PD98059 did not block this signaling defect in LPS-tolerant cells. Total ERK was not altered by LPS PreRx, ± PD98059. PD90859 inhibited TNF release in naïve cells, which was also inhibited in LPS-tolerant cells.

Conclusion: MEK is required for LPS-stimulated TNF secretion but is not necessary for expression of impaired ERK activation in LPS-tolerant macrophages.

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Liquid perfluorochemical attenuates inflammatory response in lipopolysaccharide-treated RAW 264.7 macrophages: involvement of inhibition of nuclear factor-κb activation

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It has been reported that partial liquid ventilation with perfluorochemicals (PFCs) may have an inflammatory effect. Overproduction of nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) may account for the inflammation. The purpose of this study was to test whether FC-77, a type of PFC, inhibits NO production and iNOS expression in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. RAW 264.7 macrophages were incubated with FC-77 (10%, 30%, v/v) for 24 h in the presence of LPS (1 μg/ml). We demonstrated that FC-77 significantly ($P < 0.01$) inhibited LPS-induced nitrite, a stable metabolite of NO, production, iNOS induction and pro-inflammatory cytokines formation including tumor necrosis factor-α, interleukin-6 and interleukin-1β as well as intracellular peroxides formation but enhanced the anti-inflammatory cytokine, interleukin-10, production compared with that of LPS-treated control cells. In addition, FC-77 also attenuated the nuclear factor-κB (NF-κB) activation accompanied by preventing degradation of cytosolic IκBα in LPS-treated RAW 264.7 macrophages. In conclusion, the inhibitory effect of FC-77 on LPS-induced NO release and iNOS expression in macrophages most probably by reducing pro-/anti-inflammatory cytokines ratio, free radical formation and NF-κB activation. These findings may provide an additional explanation for the anti-inflammatory activity of PFC.

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Peroxisome proliferator-activated receptor gamma; a counter-regulatory pathway for bacterial endotoxin and sepsis induced inflammation

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Peroxisome proliferator-activated receptor gamma (PPARγ) is a nuclear receptor that functions directly as a transcription factor to control gene regulation. We hypothesized that the inflammatory response to bacterial endotoxin (LPS) may be counter-regulated through a pathway mediated by PPARγ. In *in vitro* studies in rat macrophages LPS and heat killed *Escherichia coli* induce production of inflammatory mediators, which are preceded by phosphorylation of ERK, and IκBβ degradation. Treatment of the cells with PPARγ ligands, such as the cyclopentenone prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and thiazolidinediones significantly inhibit the inflammatory response. Also, 15d-PGJ_2 reduces phosphorylation of ERK and increases cellular IκBα content. In *in vitro* human umbilical vein endothelial cells and human coronary artery endothelial cells treatment with 15d-PGJ_2 reduces LPS-induced expression of intercellular adhesion molecule-1 (ICAM-1), E-selectin, and VCAM. Consistent with these findings, in *in vivo* studies activation of PPARγ by putative ligands ameliorates polymicrobial sepsis induced by cecal ligation puncture in rats. Specifically, PPARγ ligands ameliorated hypotension, reduced neutrophil infiltration in lung, colon and liver, and reduced cytokine production. These data support the hypothesis that PPARγ is an anti-inflammatory pathway and that selective PPARγ ligands may represent new therapeutic strategies to treat endotoxemia and polymicrobial sepsis.

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Piceatannol prevents lipopolysaccharide (LPS)-induced nitric oxide (NO) production and nuclear factor (NF)-κB activation through inhibiting IκB kinase (IKK)

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The effect of anti-cancer drug piceatannol, a derivative of resveratrol, on LPS-induced NO production was examined. Piceatannol significantly inhibited NO production in LPS-stimulated RAW 264.7 cells. The inhibition was due to the reduced

expression of an inducible isoform of NO synthase. The inhibitory effect of piceatannol was mediated by down-regulation of LPS-induced NF-κB activation, but not by its cytotoxic action. Piceatannol inhibited IκB kinase (IKK)-α and IKK-β phosphorylation, and subsequently IκBα phosphorylation in LPS-stimulated RAW 264.7 cells. On the other hand, piceatannol did not affect the activation of mitogen-activated protein kinases including extracellular signal regulated kinase 1/2, p38 and stress-activated protein kinase/c-Jun NH_2-terminal kinase. Piceatannol inhibited the phosphorylation of Akt and Raf-1 molecules, which regulated the activation of IKK-α and IKK-β phosphorylation. The inhibitory mechanism of piceatannol on LPS-induced NO production will be discussed.

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The inhibitory action of activated protein C (APC) on LPS-induced nitric oxide (NO) production in RAW264.7 macrophage cells

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The effect of APC, an anti-coagulant factor, on LPS-induced NO and tumor necrosis factor (TNF)-α production in RAW 264.7 murine macrophage cells was studied. APC reduced LPS-induced NO and TNF-α production in a concentration-dependent manner. LPS-induced NO production was inhibited by the pretreatment with APC 24 h before LPS stimulation and the post-treatment at 6 h after LPS stimulation. The expression of an inducible-type NO synthase (iNOS) was markedly down-regulated by APC. There was no significant difference in LPS binding between APC-pretreated and untreated cells. APC did not affect the viability of LPS-stimulated RAW 264.7 cells. APC inhibited LPS-induced nuclear factor (NF)-κB activation. APC prevented the phosphorylation of IκBα. The inhibitory action of APC on NO and TNF-α production in LPS-stimulated macrophages might be due to the impaired expression of iNOS through insufficient activation of NF-κB. APC might be useful for prevention of endotoxic shock.

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Inhibition of LPS-induced nitric oxide and cytokine production in RAW 264 cells by 2-aminopurine

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Lipopolysaccharide (LPS) induces the expression of inducible nitric oxide synthase (iNOS), followed by the large amount of nitric oxide (NO) production, through TLR4 in murine macrophages. Both MyD88-dependent and independent (TRIF-dependent) signals are necessary for iNOS expression. TIRAP, which is an adaptor molecule of TLR2 and TLR4 and up-regulates MyD88-dependent signaling, activates double stranded RNA-dependent protein kinase (PKR). We examined the effect of 2-aminopurine (AP), originally described as a specific inhibitor for PKR, on LPS-induced NO and cytokine production in RAW 264 murine macrophage-like cell line. 2-AP significantly reduced NO production and the expression of iNOS, TNF-α and IL-6. The activation of nuclear factor-κB partially inhibited by 2-AP and no inhibition was observed on the degradation of IκB. On the other hand, 2-AP significantly reduced the mRNA expression of IFN-stimulated genes including IFN-β, IP-10, IRG-1 and iNOS. Further, exogenous IFN-β retained NO production in LPS-stimulated and 2-AP treated cells. These data suggest that 2-AP inhibited IFN-β production induced by LPS, resulting in the inhibition of NO production.

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Inhibitory effects of antifungal agent amphotericin B on LPS-induced nitric oxide synthesis in mouse macrophages

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Amphotericin B is a well-known antifungal agent and has been reported to possess immunomodulating properties. The aim of the present study was to investigate the efficacy of amphotericin B as an inhibitor of lipopolysaccharide (LPS)-induced nitric oxide (NO) production in mouse macrophages. The mouse macrophage like cell-line RAW 264.7 was used in this study. LPS-induced expression of iNOS mRNA and production of NO, and lipid A-induced production of NO were all completely inhibited by treatment with amphotericin B. Interestingly, LPS-induced formation of osteoclasts and stimulation of vascular permeability in mouse skin were also inhibited by amphotericin B treatment. These results suggest that amphotericin B is a potent inhibitor of LPS-induced NO synthesis in mouse macrophages. Based on these observations, amphotericin B may prove to be an effective anti-inflammatory agent against LPS-stimulated periodontal destruction in periapical and periodontal diseases.

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Inhibition of LPS-induced macrophage activation by flavonoids luteolin and quercetin

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Flavonoids, phenolic compounds widely present in plants, are able to modify biological responses by their anti-oxidant, anti-inflammatory, and anti-allergic activities. We reported some flavonoids had suppressive effects on LPS-induced TNF production in murine macrophages. Here we show inhibitory mechanisms of flavonoids luteolin (LUT) and quercetin (QUR) on LPS-induced cytokine production by bone marrow-derived (BM)-macrophages. Pretreatment of BM-macrophages with either LUT or QUR resulted in significant suppression of LPS-induced TNF-α and IL-6 production. These suppressive phenomena were due to the inhibition of IκB degradation before NF-κB activation and phosphorylation of p38 and Akt in the MAP kinase pathway. In addition, TNF-α-induced NF-κB activation and apoptosis were blocked by pretreatment with LUT and QUE. On the other hand, they did not show any effects on PMA-induced NF-κB activation and actinomycin D-induced apoptosis. LPS stimulation caused accumulation of lipid raft demonstrable by binding of FITC-cholera toxin B subunit to ganglioside GM_1 using confocal laser microscopy. Treatment with LUT and QUR inhibited LPS-induced accumulation of lipid raft. Taken together, LUT and QUR inhibit the fluidity of cell membrane as well as activation of LPS- and TNF-α-induced signal transduction.

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Quercetin attenuates endotoxin-induced hepatotoxicity in rats: possible antioxidant mechanism

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Lipopolysaccharide (LPS) causes various pathophysiological changes that lead to septic shock and multiple organ failure. Endotoxin accumulates in tissues rich in cells of the reticulo-endothelial system such as liver and spleen. The interaction of LPS with macrophages, neutrophils and Kupffer cells results in generation of hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen. Exogenous administration of antioxidants such as coenzyme Q10 or α-tocopherol has shown to protect against tissue damage resulting from endotoxemia. Quercetin, a potent antioxidant, is one of the polyphenolic flavonoids found richly in onions, red wine and green tea. *In vitro* and animal studies have

shown that quercetin inhibits degranulation of mast cells, basophils and neutrophils. Thus the present study was aimed at investigating the protective effect of quercetin in endotoxin-induced hepatotoxicity. The rats were challenged with LPS 1 mg/kg i.p. and sacrificed after 6 h. Liver function tests (SGOT, SGPT, total bilirubin and total protein) were estimated in serum. Oxidative stress in liver was measured using lipoperoxide (MDA) levels, glutathione (GSH) content and superoxide dismutase (SOD). Quercetin (25 mg/kg i.p for 2 weeks prior to LPS challenge) significantly attenuated LPS-induced liver dysfunction and hepatic oxidative stress. The results of the present study indicate that quercetin demonstrates the hepatoprotective effect mainly due to its antioxidant mechanism.

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Quercetin attenuates endotoxin induced sickness behaviour and oxidative stress in brain

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Immune activation, either by cytokines or endotoxin, elicits a constellation of non-specific symptoms such as weakness, malaise, listlessness, fatigue, adipsia, anorexia, depression, and anxiety collectively termed 'sickness behaviour'. In most plants, quercetin and its sugar conjugates not only are the most abundantly distributed flavonoids but also represent the largest proportion of the flavonols in the plant kingdom. Quercetin acts as a potent antioxidant due to its ability to block the action of xanthine oxidase, chelation of iron and direct scavenging of hydroxyl radicals. The present study was designed to investigate the effect of chronic treatment with quercetin (25 mg/kg i.p. for 15 days) on lipopolysaccharide (LPS)-induced sickness behaviour and oxidative stress produced in brain. The hypothesis was tested through the analysis of LPS-induced behavioural changes in rats, in plus maze and open field paradigms. Other parameters such as feeding and water consumption, weight loss and organ weight index were also estimated. Oxidative stress was estimated by measuring the level of MDA and reduced glutathione in brain homogenates. Administration of LPS induced reduction in appetite, body weight, suppressed locomotor and exploratory activity and induced an anxiogenic response in the rodents. LPS also elicited a marked oxidative stress in brains of rats as is evident by increased MDA levels and decreased levels of glutathione in whole brain homogenates. Quercetin significantly attenuated LPS-induced oxidative stress in the brain and all other manifestations of sickness behaviour.

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Protective role of metallothionein in acute lung injury induced by bacterial endotoxin in mice

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Metallothionein (MT) is a protein that can be induced by inflammatory mediators and participate in cytoprotection. However, its role in inflammation remains to be established. We determined whether intrinsic MT protects against acute inflammatory lung injury induced by bacterial endotoxin in MT-I/II knock out (–/–) and wild-type (WT) mice. $MT^{-/-}$ mice were more susceptible than WT mice to lung inflammation and especially to lung edema, which were induced by intratracheal challenge with lipopolysaccharide (LPS, 125 μg/kg). After LPS challenge, MT deficiency enhanced vacuolar degeneration of pulmonary endothelial cells and type I alveolar epithelial cells, and caused focal loss of the basement membrane. LPS treatment induced no significant differences in the enhanced expression of pro-inflammatory cytokines and chemokines nor in the activation of nuclear factor-κB (NF-κB) pathway in the lung between the two genotypes. Lipid peroxide levels in the lungs were significantly higher in LPS-treated $MT^{-/-}$ mice than in LPS-treated WT mice. These results indicate that endogenous MT is a protective molecule against acute lung injury related to LPS. The effects are possibly mediated via the enhancement of pulmonary endothelial and epithelial integrity, not via the inhibition of NF-κB pathway.

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Glutamine-induced heme oxygenase-1 protects against intestinal tissue injury in acute endotoxemia

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Objective: To investigate whether glutamine (Gln) pretreatment improves the intestinal injury in rats with acute endotoxemia by virtue of its site-specific heme oxygenase-1 (HO)-1 induction in the lower intestinal tract.
Interventions: Acute endotoxemia was induced in rats by intraperitoneal injection of lipopolysaccharide (LPS; 10 mg/kg). Rats were also treated with Gln (0.75 g/kg) dissolved in lactated Ringer's solution via tail vein.
Measurements and Main Results: Gln treatment itself markedly induced HO-1 mRNA and protein in the mucosal epithelial cells in the ileum and colon, whereas its expression in the duodenum and jejunum was not influenced by the treatment. Gln treatment before LPS administration significantly ameliorated LPS-induced mucosal injury, inflammation, and apoptosis in the lower intestine, as judged by significant decreases in TNF-α gene expression, histological damage scores and expression of activated caspase-3 and an increase in gene expression of Bcl-2. In contrast, treatment with tin mesoporphyrin, a specific inhibitor of heme oxygenase activity, abolished the beneficial effect of Gln pretreatment.
Conclusions: Gln pretreatment significantly ameliorates intestinal tissue injury in rats of acute endotoxemia by virtue of its specific HO-1 induction in mucosal epithelial cells of the lower intestine. HO-1 induction may protect intestinal cells from oxidative damage by LPS via its anti-apoptotic property.

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Effect of pioglitazone on endotoxin-induced decreases in hepatic drug-metabolizing enzyme activity and expression of CYP3A2 and CYP2C11

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It has been reported that peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands ameliorate the expression of inducible nitric oxide synthase (iNOS) by endotoxin. In the present study, we investigated the effect of pioglitazone, a potent PPAR-γ ligand, on the endotoxin-induced reduction of hepatic drug-metabolizing enzyme activity and on the down-regulation of the expression of hepatic cytochrome P450 (CYP) 3A2 and CYP2C11 proteins in rats. Endotoxin (1 mg/kg) significantly decreased hepatic drug-metabolizing enzyme activity *in vivo*, as represented by the systemic clearance of antipyrine and protein levels of CYP3A2 and CYP2C11 24 h after intraperitoneal injection. Pretreatment with pioglitazone (10 mg/kg, 4 times at 10-min intervals) significantly protected the endotoxin-induced decreases in the systemic clearance of antipyrine and protein levels of CYP3A2, but not CYP2C11, with no biochemical and histopathological changes in the liver. Pioglitazone alone had no effect on the systemic clearance of antipyrine and protein levels of CYP3A2 or CYP2C11. Pioglitazone significantly protected endotoxin-induced overexpression of inducible nitric oxide synthase in the liver, but not the overproduction of nitric oxide (NO) in plasma. It is unlikely that the protective effect of pioglitazone against endotoxin-induced decreases in the hepatic drug-metabolizing enzyme activity and protein levels of CYP3A2 in the liver is due to the inhibition of the overproduction of NO.

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Protective effects of combination of propofol and low-dose dexamethasone on conscious rats treated with endotoxin

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Our previous studies reported that: (i) propofol, in addition to its mild antioxidant effect, attenuates the liver dysfunction caused by endotoxin; and (ii) the low-dose dexamethasone not only exerts anti-inflammatory effects but also attenuates renal dysfunction caused by endotoxin. In this study, we examined the therapeutic effect by combining propofol and low-dose dexamethasone (0.1 mg/kg) in conscious rats with endotoxemia. Intravenous injection of rats with endotoxin (10 mg/kg lipopolysaccharide; LPS) caused hypotension, vascular hyporeactivity and tachycardia as well as significant organ injury. The combined therapy of propofol and low-dose dexamethasone protected the conscious LPS-rat from circulatory failure, liver and renal dysfunction, metabolic acidosis, and inflammatory processes in lung and kidney tissues. These protective effects may be attributed to the attenuation of plasma TNF-α, inhibition of tissue NO production, decreases of tissue superoxide levels and myeloperoxidase activity. In addition, the survival rate at 22 h was significantly increased after this combined therapy when compared to the LPS group. These results suggest that inhibition of inflammatory responses, suppression of free radical release and amelioration of organ dysfunction contribute to the beneficial effect of combined treatment in conscious endotoxemic rats.

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Protective role of IL-6 in pulmonary hemorrhage induced by bacterial endotoxin

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The present study elucidated the role of interleukin-6 (IL-6) in hemostatic and coagulatory changes during severe inflammation induced by intraperitoneal administration of lipopolysaccharide (LPS: 1 mg/kg) using IL-6 null (–/–) mice. After LPS challenge, IL-$6^{-/-}$ mice revealed significant prolongation of prothrombin time and partial thromboplastin time and a decrease in platelet counts as compared with wild type mice. LPS treatment induced marked pulmonary hemorrhage with neutrophilic inflammation in IL-$6^{-/-}$ mice, in contrast, only mild neutrophilic infiltration in WT mice. The protein levels of pro-inflammatory mediators, such as IL-1β, macrophage inflammatory protein-1α, macrophage chemoattractant protein-1, and keratinocyte chemoattractant, in the lungs were significantly greater in IL-$6^{-/-}$ mice than in WT mice after LPS challenge. These results directly indicate that IL-6 is protective against coagulatory and hemostatic disturbance and subsequent pulmonary hemorrhage induced by bacterial endotoxin, at least partly, via the modulation of pro-inflammatory processes.

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Influence of fructose-1,6-diphosphate on endotoxin-induced lung injuries in sheep

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Lung injury induced by *Escherichia coli* endotoxin (LPS) is characterized by pulmonary edema hypertension and increased microvascular permeability resulting in the formation of pulmonary. Although the role of the neutrophils in the genesis of permeability edema appears to be predominant, relevant studies suggest involvement of other immune cells. Fructose-1,6-diphosphate (FDP) is reported to suppress neutrophil generation of oxyradicals as well as to attenuate expression and secretion of inflammatory cytokines (IL-1β, IL-6, TNF-α, NF-κB). Therefore, the effects of FDP on pulmonary and systemic hemodynamics, lung lymph protein clearance and leukocyte counts during 4 h of endotoxemia were studied in anesthetized sheep (n = 18). Some of the animals underwent thoracotomy and mediastinal lymphatic node was cannulated. Following stabilization, all sheep received *E. coli* endotoxin 5 μg/kg i.v. infusion over 30 min. Concomitantly with the endotoxin infusion, half of the

animals were selected randomly to receive an i.v. bolus of FDP (10%) 50 mg/kg followed by a continuous infusion of 5 mg/kg/min for 4 h; the rest were treated in the same manner with glucose 10% in 0.9% NaCl. Pulmonary artery pressure (PAP) and resistance in the glucose group increased from 20.8 ± 1.6 to 36.7 ± 3.2 mmHg ($P < 0.007$) and from 531 ± 114 to 1137 ± 80 dynes/s/cm^{-5}, respectively ($P < 0.005$). Despite an increase during endotoxin infusion, these parameters in the FDP group returned to control values. There were no differences in left ventricular pressures, cardiac output, heart rate and arterial oxygen tension between the groups. In the glucose group, lymph protein clearance was higher ($P < 0.01$) and blood leukocyte count lower ($P < 0.02$). The wet/dry lung tissue weight ratio (g/g) for the glucose group was 5.6 ± 0.04 and for the FDP-treated group was 4.76 ± 0.06 ($P < 0.0005$). It was concluded that FDP treatment attenuated significantly the characteristic pulmonary hypertension, lung lymph protein clearance and pulmonary vascular leakage seen in sheep infused with endotoxin probably by modulating the pro-inflammatory response of cells of the innate immune system.

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The protective effect of protamine on murine endotoxin shock

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Protamine is used clinically to neutralize the anti-coagulation effect of heparin. Its effect on mortality of LPS-injected BALB/c mice was studied. Various doses of protamine were intraperitoneally injected 24 h prior to LPS injection. The survival rates were significantly increased from 37% in vehicle group to 76% ($P < 0.01$) at 3.0 mg/mouse of protamine in a dose-related manner. For time course studies, protamine was effective when injected 48 h or 24 h before LPS injection, and was ineffective when injected 12 h, 4 h, and 0 h before LPS injection. For a possible effect on coagulation, blood was obtained daily up to 5 days after protamine injection. The plasma fibrinogen, prothrombin time, and activated thromboplastin time were statistically not different between groups of protamine and vehicle control. In conclusion, protamine has a protective effect on murine endotoxin shock when injected 24 h before LPS injection. The protection seems not to be related to coagulation system.

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Effects of L-glutamine and L-asparagine on mouse survival with endotoxin shock

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L-glutamine was reported able to protect mice against endotoxin-induced mortality. The protective effects of L-glutamine-related compounds, *i.e.* L-asparagine, L-glutamate, and L-aspartate were studied. *Escherichia coli* lipopolysaccharide (LPS) was injected simultaneously with L-glutamine (10 mg) or vehicle, into each group of 20 BALB/c mice. The same procedure was conducted for L-asparagine, L-glutamate, L-aspartate, and L-lysine. The survival rate of vehicle-control was 20%, and L-glutamine was 85% ($P < 0.001$), L-asparagine 90% ($P < 0.001$), L-glutamate 65% ($P < 0.05$), L-aspartate 60% ($P = 0.05$), and L-lysine 15% ($P > 0.5$). The doses for optimal protection from L-glutamine and L-asparagine were 1 mg and 10 mg per mouse, respectively. There was a protective effect when L-glutamine or L-asparagine was injected prior to LPS injection or 1 h after LPS, but not 3 h after LPS. In conclusion, L-glutamine, L-asparagine, L-glutamate, and L-aspartate, but not L-lysine, can specifically protect mouse against endotoxin shock. L-glutamine and L-asparagine have the best protective effect. This effect was abolished when the amino acid was given 3 h after LPS.

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Macrophage activation by trehalose 6, 6′-dimycolate (TDM) and related glycolipids via Toll-like receptor 2

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TDM, present in the envelope of *Mycobacterium tuberculosis* and related bacteria, has various immunomodulating activities. The mechanisms by which TDM and mycolic acid-containing glycolipids activate macrophages via Toll-

like receptors (TLRs) are unclear. When peritoneal macrophages were stimulated for 24 h with TDM, prepared from *M. tuberculosis* Aoyama B and coated on the surface of plastic wells, TNF-α levels were significantly higher in culture supernatants of macrophages from TLR4-deficient C57BL/10ScCr (B10Cr) than the wild-type C57BL/10ScSn (B10Sn) mice. In cultures of macrophages from TLR2 knockout mice, TDM-induced TNF-α production was significantly reduced compared with that from B10Sn mice. TDM from *M. tuberculosis* exhibited stronger TNF-α-inducing activity than TDM from *Rhodococcus* spp. and related glycolipids such as trehalose monomycolate (TMM) and glucose monomycolate (GMM). The carbon chain length of mycolic acid in rhodococcal glycolipids is approximately half of that in *M. tuberculosis*. Regarding activation of NF-κB in HEK293 cells overexpressed mouse TLR2, both TDM exhibited very weak activity. Among compounds tested, the strongest NF-κB activation was detected in TLR2-transfectant stimulated with rhodococcal TMM. Taken together, macrophage activation by TDM and mycolic acid-containing glycolipids seems to occur via the interaction with TLR2 and TLR6. Therefore, we are investigating whether cytokine gene expression in macrophages and dendritic cells induced by TDM and related glycolipids is mediated by MAPK and NF-κB signaling pathway.

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Binding specificity of Dectin-1 to 1,3-β-glucans and its role for cellular activation

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Dectin-1 is a high affinity β-glucan receptor expressed on dendritic cells and macrophages. Since co-ligation of Dectin-1 and TLR2 by zymosan, a complex of 1,3-β-glucan and unknown TLR2-ligand, gives enhanced NF-κB activation, Dectin-1 may modulate immunostimulatory activity of the leukocytes. Characterization of the biological role of Dectin-1 on the recognition of β-glucans and the subsequent immunological activities was examined by using Dectin-1 mutants and specific mAbs. Critical moiety of Dectin-1 for recognition was on β-sheet 4 region of typical C-type lectin carbohydrate recognition domain. This mutation resulted in decreased NF-κB activation in response to zymosan. Expression level of Dectin-1 protein on BMDC varied in mouse strains. The high responder strain showed higher expression of Dectin-1. Treatment with the blocking mAb also decreased the effects of β-glucans on cytokine production and anti-tumor activity. These results suggest that Dectin-1 is important receptor for inducing various immunostimulatory activities of 1,3-β-glucans.

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Structure and innate immune response of lipoteichoic acids from *Listeria monocytogenes*

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The Gram-positive bacterium *Listeria monocytogenes* is a facultative intracellular human pathogen that causes listeriosis in immune suppressed patients, pregnant women and their unborn babies. Lipoteichoic acids (LTAs) are cell membrane anchored amphiphiles recognized by the innate immune system via Toll-like receptor 2 (TLR-2). We prepared LTA from *Listeria* using butanol extraction and hydrophobic interaction chromatography leading to two distinct microheterogeneic isolates varying in chain length. The chemical structure was elucidated by gas chromatography, one- and two-dimensional nuclear magnetic resonance and mass spectrometry. Listerial LTA activates murine macrophages in a TLR2 dependent fashion as shown in knockout mice and has similar activity towards human monocytes and whole blood as compared to staphylococcal LTA. In these assays, the listerial LTA containing a longer polyglycerolphosphate was more potent than the variant with the shorter one. De-alanylated LTA exhibited a significantly reduced activity but D-alanine residues were not essential for signaling. The glycolipid anchor, obtained by degrading the entire polyglycerolphosphate, was only slightly active representing the minimal active structure with the fatty acids being essential for receptor binding. Elucidation of the structure–function relationship of listerial LTA will improve our understanding of *Listeria* pathogenesis and will possibly lead to novel intervention strategies in listeriosis.

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Isolation and characterization of lipoteichoic acid from *Streptococcus pneumoniae*

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Streptococcus pneumoniae causes pneumonia in elderly people and meningitis in children. According to the literature, pneumococcal lipoteichoic acid (LTA) has a completely different chemical structure to that of *Staphylococcus aureus*: the polyglycerophosphate in the backbone is replaced by polyribitol phosphate carrying phosphorylcholine and not D-alanine, which plays a central role in the biological activity of staphylococcal LTA. We were able to isolate LAL-negative LTA from *Str. pneumoniae* by butanol-extraction, which is more gentle than the previously used chloroform-methanol extraction: 1 g lyophilised bacteria resulted in 10 mg LTA. Dose response curves of pneumococcal and staphylococcal LTA in human whole blood induced a similar cytokine pattern (*e.g.* for TNF, IL-10 and IL-8). Neither LTA induced a significant release of IFN-γ or was inhibited by the endotoxin-specific LAL-F protein. Stimulation of bone marrow-derived macrophages of wild-type and knockout mice showed that LTA from *Str. pneumoniae* was Toll-like receptor (TLR)2 and not TLR4-dependent. First NMR data of the pneumococcal LTA indicate the presence of bound D-alanine, which might explain the similarities in the biological activity of pneumococcal and staphylococcal LTA.

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JNK is involved in the induction of COX-2 by lipoteichoic acid from *Staphylococcus aureus* in human pulmonary epithelial cells

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In this study, we address the molecular mechanism of lipoteichoic acid (LTA)-induced cyclooxygenase-2 (COX-2) expression. Promoter activity assays demonstrated that LTA from *Staphylococcus aureus* stimulated COX-2 promoter activity in a dose-dependent manner in human pulmonary epithelial cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that COX-2 mRNA level was increased after LTA treatment. Thus, LTA stimulates COX-2 expression via transcriptional activation. We also tried to elucidate the signaling pathways by which LTA activates COX-2. Jun N-terminal kinase (JNK) inhibitor SP600125, but not extracellular signal-regulated kinase (ERK) inhibitor PD98059, suppressed LTA-induced COX-2 promoter activity. In addition, dominant-negative JNK also blocked the induction of COX-2 by LTA. Collectively, our results suggest that JNK is involved in the induction of COX-2 by lipoteichoic acid from *S. aureus* in human pulmonary epithelial cells.

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Comparison of cytokine-inducing active components in the LTA fraction obtained with 1-butanol extraction from Enterococcus hirae and Staphylococcus aureus

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Lipoteichoic acid (LTA) is a macromolecular cell-surface glycoconjugate of Gram-positive bacteria. We previously reported that purified LTA obtained with hot-phenol extraction from *Enterococcus hirae* had no immunostimulating activity, but another glycolipid (AF-EH) co-existing in the LTA fraction had the activity. However, Hartung *et al*. recently reported that LTA from *Staphylococcus aureus* extracted with 1-butanol possessed the activity. In this study, we compared both LTA obtained with butanol extraction. LTA of *E. hirae* ATCC 9790 (EH-B40) and *S. aureus* DSM

20231 (SA-B40) were obtained with butanol extraction followed by hydrophobic chromatography. Cytokine inducing activity was evaluated using human PBMCs. The yield of EH-B40 from starting bacteria was almost identical to that of AF-EH. AF-EH, EH-B40 and SA-B40 showed similar SDS-PAGE profiles stained with Alcian Blue, and possessed a similar extent of IL-6 and TNF-α inducing activities. These activities were all inhibited by mAbEH1, which is a mouse anti-AF-EH mAb neutralizing the activity of AF-EH. Neutral glycolipids, which we synthesized according to the proposed structures in both LTA, failed to induce these cytokines. From these facts, it is suggested that essential partial structure(s) for the activity are shared in both LTA and more structure in addition to the neutral glycolipid is needed.

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Toll-like receptor 2-dependent NF-κB activation in response to *Porphyromonas gingivalis* FimA precursor lipoprotein

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Porphyromonas gingivalis is an etiologically important pathogen for periodontal diseases. A cell surface component, fimbriae, is important to adhere to host mucosal surface and has been reported to induce the activation of inflammatory cells and gingival epithelial cells through TLRs. To investigate the relationship between the structure and the biological activities of fimbriae, we prepared rFimA(1-383)-His tag, rFimA(47-383)-His tag, and GST-fusion-derived rFimA(47-383) proteins and exposed to NF-κB-dependent CHO/CD14 and CHO/CD14/TLR2 reporter cells. Following the removal of LPS with Triton X-114 treatment, none of those FimA proteins activated CHO/CD14 reporter cells. However, rFimA with the intact N-terminal prosequence (rFimA(1-383)-His tag), but not the mature form proteins (rFimA(47-383)-His tag, rFimA(47-383)-GST) activated CHO/CD14/TLR2 cells. Since it has been reported that N-terminal cysteine residue (C-28) of precursor FimA was modified with diacylglycerol, we prepared a mutant in which Cys-28 was substituted with Ala (rFimA[C19A](1-383)-His tag). [^{3}H]-Palmitic acid labeling revealed that rFimA with the intact N-terminal prosequence, but not the mutant rFimA[C19A], was lipidated and the mutant rFimA[C19A] failed to activate CHO/CD14/TLR2 cells. These data indicated that the precursor lipoprotein of *P. gingivalis* FimA was recognized by TLR2.

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Lipopolysaccharide (LPS) binding protein (LBP) mediates innate immune responses by triacylated and diacylated lipopeptides

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Lipopolysaccharide (LPS) binding protein (LBP) is an acute phase protein synthesized predominantly in the liver. It binds LPS of Gram-negative bacteria and transfers it via a CD14-enhanced mechanism to a receptor complex including Toll-like receptor (TLR)-4 and MD-2, initiating the release of pro-inflammatory cytokines. Lipoproteins and lipopeptides have repeatedly been shown to act as potent cytokine inducers, interacting with TLR2, in synergy with TLR1 or TLR6. In this study, we show that these compounds also interact with LBP and CD14. We employed triacylated lipopeptides, corresponding to lipoproteins of *B. burgdorferi*, mycobacteria and *Escherichia coli*, as well as diacylated lipopeptides, corresponding to, for example, MALP-2 of *Mycoplasma* spp. Responsiveness of human mononuclear cells to these compounds was greatly enhanced in the presence of human LBP. Binding of lipopeptides to LBP as well as competitive inhibition of this interaction by LPS was demonstrated in a microplate assay. Furthermore, we were able to show that LBP transfers lipopeptides to CD14 on human monocytes employing FACS analysis. These results support that LBP is a pattern recognition receptor transferring a variety of bacterial ligands including the two major types of lipopeptides, to CD14 present in different receptor complexes.

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Structure-activity relationship of signaling by synthetic lipopeptides through TLR2/TLR6 and TLR2/TLR1 heterodimers

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Bacterial lipoproteins are known to induce signaling in cells of the innate immune system through Toll-like receptor-(TLR)2/TLR1 or TLR2/TLR6 heterodimers. They are composed of di-O-acylated or triacylated S-(2,3-dihydroxypropyl)-cysteinyl residues N-terminally coupled to distinct polypeptides. Currently, it is thought that triacylated lipopeptides, like the synthetic Pam_3CSK_4, are recognized by TLR2/TLR1 heterodimers, whereas diacylated lipopeptides, like MALP2 (Pam_2CGNNDESNISFKEK), induce signaling through TLR2/TLR6 heterodimers. However, we have now found that not only triacylated lipopeptides, but also diacylated lipopeptides like Pam_2CSK_4 and the C-terminally elongated MALP2 derivative Pam_2CGNNDESNISFKEK-SK_4 (MALP2-SK4) induce cell activation in a TLR6-independent manner. Furthermore, we have confirmed that activation of murine spleen cells by Pam_3CSK_4 is at least in part TLR1-dependent, whereas Pam_2CSK_4 and MALP2-SK4 signal through TLR2 in a TLR6 as well as in a TLR1-independent manner. Finally we have characterised the N-acyl residue on receptor specificity. The TLR6-dependent MALP2 becomes TLR6-independent when an N-acyl residue of at least ten carbons such as decanoic acid is incorporated. Additionally, we have observed that these triacylated MALP2 derivatives are signaling independently of TLR1. Our results indicate that both the lipid and the peptide part of lipoproteins take part in the specificity of recognition by TLR2 heterodimers.

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Molecular interactions of lipopeptides with CD14 and TLR2

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Bacterial lipoproteins (LPs) activate innate immune cells through the pattern recognition receptor TLR2. However, the molecular mechanisms of LP recognition are not yet clear. We used a FLAG-labeled derivative of the synthetic lipopeptide Pam_3CSK_4 to study the roles of CD14 and TLR2 in binding and signaling of LP. Although the activity of Pam_3CSK_4-FLAG was TLR2 dependent, binding could only be detected to CD14. However, partial co-localization of Pam_3CSK_4-FLAG and TLR2 could be revealed by confocal microscopy. To further study molecular associations we used fluorescence resonance energy transfer (FRET) imaging technique and could show that CD14 and Pam_3CSK_4-FLAG associate with TLR2 after Pam_3CSK_4-FLAG binding. This association could be blocked by anti-TLR2 mAb or unlabeled Pam_3CSK_4 and is not seen when the inactive monoacylated $PamCSK_4$-FLAG derivative or other TLR ligands were used. In addition, fluorescence recovery after photobleaching (FRAP) analysis revealed that after Pam_3CSK_4-FLAG stimulation TLR2 mobility is decreased, indicating the formation of a low mobile TLR2 signaling complex due to its association with intracellular adaptor molecules. Thus, LP binding to CD14 is the first step in the LP recognition and signaling cascade inducing molecular associations of CD14 and LP with TLR2 and formation of the TLR2 signaling complex.

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Membrane-anchored CD14 is important for the induction of interleukin-8 by lipopolysaccharide and peptidoglycan in uroepithelial cells

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We investigated the induction of interleukin-8 (IL-8) by bacterial lipopolysaccharide (LPS) and peptidoglycan (PGN) in four bladder cancer cell lines – T24, 5637, UM-UC-3 and HT1197. T24 and 5637 cells strongly induced IL-8 after stimulation with LPS or PGN, whereas UM-UC-3 and HT1197 cells did so very weakly. The expression of CD14 at the mRNA, total cellular protein and cell surface protein levels varied among these cell lines, but the expression levels of Toll-like receptors 2 (TLR2) and 4 (TLR4) were not significantly different. The CD14 expression levels were found to correlate with the inducibility of IL-8 by LPS or PGN. Treatment of T24 and 5637 cells with phosphatidylinositol-specific phospholipase C to eliminate CD14 from the cell surface dramatically suppressed the induction of IL-8. On the other hand, UM-UC-3 cells transfected with CD14 cDNA expressed membrane-anchored CD14 and shared more efficient induction compared with untransfected control. These results suggest that the presence of the membrane-anchored, but not the soluble, form of CD14 is a strong factor in IL-8 induction in bladder epithelial cells in response to bacterial components. The presence of the membrane-anchored form of CD14 may thus be a determinant for the inflammatory response of uroepithelial cells.

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Double-stranded RNA-mediated TLR3 activation is enhanced by CD14

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A synthetic mimic of viral double-stranded RNA (dsRNA), polyinosine-polycytidylic acid (pIpC), activates Toll-like receptor 3 (TLR3), and subsequently induces production of cytokines including type I interferons. We found that bone-marrow derived macrophages from CD14-deficient ($CD14^{-/-}$) mice exhibited impaired responses to pIpC. Furthermore, pIpC-mediated TLR3 activation in HEK293 cells was enhanced by co-transfected CD14. To further investigate how CD14 is associated with pIpC-mediated TLR3 activation, we made 200 bp short, homogeneous fragments of pIpC (pIpCsf). These fragments maintain the inflammatory activity of the larger commercial pIpC. pIpCsf were then labeled with [^{32}P] or Alexa Fluor 488 (AF488) and used for ligand uptake and intracellular localization studies. [^{32}P]-pIpCsf uptake occurred in CHO/CD14 and $CHO/CD14^{+}$TLR3 cells but not in CHO/TLR3 or CHO/Vector cells, showing that uptake of pIpCsf is TLR3-independent but CD14-dependent. Similarly, deconvolution confocal microscopy revealed that AF488-pIpCsf internalization was dependent on CD14. However, AF488-pIpCsf-mediated cellular activation required TLR3. Using FACS and microscopic analyses, we observed that TLR3 was primarily localized intracellularly. Upon CD14-mediated internalization of AF488-pIpCsf, nuclear translocation of the NF-κB p65 subunit was detected as was co-localization of AF488-pIpCsf and TLR3, suggesting that TLR3 initiates signal propagation intracellularly.

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PolyG-flanked palindromic CpG DNA activates STAT1 and NF-κB through the p38 MAPK pathway to induce autocrine IFN-α/β-independent production of IFN-α, IP-10, and MIP-1α in human plasmacytoid dendritic cells

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Unmethylated CpG DNA induces Th1-dominant immune responses in vertebrates. We demonstrate here the sequence of CpG DNA, which activates human PDC, and its signaling mechanisms. Poly G-flanked palindromic CpG DNA

(designated as palGACGA) is identified as a potent ligand for TLR9 in PDC, to produce IFN-α, IP-10, and MIP-1α, in a manner independent of autocrine stimulation of IFNAR. palGACGA activates ISGF3 components (STAT1, STAT2, and IRF-9), IRF-7, and NF-κB p65/p50, accompanied with the phosphorylation of p38 MAPK. Pre-treatment of PDC with p38 MAPK inhibitor decreases the STAT1 phosphorylation, IRF-7 expression, and NF-κB activity, with the concurrent reduction of IFN-α, IP-10, and MIP-1α. Only the blockage of NF-κB activation alone also inhibits these gene expressions. Interception of endocytosis and endosomal maturation with the scavenger receptor ligands and chloroquine, respectively, prevents the palGACGA-induced expression of IFN-α, IP-10, and MIP-1α. With these results, we propose that, in PDC, palGACGA up-taken and recognized by TLR9 activates both ISGF3 components and NF-κB through the p38 MAPK. These pathways would cause, respectively and/or co-operatively, transcription of the genes which possess the ISRE and/or κB sites in their promoters, thereby inducing IFN-α, IP-10, and MIP-1α, in a manner independent of autocrine activation of IFNAR.

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In search of the TLR2 activity in peptidoglycan from *Staphylococcus aureus*

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TLR2 has been reported to interact with a broad panel of structurally non related molecules such as peptidoglycan (PG), lipoteichoic acid (LTA), lipoproteins, lipopolysaccharide, zymosan, and different proteins. In order to investigate the PG-mediated TLR2 activity in more detail, we purified and analyzed soluble PG (sPG) from *Staphylococcus aureus*. Gel permeation chromatography of TLR2-active fractions showed two characteristic TLR2-active bands in SDS-PAGE (10 kDa and 15 kDa), which were further purified by preparative SDS-PAGE followed by electro-elution. GC-MS analysis showed that both fractions lacked GlcNAc and MurNAc, thus indicating another molecule to mediate TLR2 activity which was still expressed in both fractions. We further investigated if contaminating LTA was responsible for TLR2 activity. Upon hydrophobic interaction chromatography, in fractions, co-eluting with reference LTA, neither LTA components (glycerol, phosphate, *i*-17:0) nor TLR2 activity was observed, thus ruling out LTA being responsible for the TLR2 activity. Further progress towards the identification of the TLR2 active molecule(s) will be presented.

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Recognition of 6-*O*-acylated muramyl dipeptides carrying lipid A-type or branched-type fatty acid by murine macrophages

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In pattern recognition of pathogens by TLRs, the structural requirement for their recognition is not yet clear. TLR2 and TLR4 recognize peptidoglycan (PGN) and glycolipids present in the cell wall of Gram-positive bacteria, and lipopolysaccharide (LPS) located in outer membrane of Gram-negative bacteria, respectively. Muramyl dipeptide (MDP), the minimum structure of PGN for expressing adjuvant activity, is recognized by an intracellular receptor NOD2. Mycolic acid is a characteristic branched fatty acid and a representative constituent of mycobacterial glycolipids such as trehalose dimycolate (TDM), which is recognized by TLR2. On the other hand, lipid A, a lipid part of LPS, consisting of acyloxyacyl group, is recognized by TLR4. Here we examined, among TLR2, TLR4 or NOD2, which receptor can recognize 6-*O*-acylated MDP carrying branched-acyl or acyloxyacy groups. When peritoneal exudates macrophages (PEM), obtained from C57BL/10ScSn (Sn, wild-type) mice, were stimulated with chemically synthesized 6-*O*-acylated MDP (branched-type 6-O-B30-MDP (Me) and GMD309 with acyoxyacyl group, gifts from Prof. M. Kiso and A. Hasegawa), both compounds caused significant levels of TNF-α production. In cultures of

TLR2$^{-/-}$ and TLR4$^{-/-}$ PEM, GMD-309 exhibited TNF-α-inducing activity but 6-O-B30-MDP(Me) did not. Regarding IL-6 production, both compounds showed significant activity in cultures of not only Sn but also TLR2$^{-/-}$ and TLR4$^{-/-}$ PEM, indicating that both compounds are not recognized by TLR2 and TLR4. To confirm recognition by NOD2, we are investigating using NOD2-knock-down cells.

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Systematic functional analysis of NOD2 reveals regulatory mechanisms and critical residues involved in muramyl dipeptide recognition

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Nod1 mediates p38 and p42/44 MAP kinase activation and IL-8 secretion induced by intracellular bacteria

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Nod proteins have recently been shown to serve as cytosolic pattern recognition molecules. While Nod1 detects peptidoglycan containing *meso*-diaminopimelic acid, Nod2 recognizes a muramyldipeptide which is conserved in all kinds of peptidoglycans. To further examine intracytosolic immune recognition, we used *Listeria monocytogenes* as an organism particularly amenable for studying innate immunity to intracellular pathogens. In contrast, *Listeria innocua* very poorly invades human cells, and was therefore used as a control.

Here we show that *L. monocytogenes* but not *L. innocua* substantially induce IL-8 production in human endothelial cells (HUVEC). siRNA experiments in HUVEC and Nod1-overexpression experiments in HEK293 cells demonstrate that Nod1 is critically involved in the chemokine secretion and NF-κB activation initiated by *L. monocytogenes*. Moreover, we show for the first time that Nod1 mediated activation of the MAP kinases p38 and p42/44, and TAK1 might be involved in the Nod1-dependent p38 activation induced by *L. monocytogenes*. Finally, *L. monocytogenes*- and Nod1-induced IL-8 production was blocked by a specific p38 inhibitor. In conclusion, *L. monocytogenes* induced a Nod1-dependent activation of MAP kinases and NF-κB, which resulted in IL-8 production in HUVEC. Thus Nod1 is an important component of a generalized intracytoplasmic surveillance pathway detecting intracellular pathogens such as *L. monocytogenes*.

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Up-regulation of PGRPs by chemically synthesized pathogen-associated molecular patterns in oral epithelial cells

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Peptidoglycan recognition proteins (PGRPs), a family of pattern recognition receptor molecules in innate immunity conserved from insects to mammals, recognize bacterial cell wall peptidoglycan (PGN). In human, four kinds of PGRPs (PGRP-L, -Iα, -Iβ, and -S) have been cloned. In this study, we examined the possible regulation of PGRP expression in oral epithelial cells upon stimulation with chemically synthesized pathogen-associated molecular patterns (PAMPs): tri-acyl lipopeptide (Pam_3CSSNA), lipid A (LA-15-PP), diaminopimelic acid containing desmuramyl peptides (DMPs), and muramyldipeptide (MDP). These synthetic PAMPs markedly up-regulated PGRPs, but not pro-inflammatory

cytokines, in the cells. Suppression of TLR2, TLR4, NOD1 and NOD2 specifically inhibited the up-regulation of PGRP induced by Pam_3CSSNA, LA-15-PP, DMPs, and MDP, respectively. These findings suggested that bacterial PAMPs induced the expression of PGRPs in oral epithelial cells, and the PGRPs are involved in host defense against bacterial invasion without accompanying inflammatory responses.

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Recognition of monomeric and polymeric Gram-negative peptidoglycan and signal transduction by the *Drosophila* microbial recognition receptor PGRP-LC

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Insects rely solely on innate immune responses to combat pathogenic micro-organisms. These responses include the stimulation of extracellular proteases leading to melanization and coagulation, and the activation of intracellular signaling pathways driving antimicrobial peptide production. In *Drosophila*, the IMD pathway specifically controls antimicrobial peptide induction in response to Gram-negative bacteria. In this study, we demonstrate that the *Drosophila* IMD pathway is remarkably sensitive to both polymeric and monomeric Gram-negative peptidoglycan, while much less sensitive to Gram-positive peptidoglycan. RNAi experiments revealed that different PGRP-LC isoforms are required to detect polymeric and monomeric peptidoglycans. Overexpression of PGRP-LC activates the IMD pathway and induces antimicrobial peptide expression. Using this assay, we have identified a single region within the cytoplasmic domain of PGRP-LC required for signal transduction. This region includes a short motif with similarity to the RHIM domain in mammalian RIP and TRIF proteins. In addition, we have found that IMD binds the a different region of the PGRP-LC cytoplasmic domain. However, pathway activation, by overexpression of PGRP-LC, does not require this IMD-interacting region. These results suggest that an unknown regulatory/signaling mechanism is involved in the activation of the IMD pathway by. PGRP-LC

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Synergy of peptidoglycan recognition protein (PGRP)-LE with PGRP-LC in *Drosophila* immunity

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In innate immunity, pattern recognition molecules recognize cell wall components of micro-organisms and activate subsequent immune responses, such as the induction of antimicrobial peptides and melanization in *Drosophila*. The diaminopimelic acid (DAP)-type peptidoglycan is a potent inducer of the IMD-dependent induction of antibacterial peptides. Peptidoglycan recognition protein (PGRP)-family members act as pattern recognition molecules. Loss-of-function mutations of *PGRP-LC* affect the IMD-dependent induction of antibacterial peptides and resistance to Gram-negative bacteria, whereas PGRP-LE binds to the DAP-type peptidoglycan and the gain-of-function mutation induces constitutive activation of both the IMD pathway and melanization. Here, we generated *PGRP-LE* null mutants and report that PGRP-LE acts both upstream and in parallel with PGRP-LC in the IMD pathway and shows synergy with PGRP-LC in response to *E. coli* and *B. megaterium* infections, which have the DAP-type peptidoglycan. Moreover, PGRP-LE is required for infection-dependent activation of melanization in *Drosophila*. A role for PGRP-LE in the epithelial induction of antimicrobial peptides is also suggested.

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Recombinant C-reactive protein from the horseshoe crab is a potent pathogen recognition receptor (PRR)

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C-reactive protein (CRP) was found to be the predominant PRR which recognizes endotoxin in the cell-free hemolymph of the horseshoe crab. Native CRP-2 had previously been shown to exhibit agglutination activity against the polysialic capsule of

Escherichia coli K1 but its role in bacterial clearance is not well-characterised. The antimicrobial activity of recombinant CRP-2 (rCRP-2) was tested against *Pseudomonas aeruginosa*, *E. coli* and *Staphylococcus aureus.* At nanomolar concentrations, rCRP-2 was able to agglutinate 10^7 CFU bacteria. However, the rCRP-2 was 5 times more capable of inhibiting growth of the bacteria. This bacteriostatic effect was observed for 6 h, demonstrating that rCRP-2 is stable with prolonged antibacterial potency. In addition, rCRP-2 exhibited higher specificity for Gram-negative bacteria. Pull-down experiments suggest that rCRP-2 interacts with hemocyanin, and proteins of 35 kDa and 40 kDa found in the cell-free hemolymph. This interaction was enhanced in the presence of calcium and upon infection. We propose that rCRP-2 and its interacting partners are important for effective bacterial clearance and this ensemble of proteins contribute to the powerful innate immune system of the horseshoe crab.

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Innate immunity, hemostasis and embryonic development inter-related through serine protease cascades

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Innate immunity, which predates adaptive immunity is the frontline defense which the host mounts against microbial pathogen invasion. Being present in all multicellular organisms, the innate defense uses genome-encoded receptors, to distinguish self from non-self. Invertebrate innate immunity employs several mechanisms to recognize and eliminate pathogens: (i) hemostasis to immobilize the invading microbes; (ii) lectin-induced complement pathway to opsonize the pathogen; (iii) melanization to oxidatively kill the invading pathogens; and (iv) rapid synthesis of potent antimicrobial peptides. Serine proteases play significant roles in these mechanisms, although evidence on their functions remains dismal and scattered, and only several members have been characterized, for example, the serine protease cascade in *Drosophila* dorsoventral patterning; the *Limulus* blood clotting cascade; and the silk worm prophenoloxidase cascade. Here, we seek to clarify the functional significance of serine proteases in various invertebrates. We provide compelling evidence to suggest that the serine protease cascades are integrated to play significant roles in inter-relating and networking fundamental biological processes such as early embryonic development, hemostasis and innate immune defense.

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Protease-activated receptors-mediated endothelial activation and anti-inflammatory effects of protease inhibitors

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Introduction: Protease-activated receptors are part of the family of G-protein coupled receptors (PAR1 for thrombin and PAR2 for trypsin). The objectives of the present study were to determine the status of expression of PAR on human aortic endothelial cells (HAEC), to examine the increase in expression of IL-8 and ICAM-1 on HAEC by activating PAR, and to determine effects of nafamostat mesilate and gabexate mesilate, serine protease inhibitors, on up-regulation of these pro-inflammatory factors.
Materials, Methods and Results: mRNA by RT-PCR and protein of PAR1 and PAR2 by Western blotting were constitutively expressed on HAEC without stimulants. Incubation of HAEC with PAR1, PAR2 agonist, thrombin or trypsin increased the expression of ICAM-1 and the production of IL-8 by ELISA in the concentration-dependent manner. Nafamostat mesilate and gabexate mesilate inhibited the expression of ICAM-1 and the production of IL-8 on HAEC treated with thrombin or trypsin, but didn't with PAR1 agonist peptide or PAR2 agonist peptide.
Conclusions: These results suggest that activation of PAR1 and PAR2 by thrombin and trypsin resulted in increase in ICAM-1 and IL-8 from HAEC and that nafamostat mesilate and gabexate mesilate can protect against PAR-mediated endothelial cell activation by inhibiting activity of thrombin and trypsin.

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Hepatoprotective, antiproliferative and NO regulatory effects of leaf extracts from *Toona sinensis*

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Toona sinensis is a broadleaf tree. The leaves and young shoots have been used as a vegetable in China for thousands of years. The leaves and stems of this plant have been used for the treatment of enteritis, dysentery and itch in oriental medicine. In the present study, we estimated the effect of *T. sinensis* on CCl_4-induced acute hepatotoxicity., It is shown that *T. sinensis* exerts a significant dose-dependent decline effect on serum SGOT and SGPT promotion. We found that *T. sinensis* leaf extracts possessed inhibitory effect on human hepatoma cell proliferation (HepG2 and 2.2.15 cells). Our results also demonstrated the cytotoxic effect of *T. sinensis* on various cancer cell lines with MTT and methylene blue calorimetric methods. The apoptotic cell genomic DNA strand breaks induced by *T. sinensis* were stained with the TUNEL assay. The apoptotic signal nitric oxide (NO) has a multi-regulated role in cell proliferation. In hepatocytes, NO can inhibit the main mediators of cell death – caspase protease. *T. sinensis* may change the intracellular lipopolysaccharide (LPS)-induced NO level; high NO concentrations may lead to the formation of toxic reaction products such as dinitrogen peroxynitrite that may induce hepatoma cell apoptosis.

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Ligand-regulated chimeric receptor approach reveals the diversity of Toll-like receptor function originating from transmembrane and cytoplasmic domains

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The functional differences between Toll-like receptors (TLRs) were studied by creating ligand-regulated chimeric receptors composed of the extracellular region of TLR4 and the transmembrane and cytoplasmic regions of other TLRs. They were expressed in bone marrow-derived macrophages from TLR4-deficient mice. Interestingly, the chimeras between TLR4 and either TLR3, TLR7, TLR8, or TLR9 localized intracellularly. LPS, a ligand for these chimeras, induced the production of pro-inflammatory cytokines as well as the activation of NF-κB and MAP kinases in $TLR4^{-/-}$ macrophages expressing TLR4, TLR4/TLR5, or TLR4/TLR8 but not TLR4/TLR1, TLR4/TLR2, or TLR4/TLR6 chimeras. Co-expression of unresponsive chimeras in some combinations (chimera with TLR1+TLR2 or TLR2+TLR6 but not TLR1+TLR6) resulted in LPS-responsiveness. The activity of the TLR2+TLR6 chimera pair was much higher than that of the TLR1+TLR2 pair, indicating the better function of the signaling domain of TLR6. Finally, LPS induced effective IFN-γ production and subsequent STAT1 phosphorylation in macrophages expressing full-length TLR4 but not other cell surface TLR chimeras. Taken together, these results suggest that the functions of TLRs are diversified not only in their extracellular regions for ligand recognition but also in their transmembrane/cytoplasmic regions for subcellular localization and signaling properties.

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MyD88 stabilizes mRNA for TNF and IP-10 by TIR domain-independent mechanisms

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MyD88 plays a crucial role in transducing signals of TLRs to initiate innate immune responses. It is not known how a defect in MyD88 affects IFN-γ responses. IP-10 and TNF gene expression was greatly reduced in MyD88-null macrophages in response to LPS-free IFN-γ, as compared with wild-type cells. Phosphorylation and nuclear translocation of STAT1 were not defective in MyD88-null cells. Nuclear run-on assays revealed identical transcription initiation rates between mutant and wild-type macrophages. Half-lives of TNF and IP-10 mRNA were reduced to 50% in the mutant. When co-transfected with luciferase

reporter constructs containing the 3′-UTR of TNF or IP-10 genes, MyD88 or IRAK1 increased luciferase activity in RAW 264.7 cells; while TRIF, RIP1, MKK3, MKK4 or ASK1 had no effect. The N-terminal portion of MyD88, which contains only a death domain, conferred this activity. The TIR domain of MyD88 was inactive, as were death domain containing molecules FADD and TRADD. MyD88- or IRAK1-induced luciferase activity of TNF-3′-UTR-containing constructs was inhibited by dominant negative p38 (DN-p38), but not by DN-JNK1. Thus, MyD88 appears to stabilize cytokine mRNAs by activating p38 via IRAK1. In contrast to all previously described functions of MyD88, the mRNA stabilizing effect of MyD88 does not require its TIR domain, suggesting that this novel activity is independent of extracellular signals.

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Mastoparan, a G-protein agonist peptide, differentially modulates TLR4- and TLR2-mediated signaling in human endothelial cells and murine macrophages

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Previous studies have implicated a role for heterotrimeric G protein-coupled signaling in B cells, monocytes, and macrophages stimulated with lipopolysaccharide (LPS) and have shown that G proteins co-immunoprecipitate with membrane-bound CD14. In this study, we have extended these observations in human dermal microvessel endothelial cells (HMECs) and in murine macrophages to define further the role of heterotrimeric G proteins in TLR signaling. Using mastoparan, to disrupt G protein-coupled signaling, we identified a G protein-dependent signaling pathway in HMECs stimulated with TLR4 agonists that is necessary for the activation of p38, NF-κB and IL-6 transactivation. In murine macrophages, G protein dysregulation by mastoparan resulted in inhibition of LPS-induced signaling leading to both MyD88-dependent and MyD88-independent gene expression. In addition to inhibition of TLR4-mediated mitogen-activated protein kinase (MAPK) phosphorylation in macrophages, mastoparan blunted IRAK-1 kinase activity induced by LPS, yet failed to affect phosphorylation of Akt by phophoinositol-3-kinase (PI3 kinase). These data confirm the importance of heterotrimeric G proteins in TLR4-mediated responses in cells that utilize either soluble or membrane-associated CD14 and reveal a level of TLR and signaling pathway specificity not previously appreciated.

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Porphyromonas gingivalis and *Escherichia coli* LPS differentialy activate human gingival fibroblasts and alter EGF and P_2X_7 nucleotide receptor expression

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Porphyromonas gingivalis LPS is associated with the development of rapid progressive periodontitis (RPP), which is a group of inflammatory diseases that promote the accelerated destruction of the periodontum. Furthermore, EGF action and the cytolytic release of nucleotides are important features of RPP, and gingival fibroblasts express LPS receptors (TLR2, TLR4) as well as EGF and nucleotide (P_2X_7) receptors. To study the crosstalk between these receptors, we generated a life-expanded (hTERT-transduced) human gingival fibroblast cell line (QHGF). Exposure of QHGF to *P. gingivalis* LPS and EGF for 24 h leads to a down-regulation of EGF receptor expression by ~90%, whereas stimulation with *P. gingivalis* LPS alone induces ~5-fold up-regulation of P_2X_7. Additionally, *P. gingivalis* LPS promotes a rapid degradation of the NF-κB endogenous inhibitor IκBα in QHGF, whereas *P. gingivalis* LPS poorly enhances IκBα degradation in murine RAW 264.7 macrophages. Conversely, *Escherichia coli* LPS stimulates a modest decrease of IκBα levels in QHGF, as opposed to a most robust response in RAW 264.7 macrophages. Therefore, QHGF differ from macrophages in their responsiveness to EGF, nucleotides and LPS. We hypothesize that *P. gingivalis* LPS-induced modulation of EGF and P_2X_7 receptor expression and signaling in QHGF is critical for the death/survival responses characteristic of RPP.

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Synergistic activation of human monocytic cells by NOD-ligands and TLR-ligands

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Two types of synthetic peptidoglycan fragments, diaminopimelic acid (DAP)-containing desmuramylpeptides (DMPs) and muramyldipeptide (MDP), induced secretion of interleukin (IL)-8 in a dose-dependent manner in human monocytic THP-1 cells, although high concentrations of compounds are required as compared with chemically synthesized Toll-like receptor (TLR) agonists mimicking bacterial components; TLR2 agonistic lipopeptide (Pam_3CSSNA), TLR4 agonistic lipid A (LA-15-PP), and TLR9 agonistic bacterial CpG DNA. We found marked synergistic IL-8 secretion induced by MDP or DMPs in combination with TLR agonists. Suppression of NOD1 and NOD2 specifically inhibited the synergistic IL-8 secretion induced by DMP and MDP in combination with these TLR agonists, respectively. These findings indicated that NOD2 and NOD1 are responsible for the synergistic effects of MDP and DMPs with TLR agonists, and suggested that in host innate immune responses to invading bacteria, combinatory dual signaling through extracellular TLRs and intracellular NODs might lead to the synergistic activation of host cells.

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NOD1-agonistic desmuramylpeptide (DMP) as well as NOD2-agonistic muramyldipeptide (MDP) induced endotoxin hypersensitivity in mice in a Toll-like receptor (TLR) 2-independent manner

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Mice primed with intravenous injection of DMP FK156 (Fujisawa) or MDP showed enhanced production of serum cytokines upon challenge injection (4–6 h after) of LPS or synthetic lipid A. The priming activity of MDP was also observed in TLR2 knockout and IFN-gamma-R knockout mice, but not in C3H/HeJ mice. In contrast, MDP-primed C3H/HeJ mice, but not TLR2 knockout mice, showed an enhanced response to a synthetic lipopeptide, Pam_3CSSNA. MDP administration increased the mRNA expression of MyD88 and TLR4 in the liver, lungs and spleen, but not in peripheral blood cells in the C57BL/6 mice, and increased TLR4 expression in their splenocytes. In accordance with these findings, in the MDP-primed mice, TNF-α mRNA expression in the organs was markedly increased upon endotoxin challenge. These findings suggest that NOD-agonist primed mice show enhanced responses to TLR ligands through enhanced TLR-MyD88 signaling in a TLR-independent manner.

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Alpha-galactosylceramide (GalCer) enhances LPS-induced nitric oxide (NO) production in murine peritoneal cells

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GalCer is a specific ligand for T cell receptor (TCR)-Vα14 and is known to activate NKT cells expressing TCR-Vα14. In this study, we examined the effects of GalCer in LPS-induced NO production in *in vitro* cultures of murine peritoneal macrophages and spleen cells. Simultaneous treatment with LPS and GalCer enhanced LPS-induced NO production in the culture of peritoneal macrophages but not spleen cells. GalCer alone did not affect LPS-induced NO production in peritoneal

macrophages. GalCer slightly enhanced LPS-induced NO production in RAW 264.7 macrophage cells. GalCer did not enhance LPS-induced tumor necrosis factor-α production in peritoneal cells. There was no marked difference in interferon-γ production in LPS-stimulated peritoneal macrophages in the presence or absence of GalCer. The detailed mechanism of the enhancing action of GalCer on LPS-induced NO production will be discussed.

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The serum antibody response and protective efficacy of a detoxified *Escherichia coli* (J5 mutant) lipopolysaccharide/group B meningococcal outer membrane protein (J5 dLPS/OMP) vaccine for sepsis is enhanced by CpG oligonucleotide adjuvant (CpG)

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Whereas the J5 dLPS/OMP vaccine in animals induced > 20-fold increases in serum IgG levels to J5 LPS, we observed a mean 3-fold increase in human subjects in a recent phase I clinical study of this vaccine. We, therefore, examined the ability of CpG to increase this response. Outbred mice were given vaccine alone, vaccine + CpG or CpG alone and cecal ligation/puncture (CLP) was performed 21 days later. Two weeks after the last dose of vaccine (*i.e.* before CLP), the CpG-only group had < 1 mcg/ml of anti-J5 LPS IgG. The vaccine + CpG group had 475 ± 415 mcg/ml versus 151 ± 239 mcg/ml for the vaccine alone ($P = 0.003$). At 48 h after CLP, both groups had a significant decrement in J5 LPS-specific but not in total IgG. While 0/9 of the CpG control group survived, in the vaccine-only group, 14/15 mice survived versus 15/15 in the vaccine + CpG group. Both vaccine groups had lower levels of circulating cytokines than CpG controls; however, the CpG + vaccine group had the lowest level of LPS and bacteria in the peritoneum, suggesting that the vaccine + CpG may protect against a more severe sepsis. We conclude that vaccine + CpG merits testing in human subjects.

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Higher induction of histamine-forming enzyme, histidine decarboxylase (HDC), in various tissues in muramyldipeptide-primed mice upon intravenous injection of endotoxin

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We reported previously that: (i) intravenous injection of lipopolysaccharide (LPS) into mice induced histidine decarboxylase (HDC), with a resultant elevation of histamine levels in various tissues; and that (ii) pretreatment of mice with muramyldipeptide (MDP; 4–6 h prior) induced endotoxin hypersensitivity. Here, we examined whether NOD2-agonistic MDP and NOD1-agonistic desmuramylpeptides (DMPs, FK156 and FK565; Fujisawa Pharmaceuticals) increased HDC production in response to endotoxin in BALB/c mice. MDP alone scarcely induced HDC, while DMPs significantly induced HDC in the lungs and liver. Higher induction of HDC in various tissues, especially in the lungs, was observed in MDP- or FK156-primed mice, in response to endotoxic LPS and synthetic lipid A, than in non-primed mice, while simultaneous administration of MDP and endotoxin was inactive in this respect. Further studies are being conducted to examine the possible priming activities of NOD1 and NOD2 agonists in combination with various Toll-like receptor agonists derived from bacteria.

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Synergy of CpG-oligonucleotides with muramyldipeptide in cytokine induction

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Muropeptides, as well as CpG-oligonucleotides (ODN), which imitate bacterial DNA, modulate inflammatory responses. In peripheral blood mononuclear cells no significant cytokine release could be induced by CpG (2006-PT) or MDP alone, but

a significant synergy of 100 nM MDP with 2 μM CpG (TNF:CpG: 160 ± 30 pg/ml vs MDP: 50 ± 20 pg/ml vs CpG+MDP: 3770 ± 1120 pg/ml) was found, which was only little sequence-specific but also occurred with the control GpC oligonucleotide (TNF:GpC: 290 ± 110 pg/ml vs. GpC+MDP: 2890 ± 1060 pg/ml). The synergistic effect was not TLR4-dependent as confirmed by bone marrow cells from C3H/HeJ mice, excluding endotoxin contaminations of MDP or CpG. To investigate the role of sequence and thioat-modification for the synergism, a panel of ODN, which differed in length (48-, 36-, 24- [referred as CpG], 12- and 6-ODN) and sequence (3′12-ODN, 5′12-ODN and 24-ODN-polyA), as well as DNA, isolated from *Escherichia coli*, was investigated. Our results showed that except for the 6-ODN all ODNs, as well as 1 ng/ml bacterial DNA, synergized with MDP, although a 10-fold higher concentration of the 12-ODN and polyA were necessary, indicating that a minimum length is necessary and that the CpG motif optimizes the effect.

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Cytokine production of mouse CD5$^+$ B cell hybridoma in response to LPS and IFN-γ

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The mouse TH2.52 B cell line is a hybridoma between mouse splenic B cells and mouse B lymphoma, which possesses the typical phenotype of B1 cells including CD5 expression. In the present study, we characterized the cytokine production of TH2.52 cells in response to LPS and/or IFN-γ. LPS induced the production of TNF-α and IL-6 in TH2.52 cells, like B2 cells. On the other hand, IFN-γ induced IL-2, IL-4, IL-6, TNF-α and nitric oxide. Further, TH2.52 cells became dendritic and phagocytic in response to IFN-γ, suggesting the transition to macrophage-like cells. This transition was inhibited by the addition of IL-4. Taken together, TH2.52 cells behave like B cells under LPS stimulation and like macrophages under IFN-γ stimulation. IL-4, a Th2 cytokine, counteracted the action of IFN-γ, a Th1 cytokine. B1 cells might play a role in humoral and cell-mediated immunity in LPS and IFN-γ stimulation, respectively.

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Exposure to CpG-ODN exacerbates the inflammatory response and augments intestinal permeability caused by systemic hypotension via an IFN-γ dependent route

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The effects of *in vivo* exposure to CpG-ODN on the inflammatory response and gut barrier function were studied in rats compromised by hemorrhagic shock. In addition, we defined responsiveness of peritoneal macrophages from CpG-treated animals to endotoxin (LPS), lipoteichoic acid (LTA) and CpG-ODN *ex vivo*. TNF-α concentrations were significantly elevated in supernatant of peritoneal macrophages from CpG-treated rats upon stimulation with 1 and 10 ng/ml LPS, 1 and 10 μg/ml LTA and low dose CpG-ODN (1 μM) compared to control rats. Subjecting these CpG-ODN treated rats to hemorrhagic shock markedly increased circulating IFN-γ, TNF-α, IL-6 and nitrite and caused a defective IL-10 response compared with control shock-rats. Additionally, administration of CpG-ODN impaired gut barrier function and further augmented shock-induced intestinal permeability for HRP and bacterial translocation to distant organs. Interestingly, administration of anti-IFN-γ significantly ameliorated the excessive inflammatory response caused by CpG-ODN, restored the IL-10 response and reduced intestinal permeability for HRP and bacterial translocation to distant organs. In conclusion, these results indicate that CpG-ODN impairs gut barrier function and enhanced responsiveness of macrophages. Furthermore, pretreatment with CpG-ODN induces a 'two-hit' phenomenon in animals compromised by hemorrhagic shock via an IFN-γ dependent route.

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The host defence peptide LL-37 is a multi-functional immunomodulator which interacts directly with the effector cells of the innate immune response

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LL-37 is a cationic peptide that is found primarily in the granules of neutrophils. Originally identified as an 'antimicrobial' peptide, it now appears that LL-37 may be a broad spectrum immunomodulator. LL-37 is a potent anti-endotoxic agent which blocks the release of pro-inflammatory cytokines produced by primary monocytes or monocyte-like cell lines upon stimulation with lipopolysaccharide. LL-37 also induces transcription and release of the chemokines IL-8, MCP-1 and MCP-3 in animal models and primary monocytes. Such results indicate that LL-37 may interact directly with the effector cells of the innate immune response; however, this interaction has not been well-characterized. In this study, we demonstrate that LL-37 induces phosphorylation of the mitogen activated protein kinases, p38 and ERK1/2 in a serum-dependent manner in primary monocytes and an airway epithelial cell line but not in T or B cells. In the presence of serum, LL-37 associates with monocytes and epithelial cells. Activation of these kinases is required for transcription of a number of chemokine genes and the release of IL-8. Phosphorylation of ERK1/2 and p38 does not occur in the absence of serum and may require the presence of serum components such as high density lipoproteins. This study demonstrates that LL-37 interacts with the cells of the innate immune response and activates kinases involved in growth, differentiation and proliferation.

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Hsp-27 significantly alters dendritic cell emergence from precursor macrophages

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Large heat-shock proteins(Hsp) have been reported to bind to TLR4, inducing maturation of monocyte-originated dendritic cells (DCs), thus providing an important link between innate and acquired immunity. Here, we attempted to examine a role of small Hsp-27 vs Hsp-60 in the emergence of DCs considering unique biological activities of small Hsp. Human macrophages were isolated with Ficoll-Hypaque followed by magnetic beads. DCs were generated with IL-4 and GM-CSF (4+G) for 5 days alone or with Hsp-27 or Hsp-60. To prevent any LPS contamination, polymyxin B was added to all cultures. After 5 days, cells were assessed for CD1a, CD83, CD209, CD14 and MLR reaction, to determine their antigen-presenting cell capability. Alternatively, macrophages were stimulated with Hsp-27, or left unstimulated overnight and evaluated for M-CSFR, GM-CSFR, IL-4R and IL-6R, important receptors for the differentiation of 4+G stimulated macrophages into DCs. In contrast to Hsp-60, Hsp-27 significantly reduced emergence of DCs (Mean ± SE % CD1a; 4+G = 47.8 ± 6.6; 4+G+Hsp-27 = 27.2 ± 6.6; 4+G+Hsp-60 = 40.9 ± 16.1). In overnight experiments, Hsp-27 down-regulated M-CSFR and IL-6R whereas the expression of GM-CSFR and IL-4R was increased with a concomitantly enhanced appearance of IL-4-induced CD209 on DCs. We conclude that Hsp-27 has the unique capability of inhibiting macrophage to DC differentiation but its action is mediated by an alternative mechanism to Hsp-27-mediated cytokine receptor expression.

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Innate immunity to the pathogenic fungus *Coccidioides posadasii* is dependent on TLR2 and Dectin-1

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Coccidioides posadasii is a pathogenic fungus that causes endemic and epidemic coccidioidomycosis in the deserts of North, Central, and South America. Innate immune mechanisms against the organism are not well understood. Here

we show that elicited mouse peritoneal macrophages respond to spherules (the tissue form of the fungus) by producing pro-inflammatory cytokines as measured by quantitative polymerase chain reaction of cellular transcripts as well as by ELISA assays of secreted protein. We examined the contribution of Toll-like receptors (TLRs), CD14 and MyD88 in macrophage responses to FKS by comparing cytokine responses of elicited macrophages from different knockout mice. Elicited mouse peritoneal macrophage from TLR2$^{-/-}$ and MyD88$^{-/-}$ produced significantly less TNF-α, MIP-2 and IL-6 following activation with spherules than the wild-type macrophages. In contrast, C3H/HeJ mice, which have a point mutation in TLR4, as well as TLR4$^{-/-}$ mice exhibited no defect in cytokine production as compared to the control mice. We also investigated the role of the macrophage β-glucan receptor, Dectin-1. RAW 264.7 macrophages overexpressing Dectin-1 produced more cytokines in respond to FKS, live spherules and purified β-glucan than control RAW cells. Blockage of Dectin-1 with antibodies inhibited cytokine production in elicited mouse peritoneal macrophages. Taken together, these results show that cytokine responses in mouse peritoneal macrophages to *C. posadasii* spherules are dependent on TLR2, MyD88 and Dectin-1.

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Early innate immune responses to filarial parasites and endosymbiotic *Wolbachia* are predominantly dependent on TLR2

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Lymphatic filariasis is caused by infection with *Brugia malayi*, *Brugia timori* and *Wuchereria bancrofti* and is a major health problem in tropical regions of the world with over 120 million infected individuals. The pathological consequences of infection are primarily acute and chronic lymphedema of the extremities (elephantiasis) and testicular hydroceles; however, not all infected individuals manifest severe disease. The host factors contributing to this variable disease presentation are unclear but may involve innate immunity. *Wolbachia* bacteria are found in most filarial parasites of importance to human health (*B. malayi*, *W. bancrofti*, *O. volvulus*). The obligate symbiotic organisms are concentrated in intracytoplasmic vacuoles within the hypodermal lateral cords and female reproductive organs and are transmitted in a matrilinear fashion. Filarial reproduction and molting are dependent on the presence of the bacteria, although the mechanisms of the symbiosis are not yet known.

Early studies of innate immune activation by the filarial worms focused on the role of TLR4. Using human TLR transfected HEK cell lines as well as mouse strains deficient in TLR2, TLR4 and MyD88, we show that inflammatory responses to soluble *Brugia* filarial extracts containing *Wolbachia* are instead primarily dependent on TLR2. Responses are also dependent on MyD88. These finding have important implications for future studies aimed at identifying host factors that contribute to the pathogenesis of clinical filarial diseases.

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Cross-talk between Nod1, Nod2 and Toll-like receptors (TLRs) for cytokine production by macrophages in response to Gram-positive bacteria

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TLRs are molecules with highly conserved structures involved in the sensing of microbial-derived compounds. Nod1 and Nod2 belong to a family of intracytoplasmic receptors and recognize distinct peptidoglycan-derived muropeptides. Macrophages possess phagocytic properties and their response to whole bacteria may be initiated both by TLRs at the cell surface, and intracellularly by Nod proteins. We compared the role of these receptors following interaction of whole bacteria with macrophages and analyzed pro- and anti-inflammatory cytokine production. Using knockout mice, we determined that TLR4 and TLR2 contribution was predominant in the induction of TNF and IL-10 by Gram-negative bacteria. In contrast, the absence of TLR2 (and/or

TLR4) did not affect the response to Gram-positive bacteria. However, in the case of TLR2/4$^{-/-}$ mice, the phagocytic property of macrophages was essential to cytokine production in response to heat-killed *Staphylococcus aureus* (HKSA) that was profoundly inhibited by cytochalasine D. By transfecting RAW 264.7 cells with dominant negative forms of Nod1 and Nod2, we showed that Nod molecules contribute to NF-κB activation and cytokine production in response to HKSA. Finally, LPS and HKSA up-regulate the expression of Nod1 and/or Nod2 mRNA, demonstrating a cross-talk between Nods and TLRs at the transcriptional level.

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Pattern recognition receptor deficiency compromises host resistance to pneumonic tularemia

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Background: *Francisella tularensis*, a category A select agent, is extremely infectious, easily disseminated and causes significant illness and death. Mechanisms underlying tularemia pathogenesis remain ill-defined. Herein, we demonstrate that CD14, TLR2, and TLR4 (pattern recognition receptors, PRRs) mitigate host responses to *F. tularensis* LVS.
Methods: Wild-type and PRR$^{-/-}$ mice were intranasally infected, tissues were harvested for histological evaluation and enumeration of bacteria, and sera was collected to measure cytokine/chemokine and antibody levels.
Results: PRR$^{-/-}$ mice were more susceptible and succumbed to infection more rapidly compared with controls. Control and PRR$^{-/-}$ mice had pulmonary lesions composed primarily of neutrophils and alveolar macrophages; however, the lungs of PRR$^{-/-}$ mice were significantly more inflamed and had higher bacterial burdens. Remarkably, although the pro-inflammatory cytokines TNF-α, IFN-γ, and MCP-1 were elevated in infected mice, no significant differences were observed between control and PRR$^{-/-}$ mice. The pattern of humoral immunity was complex and varied in different PRR$^{-/-}$ mice. CD14$^{-/-}$ mice exhibited lower francisellae-specific IgM but an IgG response comparable to controls, whereas TLR4$^{-/-}$ mice showed significantly higher IgG levels compared to controls.
Conclusions: PRR signaling mitigates the clinical course and severity of pneumonic tularemia and elucidation of downstream events may identify novel immunotherapeutic targets.

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Peripheral immunity in patients with hepatitis C virus (HCV) infection: different roles of endotoxins (LPS) and β-glucans

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HCV$^+$ patients are characterized by depressed phagocytosis and killing of polymorphs and monocytes and T-cell mediated antibacterial activity. Here, we report that a very high percentage of HCV-patients are endotoxemic and β-glucanemic. We speculated that deficits of the innate immunity might facilitate entry of Gram-negative bacteria and fungi into the HCV$^+$ host. On this basis, HCV-patients were subdivided into two subsets: LPS$^+$/β-glucan$^+$ and LPS$^-$/β-glucan$^+$. In the LPS$^-$/β-glucan$^+$ subset, we found a different pattern of peripheral immune response, when data were compared to those from the LPS$^+$/β-glucan$^+$ counterpart. In fact, here we describe in the LPS$^-$/β-glucan$^+$ patients an expansion of the T regulatory cells (CD4$^+$CD25$^+$ cells), which correlates with a reduced frequency of TNF-α$^+$ and IL-1β$^+$ monocytes. In the same patients, we detected a reduction of CD8$^+$ memory cells, likely including cytolytic lymphocytes which are specific for HCV viral epitopes. These laboratory findings coincide with less hepatic damage, *e.g.* lower values of transaminases and of total and direct bilirubin in comparison with LPS$^+$/β-glucan$^+$ individuals. In conclusion, β-glucans, if present in the host and, according to their serum concentration, may exert an anti-inflammatory activity in the course of HCV disease.

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MyD88 is pivotal for the early inflammatory response and subsequent bacterial clearance in *Chlamydia pneumoniae* pneumonia

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Chlamydia pneumoniae is an important respiratory tract pathogen that is associated with a wide range of respiratory tract infection and a number of chronic diseases. Recently, *C. pneumoniae* has been associated with coronary artery disease and atherosclerosis. In this study, we investigate the involvement of Toll-like receptor (TLR) signaling in *C. pneumoniae* induced pneumonia. MyD88-deficient mice are severely impaired in their ability to mount an acute inflammatory response toward *C. pneumoniae* as compared to wild-type and TLR4-deficient mice. Albeit a comparable bacterial burden in the lungs of infected mice 5 days after infection, MyD88-deficient mice show only minor sign of pneumonia and reduced expression of inflammatory mediators. In contrast, TLR4-deficient mice show indistinguishable signs of pneumonia compared to wild-type mice. In contrast, MyD88-deficient mice are unable to clear bacteria and show a severe, chronic inflammation with elevated systemic IL-1β and local IL-1β and IFN-γ concentrations leading to decreased survival 14 days after infection with *C. pneumoniae* while wild-type and TLR4-deficient mice recovered from acute pneumonia. These data indicate that MyD88 is essential to recognize *C. pneumoniae* infection and initiate an effective immune response leading to the clearance of bacteria from infected lungs.

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Impaired hepatic granuloma formation with normal host defense in MyD88-deficient mice

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Granulomas are characterized histologically by a nodular collection of macrophages with occasional admixture of epithelioid cells, multinucleated giant cells, and other immunocompetent cells. Cytokines and chemokines play important roles in the recruitment of these cells in granulomas, and MyD88 plays a major role in TLR/IL-1 receptor family signaling. In the present study, the roles of MyD88 on hepatic granuloma formation and host defense against BCG were examined. MyD88-deficient ($MyD88^{-/-}$) and wild-type ($MyD88^{+/+}$) mice were infected with BCG via the tail vein. The number and the size of hepatic granulomas of $MyD88^{-/-}$ mice were remarkably decreased. Immunohistochemical examination using two kinds of monoclonal antibodies to macrophages, BM8 and Mac-1, showed that BM8 or Mac-1 positive cells decreased within and out of granulomas in the $MyD88^{-/-}$ mice. The mRNA levels of IL-1β, TNF-α, IFN-β and iNOS in the hepatic tissue were lower in the $MyD88^{-/-}$ mice. Otherwise, the colony assay showed that there was no significant difference between $MyD88^{-/-}$ and $MyD88^{+/+}$ mice. These results indicate that signaling through MyD88 is important for the normal development of hepatic granulomas, but that MyD88 does not influence the host defense of BCG infection.

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Endotoxin-independent induction of innate immunity by IL-2 expressing recombinant *Salmonella typhimurium*

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Attenuated *Salmonella* strains are excellent vectors for the delivery of immunogenic proteins from a host of pathogens. The ability to fine-tune the immune response to these multivalent vaccines would be a distinct advantage. We studied the effect of expressing IL-2 by a recombinant *Salmonella typhimurium* strain (designated GIDIL2) on host immunity. Accumulated evidence has demonstrated that administration of GIDIL2, but not the parental strain, leads to the rapid induction of innate immune responses, including activation of NK cells and neutrophils and a concomitant up-regulation of NOS2 gene expression and NO production. This occurred independently of LPS-responsiveness and IFN-γ production, demonstrating its independence of the typical LPS/IFN-γ activation pathway. Importantly, GIDIL2-induced innate immunity afforded protection against lethal homologous and heterologous challenges and this protection was abrogated in mice receiving a NOS2 inhibitor. These findings demonstrate that expression of IL-2 has a profound influence on the early immune response and highlight the importance of innate immunity in the control of early bacterial proliferation.

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Sublethal infection of C57BL/6 mice with *Salmonella typhimurium* leads to an increase in TLR1, TLR2 and TLR9 mRNA as well as a decrease in TLR6 mRNA levels

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Toll-like receptors (TLRs) selectively recognise pathogen-associated molecular patterns (PAMPs) produced by either Gram-negative or Gram-positive bacteria to initiate an innate immune response. We have investigated the expression of TLRs 1, 2, 4, 5, 6 and 9, the TLR4 associated molecule MD2, adapter molecules MyD88 and TIRAP/Mal as well as the inflammatory mediators inducible nitric oxide synthase (iNOS) and tumour necrosis factor α (TNF-α) by real time PCR in C57/BL6 mice. The mice were infected with *Salmonella enterica* Typhimurium (*S. typhimurium*) M525P. This resulted in a sub-lethal infection with bacterial numbers reaching a plateau from days 4–7 onwards. In general, higher basal RNA levels were observed in spleen compared to liver. During infection, RNA expression levels in spleen were unchanged, except for a reduction in TLR6 mRNA expression. In contrast, in liver the RNA levels of TLR1, TLR2, TLR9, iNOS and TNF-α show a large increase in response to infection.

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A non-tolerizing dose of Gram-negative bacterial lipopolysaccharide primes anti-bacterial host defenses: a model for the role of endotoxin in innate immunity

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We previously observed that LPS-hyporesponsive C3H/HeJ mice were highly susceptible to lethal infection with *Escherichia coli* O18:K1, but administration of TNF and IL-1 restored antibacterial host defenses (ABHDs). To further test the hypothesis that LPS-responsiveness is required for ABHD, we gave a single, relatively low dose (35 mcg) of LPS i.p. to outbred mice and challenged them at 48 h with 3–5 LD_{50} of *E. coli*. Compared to controls, LPS-treated mice had improved clearance of *E. coli* from the blood, increased bacterial killing and enhanced survival. This LPS dose did not prevent lethality when 500 mcg of LPS was given i.p. at 48 h. In contrast, when an LPS regimen was administered that did prevent death after that same lethal LPS dose, there was no enhanced ABHD. When mice were pretreated with a

greater but sublethal dose of LPS, there was no enhanced ABHD. We conclude that LPS primes ABHD in a dose-dependent manner and by a mechanism independent of tolerance induction. By examining the innate host responses over the spectrum of beneficial (priming of ABHD) to lethal doses of LPS, it may be possible to identify those host innate immune responses that initially become dysregulated during the development of sepsis.

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CD154 is essential for protection against Gram-negative *Salmonella* infections

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Salmonellae are intracellular pathogens which, upon infection, reside inside the host's phagocytes. Activation of macrophages during the acute phase of the disease is essential for early control of bacterial replication and subsequent development of Th1 immunity. In this study, we assessed the requirements for the initiation of anti-*Salmonella* immune responses by using mice deficient in CD154 protein ($CD154^{-/-}$). Our findings demonstrate that compared to C57BL/6 controls, $CD154^{-/-}$ mice were highly susceptible to an infection by an attenuated strain of *Salmonella enterica* serovar Typhimurium, as evidenced by a significantly decreased survival rate. Moreover, the production of IL-12, TNF-α, IFN-γ and NO was markedly decreased in $CD154^{-/-}$ mice. The heightened susceptibility to infection was not due to a substantial increase in systemic bacterial burden but, instead, was associated with considerable histopathological liver damage, brought about as a result of defective macrophage recruitment. We conclude that direct recognition of *Salmonella* by host's macrophages is insufficient for protection, and that intercellular communications via the CD40–CD154 pathway play a critical role in the induction type 1 cytokine responses and protection against primary *Salmonella* infections.

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Correlation between LPS-resistance and the resistance toward cytotoxic effects of *Salmonella* infection in mouse macrophages

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LPS induced macrophage cell death in the presence of a protein synthesis inhibitor, cycloheximide (CHX). However, the macrophages with altered or no responsiveness to LPS did not die. In this study, we examined relationships between the LPS-induced cell death and the cytotoxicity during *Salmonella* infection in JA-4 cells, a subline of J774.1 macrophage-like cell line, and LPS1916 cells, an LPS-resistant mutant originated from JA-4, and also in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. As shown previously, JA-4 cells and C3H/HeN macrophages released lactate dehydrogenase (LDH) after incubation with both LPS and CHX at 37°C for 4 h, while LPS1916 cells or C3H/HeJ macrophages did not. In JA-4 cells, *Salmonella typhimurium* infection induced apoptosis, as revealed by caspase-3 activation and TUNEL staining, followed by LDH release. Incorporation of [^{35}S]-methionine/cysteine into the macrophages was also inhibited by *S. typhimurium* infection, suggesting that LPS from *Salmonella* together with protein synthesis inhibition might be involved in the induction of the macrophage cell death. However, *S. typhimurium* infection induced LDH release similarly in the LPS1916 mutant cell line, C3H/HeN as well as C3H/HeJ mouse macrophages. These results suggest that LPS-resistance of macrophages is not closely correlated with the resistance of the macrophages toward *S. typhimurium* infection.

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Effects of dexamethasone on *Salmonella* infection *in vivo*

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Dexamethasone is a glucocorticoid analogue that has anti-inflammatory effects and is sometimes used to treat sepsis. It inhibits production of pro-inflammatory mediators such as nitric oxide, tumour necrosis factor α and prostaglandins. Various studies have shown that glucocorticoids inhibit lipopolysaccharide-induced inflammation, but little has been published on the effect of glucocorticoids on *Salmonella* infection in which lipopolysaccharide plays a key role. In this study, we pre-treated male Balb/c mice by subcutaneous implantation of pellets that release dexamethasone at a constant rate over 21 days and infected with *Salmonella enterica* serovar Typhimurium strain M525P. The bacterial load in spleen and liver were analysed at various time points. We found that dexamethasone promotes bacterial growth during the early phase of infection and, depending on the dexamethasone dose, the bacterial growth reaches a plateau at higher bacterial load per organ than in placebo-treated mice. This suggests that glucocorticoids are permitting accelerated bacterial growth by inhibiting the lipopolysaccharide-stimulated early innate immune response that controls infective load. We are currently conducting RT-PCR and Western blotting analyses for inflammatory mediators to investigate this hypothesis. Glucocorticoids also inhibit neutrophil recruitment therefore we are also analysing blood smears and organ sections to study lesion formation and leukocyte profiles.

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Differential induction of SOCS3 by JAK/STAT-dependent and independent pathways during sepsis

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The suppressor of cytokine signaling (SOCS) proteins have been identified as an important feed-back regulative mechanism in immune system. Studies indicate that SOCS proteins can be induced via traditional JAK/STAT (by cytokines/hormones) and TLR (by microbes) pathways. Our previous data showed that SOCS3 protein was up-regulated in various cells/tissues after polymicrobial sepsis induced by cecal ligation and puncture (CLP). However, the lack of TLR4/MyD88 signaling only partially decreased SOCS3 expression in a tissue-selective manner. Therefore, the aim of this study was to determine the contribution of the JAK/STAT vs TLR/MyD88 pathways on SOCS3 induction in sepsis. $STAT1^{-/-}$, $MyD88^{-/-}$ and their background mice were subjected to CLP or sham-CLP procedure. 24-h later, SOCS3 expression was determined by Western blot in peritoneal leukocytes, spleen and lung harvested from these mice. Our results show that, in $MyD88^{-/-}$ mice, SOCS3 was down-regulated in peritoneal leukocytes but no change in spleen and lung compared to background after CLP. To the contrary, SOCS3 was partially decreased in peritoneal leukocytes and strongly diminished in both spleen and lung in septic $STAT1^{-/-}$ mice. These data suggest that microbial stimulation of SOCS3 expression via TLR4 signalling is tissue-limited in septic mice and additive upon basal JAK/STAT activation.

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Preventative effects of Ca^{2+} channel blockers on TNF-α induced cytotoxicity approached from endotoxin challenge

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We examined the role of intracellular Ca^{2+} in the mechanism of the preventive effects of the Ca^{2+}-channel blockers against TNF-α induced cytotoxicity during endotoxemia. The membrane protein damage in liver was found mostly in

the molecular weight (MW) range 60–150 kDa in endotoxemic mice, and was markedly injured near 140 kDa (MW of Ca^{2+}-ATPase in liver plasma membrane). These findings suggest that the depression of Ca^{2+}-ATPase activity in liver plasma membrane damage caused by free radicals in endotoxemic mice brings about a decline in exclusion of Ca^{2+} from liver cytoplasm. The administration of verapamil (10 mg/kg, s.c.) clearly prevented the liver lipid peroxide formation arising from endotoxin challenge. Cellular Ca^{2+} has been shown to be a key messenger involved in the initiation and triggering of cell death. It seems that intracellular Ca^{2+} may modulate not only oxidative stress, but also cellular function in endotoxemia. Treatment with verapamil (30 μM) markedly inhibited endotoxin(1 μg/ml)-induced TNF-α production in J774A.1 cells. On the other hand, the preventative effect of verapamil (1–10 μM) on the cytotoxicity of TNF-α in L929 cells was concentration-dependent. Similarly, the short-acting Ca^{2+}-channel blocker nicardipine(1–10 μM) significantly inhibited the TNF-α induced cytotoxicity. On the other hand, 10 μM amlodipine (long-acting Ca^{2+}-channel blocker) clearly inhibited this process. These findings suggest a preventive effect of Ca^{2+}-channel blockers on TNF-α induced cytotoxicity in fibroblast cells through Ca^{2+} mobilization by these drugs.

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Effect of prostaglandin E receptor subtype EP4 selective agonist on endotoxin-stimulated secretion of tumor necrosis factor-α by macrophages in acute ethanol-loaded rats

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Endotoxin and pro-inflammatory cytokines from activated macrophages are considered to play an important role in the progression of alcoholic liver disease and multiple organ failure. Recently, a prostaglandin receptor subtype EP4 agonist with cytoprotective effect has been developed. In this study, we examined the efficacy of an EP4 agonist ONO-AE1-437 on endotoxin-stimulated tumor necrosis factor (TNF)-α secretion of Kupffer cells, splenic macrophages and alveolar macrophages in control and acute ethanol-loaded rats (5 mg/g body weight ethanol, intraperitoneally). After the preculture in the medium containing 0, 0.1, 1, 10, 100 nmol/l of ONO-AE1-437, TNF-α secretion of these cells stimulated by 100 ng/ml lipopolysaccharide (*Escherichia coli* O55:B5) was determined for 3 h. The amount of TNF-α secreted from alveolar macrophages was highest both in the control and acute ethanol-loaded rats. Acute ethanol load enhances TNF-α secretion of splenic macrophages. The addition of ONO-AE1-437 significantly inhibited TNF-α secretion of Kupffer cells and splenic macrophages both in the control and acute ethanol-loaded rats. Alveolar macrophages were less affected. These results suggested that an EP4 agonist ONO-AE1-437 suppressed excess TNF-α secretion from macrophages stimulated by endotoxin and decreased endotoxin-related organ disturbance in patients with severe alcoholic hepatitis.

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Prolonged Toll-like receptor stimulation leads to down-regulation of IRAK-4 protein

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Interleukin-1 receptor-associated kinase (IRAK)-4 is a key mediator in the Toll-like receptor (TLR) signaling. We found that stimulation of TLR2, TLR4, or TLR9 caused a decrease in IRAK-4 protein without affecting its mRNA level in a mouse macrophage cell line, RAW 264. The decrease in IRAK-4 was accompanied by the appearance of a smaller molecular weight protein (32 kDa) that was recognized by an anti-IRAK-4 antibody raised against the C-terminal region. The decrease in IRAK-4 and the appearance of the 32 kDa protein occurred with slower kinetics than the activation of IRAK-1, and were suppressed by inhibitors of the proteasome, inducible IκBα phosphorylation or protein synthesis. These results indicate that prolonged stimulation of TLRs causes a down-regulation of IRAK-4 protein, which may be mediated through cleavage of IRAK-4 by a protease induced by the activation of NF-κB.

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Revisiting cross-tolerance with highly purified LPS

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Knowledge about endotoxin tolerance and cross-tolerance between LPS and different TLR ligands have been essentially achieved with conventional LPS, extracted according to the Westphal's method, and purified according conventional methods. However, more recent investigations with highly purified LPS led to contradictory results. We compared the cross-tolerance phenomenon with conventional *Escherichia coli* LPS (Sigma) and a highly purified *E. coli* LPS. We ensured that conventional LPS activates both peritoneal macrophages from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice while highly purified LPS was only active on TLR2$^{-/-}$ macrophages. Human monocytes were pre-treated overnight with TLR ligands (tolerization), washed and challenged for the next 24 h with TLR ligands and TNF production was assessed in the supernatants. Conventional LPS was tolerizing human monocytes for a further challenge with both LPS. In contrast, highly purified LPS had (depending upon concentrations) weak or no capacity to tolerize monocytes to a challenge with conventional LPS. Pre-treatment of human monocytes with specific TLR2 ligands (Pam_3CysSK_4 or Pam_2CysSK_4) moderately tolerized human monocytes to both LPSs. In contrast, pre-treatment with conventional and highly purified LPS led to divergent results in response to a challenge with TLR2 ligands.

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TLR3 ligand stimulation induces unique dendritic cells with cross tolerance to LPS but not TLR9 ligands

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Prior exposure to TLR ligands can lead to cross tolerance with the desensitization of immune cells to a subsequent LPS challenge. We investigated whether TLR3 or TLR4 ligand stimulation (dsRNA, poly(I:C) and LPS, respectively) induced cross tolerance of dendritic cells (DCs), by examining their expression of TLRs, as well as their subsequent response to TLR4 (LPS) and TLR9 (CpG-DNA) specific ligands. Both LPS and poly(I:C) significantly down-regulated TLR4/MD-2 DC cell surface expression on immature DCs. While poly(I:C) significantly up-regulated cell surface, but not intracellular, TLR9 expression, LPS significantly increased intracellular, but not cell surface, TLR9 expression. As expected, LPS-treated DCs displayed tolerance to subsequent LPS and CpG-DNA stimulation with those DCs secreting significantly decreased levels of IL-12 p40 and p70. However, poly(I:C)-treated DCs retained the ability to produce IL-12 with subsequent CpG-DNA, but not LPS stimulation. Inhibition of DC endocytosis with chloroquine completely inhibited IL-12 and IL-10 production by LPS-treated DCs in response to subsequent CpG DNA-stimulation, while it failed to delete their production in poly(I:C)-treated DCs. These results suggest that poly(I:C) may induce a novel DC phenotype that preserves the capacity of cytokine production to subsequent CpG DNA-stimulation.

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IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells

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Recognition of microbial products through Toll-like receptors (TLRs) triggers the expression of several cytokines that regulate innate and adaptive immunity. Signaling by various TLRs is not equivalent and leads to differential gene induction. This study analyzed the response of human dendritic cells (DCs) and PBMCs stimulated with agonists of

TLR2, TLR3, TLR4, TLR5, and TLR7, first individually and then in combination. Several cytokines were equally induced by all TLR agonists, but four genes, IFN-β, IP-10, IL-12p35 and IL-15 showed a very restricted pattern of induction. Thus, each TLR appears to possess a distinctive ability to activate DCs or PBMCs suggesting that TLR-mediated responses cannot be simply catalogued as resembling either TLR2 (MyD88-dependent) or TLR4 (MyD88-independent), and that other signaling modalities may exist. The analysis of DC and PBMC activation by combinations of TLR agonists revealed that TLR2 agonists are able to block the induction of IP-10, IL-12p35, and IFN-γ but not IL-15 and IFN-β, by TLR3 and TLR4. TLR2 stimulation led to rapid release of IL-10 that is responsible for inhibition of IP-10 and IL-12p35 induction. Cross-talk between different TLRs may modify the primary responses of TLRs to their agonist, adding a further level of complexity to the regulation of innate immunity.

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Yersinia V antigen induces both TLR homo- and heterotolerance in an IL-10-involving manner

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The virulence antigen (LcrV) of pathogenic Yersiniae, a released non-lipidated protein, is able to 'silence' macrophages against a second stimulus with the TLR2-agonist zymosan A in a CD14- and TLR2-dependent fashion via IL-10 induction. Moreover, injection of LcrV into mice leads to LPS tolerance. In this study, we analysed the potential of LcrV as an inducer of TLR homo- and heterotolerance, *i.e.* tolerance against a challenge stimulus using the same or a different TLR as LcrV. We show that rLcrV induces a refractory state both in human MonoMac 6 and in murine peritoneal macrophages against itself (autotolerance) as well as against the TLR2- and TLR4-agonistic stimuli bacterial lipoprotein Pam_3Cys and LPS, respectively. This LcrV-induced immunotolerance is most likely not due to changes in TLR2 or TLR4 expression as shown by FACS analysis and Western blot. In contrast, LcrV-mediated IL-10 production is of prominent importance for LcrV-induced immunotolerance, since LcrV-induced auto-, homo- and heterotolerance is highly impaired in IL-$10^{-/-}$ macrophages. Moreover, the involvement of IL-10 in TLR homo- and heterotolerance induction seems to be a more general phenomenon as shown by experiments using different TLR agonists in IL-$10^{-/-}$ macrophages.

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Toll-like receptor 4-deficiency reduces atherosclerosis and alters plaque phenotype in ApoE null mice without changing circulating cholesterol levels

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Toll-like receptors (TLRs) play an essential role in recognition of micro-organisms and in the initiation of an innate immune response. Recently, TLR expression has been reported in murine and human lipid-rich atherosclerotic lesions but the causal role of TLR or the common TLR-signaling molecule MyD88 signaling in atherogenesis remains unclear. To determine whether TLR4 is causally related to atherogenesis we generated double knockout mice by crossing ApoE$^{-/-}$ with TLR4$^{-/-}$ mice. Mice were fed with a high cholesterol diet for 6 months. Extent of aortic atherosclerosis was measured in en-face aorta preparations after oil-red O staining. Aortic sinus lipid, macrophage content and COX-2 expression was measured after oil-red O staining and immunohistochemistry. TLR4-deficiency was associated with a nearly 25% reduction in aortic atherosclerosis, a 55% reduction in lipid content of aortic sinus plaques, reduced macrophage infiltration into aortic sinus plaques and reduced expression of COX-2 in aortic sinus plaques. TLR4-deficiency had no significant effect on cholesterol levels or lipid profiles. Serum concentration of the chemokine MCP-1 was significantly reduced in ApoE$^{-/-}$, TLR4$^{-/-}$ mice. These data suggest an important role for TLR4 and the innate immune system in the development of atherosclerotic plaques in a murine model of hypercholesterolemia induced atherosclerosis.

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Generalized chronic periodontitis is associated with single nucleotide polymorphisms (SNPs) of Toll-like receptor (TLR) 4 in humans

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Periodontitis is an inflammatory disease affecting the connective tissue surrounding the teeth, eventually leading to tooth loss. A number of pathogens implicated in the pathogenesis of chronic periodontitis have been shown to interact with Toll-like receptors (TLRs) leading to the release of pro-inflammatory cytokines, such as IL-1 and TNF-α, which have been shown to aggravate disease. In this study, we investigated whether genetic variations of TLR2 and TLR4 act as risk factors for periodontitis. We investigated 197 individuals suffering from generalized periodontitis for the presence of the polymorphisms Asp299Gly and Thr399Ile of TLR4 as well as Arg753Gln of TLR2. We found a significantly higher frequency of SNPs of TLR4 as compared to matched controls (odds ratio 3.650;, 95% CI 1.573–8.467; $P \leq 0.0001$), while no difference was observed for TLR2. TLR4 SNPs were correlated with chronic periodontitis (odds ratio 5.562; 95% CI 2.199–14.04; *P* 0.0001), but not with aggressive periodontitis. This observation was not dependent on smoking status. These data demonstrate that genetic variants of TLR4 act as risk factors for the development of generalized chronic periodontitis in humans.

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Human newborn blood monocytes demonstrate low TNF-α but high IL-6 responses to an array of Toll-like receptor ligands: role of a low-molecular weight plasma inhibitor of TNF-α release

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Human newborns suffer a relatively high frequency and severity of microbial infection and are prone to leukopenia in the face of overwhelming bacterial sepsis, yet the mechanisms underlying their susceptibility are incompletely defined. We have discovered a marked 1–3 log impairment in Toll-like receptor agonist-induced release of the pro-inflammatory cytokine TNF-α by human newborn blood monocytes in response to tri-acylated and di-acylated bacterial lipopeptides (TLRs 1/2 and 2/6, respectively), LPS (TLR4), flagellin (TLR5), and imiquimod (TLR7). In marked contrast, TLR agonist-induced release of IL-6, a cytokine with anti-inflammatory and Th2-polarizing properties, was greater in neonates than adults. Studies of the kinetics of cytokine release and a neutralizing anti-IL-6 mAb suggest that low TLR agonist-induced TNF-α release is not due the presence of high IL-6 concentrations. Remarkably, unstimulated newborn plasma confers substantially reduced BLP-, LPS-, and imiquimod-induced TNF-α release on adult monocytes, reflecting the presence in neonatal blood plasma of a soluble, low-molecular weight factor (< 10 kDa) that inhibits TLR agonist-induced TNF-α release. The polarization of neonatal mononuclear cells towards IL-6 release coupled with limited TNF-α release to many TLR agonists is likely to profoundly modulate innate and adaptive immune responses in human newborns.

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Deficient cytokine responses in human IRAK-4 mutant primary cells are secondary to dysregulated TIR-IRAK-TRAF6 signalling and defective, cell-type specific MAPK responses

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Pattern recognition by Toll-like receptors (TLRs) is important in initiating protective immune responses. IRAK-4 is required for various responses to TLR and interleukin-1 receptor (IL-1R) signalling via Toll-IL-1R (TIR) domains, but its precise role remains unclear. We describe a child homozygous for the Q293X mutation in IRAK-4, presenting with recurrent *Streptococcus pneumoniae* bacteremia. We demonstrate that despite profoundly deficient cytokine responses, our patient's cells retain partial TIR signalling. We describe cell-type specific defects in response to LPS, IL-1β and TNF-α in peripheral blood mononuclear cells and primary fibroblasts, suggesting possible differences in the role of IRAK-4 in these cell types. Defective cytokine responses to these agonists were observed to fall into two categories: (i) failure of transcription (*e.g.* IL-6); and (ii) transcription with failure to translate (*e.g.* IL-8), and were correlated with phosphorylation of IκBα, p38 and JNK, and NF-κB nuclear translocation. Finally, using IRAK-4 deficient leukocytes, we demonstrate a hyperphosphorylative capacity of IRAK-1 in unstimulated cells, diminished in response to LPS exposure, and abnormal osteoclastogenesis in monocytes exposed to MCSF in the absence of RANKL. Thus, we present evidence of dysregulated TIR-IRAK-TRAF6 signalling secondary to IRAK-4 mutation, with defective, cell-type specific MAPK responses and consequent deficient cytokine responses.

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Innate immune responses of well-differentiated human airway epithelia – an informatics approach

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We prepared and sequenced a cDNA library from well-differentiated human airway epithelia and used this focused gene set to make a custom Affymetrix genechip. This ~23,000 gene set includes ESTs not represented on commercial arrays. Primary air-liquid interface epithelial cultures were treated for 24 h with a cocktail of IL-1, TNF-α, and IFN-γ and mRNA harvested and prepared for microarray hybridization. Expression profiling revealed constitutive or inducible expression of TLRs 1, 2, 3, 4, and 5. Expression levels for TLRs 2, 3, and 4 were induced in response to cytokines. Many genes with known or putative innate immune function were identified with constitutive or inducible expression. Two lipid transfer/LPS binding protein family members, PLUNC and LPLUNC1, showed constitutive, high level expression. Genes expressed in basal and induced conditions included MHCI, MHCII, uteroglobin, lipocalin-2, SLPI, β_2-microglobulin, and C3. Inducible genes included STAT1, HBD-2, IL-8, CCL8, CCL20, CXCL9, CXCL10, SERPINA3, SERPINB7, and DUOX2. Of 4330 ESTs not represented on commercial arrays, 1338 were detected with a present call in both control and treated airway epithelia. Large scale expression profiling in airway epithelia provides novel insights into the repertoire of responses of this mucosal surface, reflecting its dynamic role in innate immunity.

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Flow stress inhibition of TLR2 expression in human coronary artery endothelial cells

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Innate immune system activation is associated with atherosclerotic lesion development. The specific sites of lesion development are believed to be defined by the shear stress of blood flow. Consequently, we investigated the responsiveness of human coronary artery endothelial cells (HCAECs) to Toll-like receptor (TLR) 2 and TLR4 agonists in an *in vitro* model of chronic laminar flow. HCAECs under chronic laminar flow were found to be normally responsive to LPS (and TNF) in terms of E-selectin expression but were found to be hyporesponsive to stimulation with the specific TLR2 ligands macrophage activating lipopeptide-2, PAM_2-Cys and Lip19; this was observed to be due to down-regulation of TLR2 transcription and protein expression. We found that laminar flow induced SP1 serine phosphorylation by protein kinase CK2 and thereby blocked SP1 binding to the TLR2 promoter, which is required for TLR2 expression. This regulatory mechanism also blocked LPS and TNF induced TLR2 up-regulation in HCAECs and could be important for suppression of other flow sensitive endothelial proteins. These results extend the role of flow in controlling endothelial responsiveness. Given the current evidence that Toll-like receptors are pro-atherogenic, flow suppression of TLR2 expression may be atheroprotective.

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TLR4 display on human monocytes is increased in septic patients

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Sepsis is a serious condition, most often occurring as a complication of bacterial infections. The Toll-like receptors (TLR) 2 and TLR4 have been identified as key molecules in response to Gram-positive and Gram-negative bacteria. This study aimed to assess possible alterations of the surface display of TLR2 and TLR4 on monocytes and granulocytes derived from patients with sepsis in comparison with healthy controls. We have utilized flow-cytometry to determine the presence of TLR2 and TLR4 on the cell surface at baseline and in response to LPS (40 ng/ml) *in vitro*. We found no significant differences of TLR2 display on monocytes and granulocytes from septic patients compared to controls. Surface display of TLR4 on monocytes from septic patients at baseline was significantly higher than in healthy controls but there was no further response to LPS, whereas controls showed a significant increase of TLR4 display on the cell surface after LPS stimulation. In contrast, TLR4 baseline cell surface display on granulocytes was significantly lower in septic patients than in controls and there was no response to LPS in both groups. Our data suggest a complex relationship between TLR4 display and bacterial challenge *in vivo* and *in vitro*.

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The expression of TLR2 and TLR4 during upper abdominal surgery

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We have demonstrated that surgical stress rapidly induced transient hyporesponsiveness of blood cells to endotoxin and that monocyte mCD14 and HLA-DR expression reduced from the early period of surgery under general anesthesia. The aim of this study was to examine the expressions of Toll-like receptor (TLR) 2 and TLR4 during surgery. After obtaining approval from our committee on human research and informed consent, the blood samples were obtained from the patients of upper abdominal surgery before incision (Pre-OP), at 2 h after incision (OPE2h), the end of surgery (Post-OPE) and 24 h

after incision (OPE24h). The expression of TLRs was measured by monoclonal antibody staining and flow cytometry. Both expression of TLR2 and TLR4 significantly decreased from OPE2h and recovered slightly at OPE24h. These results may in part explain the impairment of endotoxin sensitivity and host-defense mechanisms during an operation.

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Soluble CD14, a new sepsis diagnostic marker

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Introduction: For detection and detection of the severity of sepsis, we present a new form of soluble CD14, named sCD14-ST, which was first discovered as a small molecular weight of CD14 specific for sepsis.
Methods: Using of antigens against 16 amino acid residue of h-CD14, sCD14-ST were found out recently. sCD14-ST ELISA kit was developed as a two-step sandwich method using two types of anti-CD14 antibodies, and this kit detects only small size sCD14 specifically. At the same time, CRP, endotoxin, IL-6, PCT or SOFA scores were evaluated to make utility contrast.
Results: Plasma levels of sCD14-ST were compared with sepsis (55 specimens), SIRS (80 specimens), or healthy control (75 specimens). The median concentration of control, sepsis or SIRS were 19.6 (ng/ml), 723 and 41.4, respectively. By receiver operating characteristic curve analysis, sCD14-ST was the best evaluating ability for sepsis diagnosis (sepsis vs normal + SIRS; ROC area 0.865) in comparison with CRP, endotoxin, IL-6, PCT or SOFA scores. By clinical kinetics analysis, sCD14-ST was waked up in early stage (< 6 h after onset), declined on parallel with SOFA scores. *Conclusion*: sCD14-ST is a useful parameter for diagnosis.

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In carrageenan-primed endotoxin shock model mice, the serum level of soluble CD14 correlates to progression of the lethal toxicity

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CD14 is expressed on monocytes and neutrophils and acts as recognizing protein for lipopolysaccharide (LPS). CD14 enhances sensitivity to LPS and involves to onset of endotoxin shock. There are some reports suggesting the relationship between soluble CD14 (sCD14) and inflammatory diseases, but only few evidences are reported on serum levels of sCD14 especially in mice. We constructed the assay system that measures the serum level of sCD14 by Western blotting. Using this system, we elucidated that serum level of sCD14 increased in endotoxin shock model mouse, which are primed and triggered with iota-carrageenan and LPS, respectively. The serum level of sCD14 increases along with progression of the disease in the state. And all mice died within a day. On the other hand, in the mouse that administered saline, carrageenan or LPS alone, we could not observe remarkable increase of the serum level of sCD14. These groups did not show lethal toxicity. The results may suggest that increasing serum level of sCD14 conjecture the lethal toxicity in carrageenan-primed endotoxin shock.

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Effect of nitric oxide on β-glucan/indomethacin induced septic shock

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We have previously shown that repeated administration of non-steroidal anti-inflammatory drugs (NSAIDs) to β-glucan, a biological response modifier, administered mice induced severe lethality. Lethality was strongly related to the translocation of enterobacterial flora to the peritoneal cavity and mal-adjustment of the cytokine network. Several reports suggest nitric oxide

(NO) plays an effective or detrimental role in septic shock. In the present study, we examined the effect of NO, an inflammatory mediator, on β-glucan/indomethacin (IND) induced septic shock by inhibiting its synthesis with N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective NO synthase (NOS) inhibitor. β-Glucan/IND treated mice enhanced lethal toxicity by administering L-NAME. Aminoguanidine, a selective inducible NOS inhibitor, induced similar toxicity. β-Glucan/IND and L-NAME treated mice contained a significantly higher number of bacteria in various organs. Additionally, TNF-α, IL-1β, IL-6 syntheses were enhanced in peritoneal exuded cells culture and mice lead to liver injury. These results suggest that significant failure of bactericidal activity of macrophages by administration of NOS inhibitor enhanced rate of enterobacterial invasion to peritoneal cavity and resulting survival, and then mice result in systemic inflammatory response syndrome. The production of NO, therefore, provides a protective function in β-glucan/IND induced sepsis.

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Comparison between dobutamine and terbutaline in multiple organ dysfunction syndrome (MODS) of peritonitis-induced septic shock in rats

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Dobutamine (DB) and terbutaline (TB) are well used in clinics, in particular, dobutamine has been used in patients with shock. Septic shock-induced MODS was performed by cecal ligation and puncture (CLP) in the Wistar rat. We evaluated effects of DB (a β_1-adrenoceptor agonist) and TB (a β_2-adrenoceptor agonist) in CLP-induced sepsis rats. The changes of hemodynamics, blood sugar, rectal temperature, blood gas (pH, PCO_2, PO_2, base excess (BE), HCO_3^-), electrolytes, hepatic (GOT, GPT, ALB, TP, LDH) and renal (BUN, CRE) function as well as the plasma nitrate (an indicator of NO) were examined. Animals were divided into six groups, *i.e.* sham operation (SOP), SOP+DB, SOP+TB, CLP, CLP+DB and CLP+TB. SOP or CLP with or without DB (0.3 mg/kg/min, at 3 h) or TB (0.3 mg/kg, at 3 h and 9 h) were administered intravenously. At 18 h after CLP, animals were sacrificed and lungs, livers and kidneys were excised to perform the pathological studies, iNOS expression analysis and superoxide analysis, whereas thoracic aortas were used to analyze superoxide and the vascular reactivity to norepinephrine (NE) and acetylcholine. Our results showed that TB, but not DB: (i) attenuated hyporeactivity to NE (both *in vivo* and *ex vivo*) and delayed hypotension; (ii) reduced GPT, GOT, LDH, CRE, BUN and hypoglycemia and increased ALB and TP; and (iii) diminished plasma NO and tissue O_2^- levels, but had no significant effect on the changes of electrolytes in rats treated with CLP. Thus, TB attenuates liver and kidney dysfunction and circulatory failure in rats with peritonitis-induced septic shock.

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Thaliporphine increases survival rate and prevents multiple organ dysfunction in LPS-induced endotoxemia

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We have investigated whether a phenolic aporphine alkaloid from the Chinese herb thaliporphine, an antioxidant with α-1 antagonist activities, has beneficial hemodynamic effects in endotoxemic rats and to delineate the mechanism by which thaliporphine ameliorates the circulatory failure, multiple organ dysfunction and mortality caused by endotoxic shock. Treatment of rats with thaliporphine significantly attenuated the delayed hypotension. But only high dose (1 mg/kg) of thaliporphine could decrease the LPS-induced tachycardia. LPS induced increase of nitric oxide and superoxide anion production was prevented by pretreatment with high-dose thaliporphine. Low dose (0.3 mg/kg) of thaliporphine significantly decreased the NOS II protein expression induced by LPS in lung. LPS treatment resulted in a rapid increase of TNF-α and IL-10 to peak levels at 60 min. However, only the increase of TNF-α was significantly reduced by pretreatment with thaliporphine. Thaliporphine could prevent multiple organ dysfunction in LPS-rats. On the other hand, it also had partial trend to stable the blood glucose level and to increase rectal temperature lowered by LPS. In survival study, our results demonstrated that thaliporphine increased 72-h survival rate dose dependently. These results show that thaliporphine prevents endotoxin-induced circulatory failure, multiple organ dysfunction, and increases survival rate. These beneficial effects may be related to the suppression of TNF-α, nitric oxide and superoxide anion production.

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Effect of lipopolysaccharide(LPS) on the pharmacokinetics of digoxin in rats

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Purpose: To understand the pharmacokinetics for drug treatment under various pathological conditions, we developed an endotoxin shock model and evaluated the pharmacokinetics of digoxin in the LPS-treated rats.
Method: LPS (5 mg/kg) was injected intraperitoneally into Wistar/ST rats; after 1–5 days, digoxin (0.1 mg/kg) was administrated orally to the rats with or without LPS pre-treatment. Blood samples were collected after administration of digoxin, and plasma digoxin concentration was measured by fluorescence polarization immunoassay.
Results: In this model, blood pressure, body temperature, TNF-α, and IL-1β were recovered to the original level within 1 day after LPS-treatment. However, on days 1 and 2 after LPS-treatment, the peak plasma concentration (C_{max}) of digoxin was significantly higher (1.4- and 1.6-fold, respectively), and area under the concentration time curve (AUC) was significantly higher (1.9- and 2.3-fold, respectively) than the untreated control. In addition, oral clearance (CL/F) decreased significantly (0.54- and 0.35-fold, respectively than the control). On day 3, CL/F decreased significantly (0.44-fold), while AUC and C_{max} increased slightly. However, on day 5, no difference was observed any more in C_{max}, AUC, or CL/F to the control.
Conclusion: Digoxin concentration is being increased under endotoxin shock even later than when the indicators of the shock become recovered.

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Cardiovascular effects of endotoxin derived from *Escherichia coli* vs *Klebsiella pneumoniae*

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The purpose was to compare cardiovascular effects of endotoxin (LPS) derived from *Escherichia coli* and *Klebsiella pneumoniae*, and examine oxidative stress. Wistar rats were anesthetized by pentobarbital. Echocardiogram and blood pressure (BP) were monitored after *E. coli* (10 mg/kg), *K. pneumoniae* (2 mg/kg), or saline. *K. pneumoniae* induced abrupt fall of BP immediately, while *E. coli* induced two-peaked fall of BP. Both *K. pneumoniae* and *E. coli* induced a decreased LV dimension, an increased LV wall thickness and an enhanced LV fractional shortening. Echocardiographic findings were normalized at 4 h after *K. pneumoniae*, while these persisted until 7 h after *E. coli*. Both echocardiographic changes and plasma cardiac troponin T levels were greater in rats with *K. pneumoniae* than in rats with *E. coli*. Both edema and congestion were evident in LV myocardium in rats with *K. pneumoniae*. Glutathione redox ratio showed increased oxidative stress in liver in rats with *K. pneumoniae*, in spite of no change in LV myocardium in rats with either *K. pneumoniae* or *E. coli*. LPS from *K. pneumoniae* and *E. coli* induced similar cardiovascular alterations characterized by a decrease in LV preload and a comensatory enhanced LV systolic function, while the extents were greater in rats with *K. pneumoniae*. Oxidative stress appears to play little role in LPS-induced cardiovascular effects.

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Profound functional and structural cardiovascular alterations induced by endotoxin derived from *Pseudomonas aeruginosa*

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Purpose: We examined functional and structural cardiovascular alterations induced by endotoxin (LPS) derived from *Pseudomonas aeruginosa*.

Methods and Results: LPS derived from *P. aeruginosa* (2 mg/kg, $n = 22$), or saline ($n = 22$) was administered to 8-week-old male Wistar rats. Echocardiogram, arterial pressure (AP), plasma concentrations of tumor necrosis factor-α (TNF-α), and troponin T (TnT) were monitored serially. Left ventricular (LV) systolic function as assessed by LV fractional shortening (FS) was enhanced modestly early after LPS administration. Subsequently, LVFS was progressively depressed after 4 h and accompanied by LV dilatation as well as elevated AP. Severe LV structural alterations were evident characterized by an edema, congestion, and intramyocardial bleeding. Plasma TNF-α level was elevated from 1 to 4 h after administration(0.5 h, 127.9 ± 34.75 pg/ml; 1 h, 882.1 ± 202.34 pg/ml; 2 h, 317.2 ± 119.90 pg/ml;, 3 h, 294.9 ± 119.55 pg/ml; 4 h, 47.2 ± 18.49 pg/ml). Plasma TnT level was increased (baseline, 0.005 ± 0.0 ng/ml; 1 h, 0.005 ± 0.0 ng/ml; 4 h, 0.160 ± 0.005 ng/ml; 7 h, 0.783 ± 0.531 ng/ml control not detected at any points). Glutathione redox ratio was unchanged after administration of LPS.
Conclusion: Progressive functional and structural LV alterations was induced by LPS derived from *P. aeruginosa* in spite of the absence of activated oxidative stress.

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Components of diesel exhaust particles diversely enhance acute lung injury related to lipopolysaccharide

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Diesel exhaust particles (DEP) synergistically aggravate acute lung injury related to lipopolysaccharide (LPS) in mice. We examined the effects of the organic chemicals (DEP-OC) and the residual carbonaceous nuclei (washed DEP) in DEP to identify the responsible components for synergistic aggravation. DEP-OC or washed DEP enhanced the infiltration of neutrophils in bronchoalveolar lavage fluid in the presence of LPS. Washed DEP combined with LPS synergistically exacerbated pulmonary edema and induced alveolar hemorrhage, which was concomitant with the increase in interleukin-1β, macrophage inflammatory protein-1α, macrophage chemoattractant protein-1, and keratinocyte chemoattractant in lung, whereas DEP-OC combined with LPS did not. The gene expression for Toll-like receptors 2 and 4 was increased by the combined treatment with washed DEP and LPS. These results suggest that washed DEP predominantly contribute to the aggravation of LPS-related lung injury rather than DEP-OC. The aggravation may be mediated through the expression of pro-inflammatory molecules and Toll-like receptors.

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Effects of components of diesel exhaust particles on systemic inflammatory response related to acute lung injury in mice

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Previously, we have demonstrated the enhancement of acute lung injury related to bacterial endotoxin by components of diesel exhaust particles (DEP; Yanagiswa *et al*. *Thorax* 2003; **58**: 605). The present study elucidated the effects of DEP components on systemic inflammatory response with circulatory translocation of lipopolysaccharide (LPS) after the intratracheal challenge of LPS *in vivo*. ICR mice were administered intratracheally of vehicle, LPS, organic chemicals (DEP-OC), residual carbonaceous nuclei (washed DEP), DEP-OC + LPS, or washed DEP + LPS. Fibrinogen and LPS levels in circulatory plasma were evaluated. LPS levels were evaluated using a kinetic LAL assay with endotoxin-specific chromogenic reagent (Endospec ES-test MK). LPS challenge significantly increased both fibrinogen and LPS levels as compared to vehicle challenge. DEP-OC + LPS challenge further increased fibrinogen levels with significance and LPS levels without significance as compared to LPS challenge. On the other hand, washed DEP + LPS instillation significantly increased both values as compared to LPS instillation. In conclusion, inhalation of DEP components aggravate translocation of LPS from respiratory system to circulatory system, which is prominently shown by washed DEP rather than DEP-OC. DEP components may influence systemic inflammatory responses.

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LK-4, an analogue of dextromethorphan, prevents circulatory failure in rats with endotoxemia

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Dextromethorphan (DM), an antitussive agent, has been claimed to have anti-inflammatory and immunomodulatory effects. LK-4, an analogue of DM, possesses greater potency of anticonvulsant/neuroprotective actions than DM has. Thus, the aim of this study was to evaluate the effects of LK-4 on sepsis induced by i.v. administration of LPS in anesthetized Wistar rats and by intraperitoneal administration to conscious ICR mice. LK-4 was treated at 30 min after LPS administration. DM (3 and 5 mg/kg, i.v.) significantly attenuated the deleterious hemodynamic changes (*e.g.* hypotension and tachycardia) in rats treated with LPS. Meanwhile, LK-4 (3 mg/kg) significantly inhibited the elevation of plasma TNF-α and IL-10 levels, as well as GOT and GPT, BUN and creatinine values caused by LPS. An overproduction of NO and superoxide anions by LPS was also reduced by LK-4. The infiltration of neutrophils into the lung and liver from the 6-h LPS-treated rats was also reduced by LK-4. LK-4 also significantly improved the survival rate in mice treated with a lethal dose of LPS. In conclusion, the beneficial effects of LK-4 on LPS-induced sepsis result from its anti-inflammatory and antioxidant effects. LK-4 may act as a potential therapeutic agent for sepsis in the future.

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Endotoxin clearance and its relationship to multiple organ disturbances in rats with liver cirrhosis

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Little is known about the relation of endotoxemia and pro-inflammatory cytokinemia to multiple organ failure in liver cirrhosis. Clearance of endotoxin and release of cytokines by various macrophages were studied in an experimental model. Male Sprague-Dawley rats were given 0.04% thioacetamide orally for 6 months. Organ distribution of infused [^{3}H]-endotoxin was analyzed at 30 min or at 24 h. Secretion of tumor necrosis factor (TNF)-α and IL-10 by Kupffer cells, splenic and alveolar macrophages (5 x 10^4 cells/ml) stimulated with 100 ng/ml lipopolysaccharide (*Escherichia coli* O55:B5) from cirrhotic and control rats were determined. In cirrhotic rats, endotoxin clearance was disturbed and serum TNF-α and creatinine were elevated at 24 h. TNF-α secretion of alveolar macrophages was significantly greater than that of other macrophages both in cirrhotic and control rats. TNF-α and IL-10 release by the Kupffer cells were decreased in cirrhotic rats compared with control rats. TNF-α release by alveolar and splenic macrophages was increased in cirrhotic rats, although IL-10 release were not changed. In liver cirrhosis with bacterial translocation or bacteremia, disturbed endotoxin clearance together with imbalance between pro-inflammatory and anti-inflammatory cytokine release by macrophages may play an important role in the development of multiple organ disturbances.

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Role of TGF-β1 in endotoxin-induced hepatic failure after 90% hepatectomy in rats

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Postoperative infections after hepatectomy sometimes lead to lethal hepatic failure, while the mechanism of the hepatic failure remains unexplained. Wistar rats underwent 90% hepatectomy, and were then divided into three groups: (i) the SAL group, injected with normal saline; (ii) the LPS group, injected with lipopolysaccharide (LPS) every day for one week; and (iii) the LPS plus TGF-Ab (LPS+TGF-Ab) group, injected with LPS with anti-transforming growth factor β1 (TGF-β1) antibody. We investigated survival rates, TGF-β1 expression in the liver, liver regeneration by proliferating cell nuclear antigen labeling index, hepatocyte apoptosis by single stranded DNA labeling

index, and perisinusoidal fibrosis using Masson's trichrome staining. In survival rates, the LPS group (30.4%) was significantly lower than the SAL group (84%) and tended to be lower than the LPS+TGF-Ab group (49.4%). Liver regeneration in the LPS group was significantly lower than in the other groups. In the LPS group, apoptosis of hepatocyte and perisinusoidal fibrosis were significantly more remarkable, and TGF-β1 expression was significantly higher than in the SAL group. TGF-β1 enhanced by LPS plays an important role in the mechanism of hepatic failure by infections after hepatectomy, especially in inhibition of liver regeneration, and induction of hepatocyte apoptosis and perisinusoidal fibrosis.

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Gene expression of liver regeneration after partial hepatectomy in mice lacking type 1 tumor necrosis factor receptor

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Aim: To investigate whether TNF-α is necessary for hepatocyte proliferation, we studied liver regeneration after partial hepatectomy in mice lacking TNF receptor-1.
Methods: TNF receptor type-1 knockout mice and wild-type mice were subjected to two-thirds partial hepatectomy (PHx). Riken cDNA microarray analysis was performed on liver samples from mice undergoing PHx to compare clearly differentiated mouse PHx models (TNFR-1 knockout mice – K group, and wild-type mice – W group).
Results: The cumulative survival after PHx in K group was lower than in W group. The time to regain the liver weight in K group was 14 days and 7 days in W group. The Ki67 expression in K group at 4 days was lower than in W group. LPS, Toll-like receptor 4 precursor and MAPK 8 interacting protein in K group was higher than in W group. For cell cycle-regulated genes, cyclin D1, NF-κB light chain and TNF receptor super family membrane 1a in K group was lower than in W group.
Conclusions: Lack of TNF-α signaling through TNFR-1 delays the initiation of liver regeneration after partial hepatectomy. It is suggested that hepatocyte proliferation can be induced by an LPS-JNK pathway instead of TNF-α and IL-6.

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Lack of manipulations of gastrointestinal tract predominantly attenuated postoperative endotoxemia and further immune response after laparoscopic surgery in comparison to open surgery

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Our previous studies demonstrated that the loss of gut barrier function together with endotoxin translocation might be the major cause for postoperative acute phase reactions after abdominal surgery. Small bowel manipulation during digestive surgery initiates an endotoxin translocation and further inflammatory response in the intestine. The less surgical manipulation of laparoscopic procedures may minimize trauma to the peritoneum; therefore, it could be decreasing the gut's inflammatory response after surgery. We applied a swine gastrectomy model with different surgical techniques (OPEN: distal gastrectomy with finger manipulation; NMP: operated as in the OPEN without finger manipulation; LADG: laparoscopically assisted gastrectomy) to compare the postoperative inflammatory reactions in the local or systemic sites. Plasma levels of either endotoxin assayed with the *Limulus* amebocyte lysate test or pro-inflammatory cytokines (IL-1β, IL-6) determined by ELISA of OPEN were significantly elevated compared with those of NMP or LADG. The cytokine mRNA levels were measured by LightCycler Real-time PCR and were increased in the OPEN compared with the other two groups. This study demonstrates for the first time that the lack of surgical manipulation during the laparoscopic surgery plays a pivotal role for decreasing the postoperative endotoxemia and further inflammatory response.

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Inhibition of experimental asthma by innate immunity: role of indoleamine 2,3-dioxygenase

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Epidemiological evidence supports that reduced exposure to microbial stimuli plays a major role in the increasing prevalence of Th1 (autoimmune diseases), and Th2 (allergic asthma) in Westernized countries. The mechanism for this observation has not yet been completely determined. In this study, we show that the administration of certain Toll-like receptor (TLR) ligands induces high levels of indoleamine 2,3-dioxygenase (IDO), which is the rate-limiting enzyme of tryptophan catabolism, in various organs via the activation of innate immunity. Pulmonary IDO activity induced by immunostimulatory sequence oligodeoxynucleotides (ISS-ODN), TLR-9 ligand, inhibits Th2-driven experimental asthma by induction of apoptosis in the effecter Th2 cells in the lung. IDO activity expressed by resident lung cells, *i.e*. epithelial cells, rather than by pulmonary dendritic cells (DCs) suppressed lung inflammation and airway hyper-reactivity. IDO activity induced by ISS-ODN in lungs also inhibits Th1-mediated lung inflammation. Our findings provide evidence for a potential pathway by which the activation of innate immunity can inhibit adaptive Th cell responses.

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Burn injury and Gram-positive infection modulate PMN responses and microvascular permeability to exacerbate host morbidity/mortality

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Early inflammatory responses in burn patients (PMN activation and vascular permeability) evidently serve adaptive roles and allow for resolution of inflammation without flagrant patient morbidity and mortality. However, infections superimposed on burn-induced inflammation may cause such responses to contribute to multiple organ/system failure and death. We have examined blood and/or tissue PMN O_2^-/elastase expression, apoptosis and vascular albumin permeability in rat models of burn injury (30% TBSA, B), intra-abdominal Gram-positive (*E. faecalis*, EF) sepsis, and a burn plus *E. faecalis* (B+EF) injury condition. While there was no significant effect of B or EF on animals' cardiovascular responses (CVRs) or survival, B+EF resulted in gross disturbance in CVRs and a substantial animal mortality. We found f-MLP-stimulated O_2^- production to be elevated equally in PMNs from B and B+EF but not EF rats. Albumin transport (permeability) across intestinal/lung vasculature was higher in EF and B+EF but not in B rats. These findings suggest that morbidity and mortality in B+EF rats was a result of combination of exaggerated vascular permeability (mainly resulting from EF) and PMN activation (resulting mainly from B). Thus morbidity and mortality in burn injury with Gram-positive infectious complication may be due to combination of effects of PMN hyperactivation and disturbed vascular permeability that individually may be innocuous.

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Hepatitis C virus

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Hepatitis C is one of the five identified viruses which attack and damage the liver and is the most serious of all. However, hepatitis C is spread through receiving organs from donors whose blood contains hepatitis C virus, getting pricked with a needle that has infected blood on it, frequently being exposed to blood products such as those used to treat hemophilia. Engaging in high risk of sexual behavior, using an infected person's toothbrush that may have blood on it, injecting illegal drugs.

An infection of hepatitis C can be recognized by the following symptoms: loss of appetite, fatigue, fever-nausea, dark-yellow urine and jaundice.

The chronic hepatitis C lies dormant for some time before symptoms appear and some patients with hepatitis C will have symptoms that prompt them to seek medical attention, *i.e.* diarrhea, tenderness in the right upper quadrant of the body, and pain in the stomach.

The treatment for hepatitis patients is interferon-α, roferon-A. Patients of hepatitis C must inject interferon themselves, 3 times a week; usually the drug reduces hepatitis C virus to low levels in the blood. However, combination therapies rather than single drug may offer the best potential for effective treatment. A major study area is development of cell culture through which scientists can study the virus outside the human body.

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Rapid detection of botulinum neurotoxin-F by capillary electrophoresis with laser-induced fluorescence detector

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A rapid assay was developed using the proteolytic activity of Botox/F to detect this toxin from biological and environmental samples. The assay was developed using capillary electrophoresis (CE) to separate a 5-carboxyfluorescein-N-hydroxysuccinimide ester (5-CF) labeled peptide that corresponds to residues 37–75 of VAMP protein and its cleavage product by the Botox/F toxin. The optimum conditions for cleavage were found to be 34°C and pH 7.6. CE separation was performed on a Prince CE-750 system equipped with ZETALIF laser-induced fluorescence detector. Using a fused silica capillary of 75 μm ID x 365 μm OD x 80 cm length and a borate buffer (50 mM, pH 9.2) with 0.3% (w/w) polyethylene glycol, the 5-CF labeled peptide and its cleavage product were separated within 15 min. The sensitivity of this method was found to be 0.05 ng/μl. The total analysis time for one sample is less than 1 h, which provides significant improvements in terms of small sample volume per analysis, speed and sensitivity compared with the standard mouse neutralization assay. Furthermore, since this method is based on the proteolytic activity of Botox, it can directly tell whether the samples contain active Botox/F. This could be an additional advantage when compared with the immunoassay methods.