# Toll-like Receptor 4-Deficiency Reduces Atherosclerosis and Alters Plaque Phenotype in Apo E Null Mice

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**Background:** Toll-like receptors (TLR) play an essential role in recognition of microorganisms 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.

**Objective and Methods:** To determine whether TLR4 is causally related to atherogenesis we generated double knockout mice by crossing $ApoE^{-/-}$ with $TLR4^{-/-}$ mice on C57Bl6 background. Both, double knockout and $ApoE^{-/-}$ mice were fed a high cholesterol diet and sacrificed at six month of age. Extent of aortic atherosclerosis was measured in en-face preparations of descending aorta after Oil-red O staining using computerized morphometry. Aortic sinus lipid, macrophage content and COX-2 expression was measured after oil-red O staining and immunohistochemistry. Systemic cytokine and chemokine concentrations were measured by ELISAs.

**Results:** TLR4-deficiency was associated with a nearly 25% reduction in aortic atherosclerosis (% aortic surface covered by plaque). Furthermore, TLR4-deficiency was associated with a 55 % reduction in lipid content of aortic sinus plaques (% of plaque area). TLR4-deficiency had no significant effect on plasma cholesterol levels or lipid subfractions by HPLC. Immunohistochemistry also showed reduced expression of COX-2, an inflammatory gene, in aortic sinus plaques of $ApoE^{-/-}$;$TLR4^{-/-}$ mice as compared to profound COX-2 expression seen in $ApoE^{-/-}$;$TLR4^{+/+}$ mice. Macrophage infiltration in $ApoE^{-/-}$;$TLR4^{-/-}$ mice was also significantly reduced by 65 % compared to control mice. Serum concentration of the chemokine MCP-1 was significantly reduced in the double knockout mice compared to control mice.

**Conclusions:** 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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# Influence of endotoxic activity of *Helicobacter pylori* LPS on physicochemical parameters

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The Gram-negative bacterium *Helicobacter pylori* (*Hp*) is recognized as a primary cause of chronic gastritis and is associated with development of peptic ulcers and gastric adenocarcinoma. The properties of LPS within the outer membrane, which has been found to induce significantly lower immunological responses than enterobacterial LPS, have been suggested to contribute to the ability of *Hp* to cause persistent infection compared to more aggressive pathogens. We performed a physico-chemical analysis of rough and smooth-form LPSs from *Hp*, its lipid A, and dephosphorylated forms of LPS and lipid A. In particular, we have investigated the acyl chain melting behaviour of the different samples using Fourier-transform infrared spectroscopy (FTIR), the inclination angle of the lipid A backbone plane against the membrane plane by dichroic measurements via attenuated total reflectance (ATR) FTIR with polarized light, the aggregate structure of some samples by synchrotron radiation X-ray diffraction, and the LBP-induced intercalation of the samples into a phospholipid membrane (corresponding to the composition of the macrophage membrane) by fluorescence resonance energy transfer spectroscopy (FRET). These results have been correlated to those obtained in a bioassay, endotoxin-induced production of cytokines in human mononuclear cells, and in a CHO cell system for testing TLR-2 and -4 reactivity. The endotoxins from *Hp* show in several aspects a similar behaviour to those of enterobacterial endotoxins with respect to intercalation and tendency for particular aggregate structures, but also some deviations such as phase transition behaviour and low TLR-reactivity. These data may be a basis for an understanding of the reduced cytokine-producing capacity of these compounds as compared to those from an enterobacterial source.

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# *Haemophilus parainfluenzae* lipooligosaccharide: analysis of structure, toxicity, and role in infectious disease

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*Haemophilus parainfluenzae* is a Gram negative coccobacillus frequently associated with the oropharaynx of human hosts. *H. parainfluenzae* has been implicated in several human diseases including infective endocarditis, biliary disease, and exacerbations of chronic obstructive pulmonary disease (COPD). Although *H. parainfluenzae* had been isolated from clinical samples, it has not been widely accepted as a human pathogen due to its role as normal flora. The ability of *H. parainfluenzae* to persist in a host and cause disease may be due in part to the dominant cell surface antigen, lipooligosaccharide (LOS). *H. parainfluenzae* LOS characterization was performed through toxicity studies, adherence assays, and cytokine induction studies. These studies revealed no significant differences when compared to nontypeable *Haemophilus influenzae* 2019, a known respiratory pathogen, suggesting that *H. parainfluenzae* LOS may be an important virulence factor of the bacterium. Elucidation of *H. parainfluenzae* LOS structure revealed a terminal D-glycero-D-manno heptose, which is absent from *H. influenzae* LOS and could play a role in adherence to host cells. Genetic and structure analysis has demonstrated the presence of phosphorylcholine, a molecule involved in surface restructuring, on the carbohydrate portion of *H. parainfluenzae* LOS. The *lic* genes from *H. parainfluenzae* have been identified and characterized.

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# Characterization of monoclonal antibodies to soluble MD-2 and development of an ELISA assay for detection of soluble MD-2 in human sera.

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MD-2 is a 20 Kd glycoprotein which binds to the extracellular domain of TLR4, a member of toll-like receptor protein essential for the innate immune response to lipopolysaccharides. In transfected cells over expressing MD-2, it exists as a complex with TLR4 on the cell surface as well as a soluble protein secreted into the medium. Soluble MD-2 (sMD-2) binds LPS tightly and confers LPS responsiveness to cells expressing TLR4 alone. Here we developed a panel of monoclonal antibodies to sMD-2. They recognized at least 8 distinct epitopes on sMD-2 by several different criteria. None of these Mabs binds to cell surface, which suggests that the structure of sMD-2 and MD-2 that complexes with TLR4 may be different. We developed an antibody captured ELISA assay for the detection of sMD-2 and use this assay to measure sMD-2 in human sera. We found that normal human sera contained between 0 to 60 ng/ml of sMD-2. We measured the level of sMD-2 in sera of normal patients and patients that were admitted to ICU clinics. We found that the ICU patients had significantly more sMD-2 in their sera than the normal controls. Significantly difference was found between the MD-2 serum level of sepsis patients and normal patients. The Mabs to sMD-2 are valuable reagents to study sMD-2 which could play significant role in innate immunity to microbial infections.

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# The rapid identification of novel scaffolds for endotoxin-sequestering compounds

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Lipopolysaccharides (LPS), otherwise termed 'endotoxins', are an integral part of the outer leaflet of the outer-membrane of Gram-negative bacteria. Lipopolysaccharides play a pivotal role in the pathogenesis of 'Septic Shock', a major cause of mortality in the critically ill patient, worldwide. The sequestration of circulatory endotoxin may be a viable therapeutic strategy for the prophylaxis and treatment of Gram-negative sepsis. We have developed a novel high-throughput screening method for the rapid identification of endotoxin-neutralizing agents amongst a focused library comprising of pre-selected candidates, each of which possess the pharmacophore for LPS recognition. The readouts of the primary fluorescence-based rapid screen are correlated with both the biological potency and the enthalpy of interaction. By performing *in vitro* toxicity screens in tandem with the bioassays, lead compounds of interest can be easily identified for further systematic structural modifications and SAR studies. Further the development of virtual screening methods will enable the prediction of novel, high-affinity LPS binding molecules.

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# Novel automated screening methods for the rapid assessment of anti-endotoxic activities: Evaluation of acyl-spermines

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Septic shock is a clinical syndrome caused by an uncontrolled immune response to endotoxin, or lipopolysaccharide (LPS) a structural component of the Gram-negative bacterial outer membrane. The structurally highly conserved lipid A moiety is the toxic center of LPS, and is therefore a logical therapeutic target for drug design. Dicationic molecules with a distance of ~ 14 Angstroms between the cationic functions bind lipid A via simultaneous ionic interactions with the phosphate groups of Lipid A. To further sequester and biologically neutralize Lipid A, an additional hydrophobic group is necessary. These requirements are fulfilled in hydrophobic polyamines which, in preliminary studies, showed considerable promise as potent, yet nontoxic LPS neutralizers. A series of mono- and *bis*-homologated acyl-spermine compounds were synthesized in order to examine the following questions pertaining to the binding to and neutralization of LPS: 1. What is the optimal length of hydrophobic groups? 2. What is the optimal number (and position) of hydrophobic groups? 3. Are there structural correlates of toxicity? 4. Can we design active, yet nontoxic molecules?
A detailed examination utilizing automated rapid-throughput screening assays for the quantitative assessment of binding affinity, LPS-neutralizing activity, surface activity and *in vitro* cytotoxicity were implemented to evaluate these questions. We have identified the optimal acyl-chain length for effective binding to, and neutralization of LPS (mono: $C_{14}$-$C_{16}$; *bis*: $C_8$-$C_9$). In general, the *bis* $C_8$-$C_9$ are preferable to the mono $C_{14}$-$C_{16}$ compounds in terms of activity and toxicity. Much of the toxicity stems from surface activity, as expected. The future challenge lies in designing less surface-active scaffolds while preserving the biological activity.

110C

# Potent endotoxin sequestration properties of novel hydrophobic lysine-spermine conjugates

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We have examined in detail the interactions of a focused library of hydrophobic lysine-spermine conjugates with LPS. The library was constructed so as to be able to test specific hypotheses pertaining to the nature of the appendage (hydrophilic versus hydrophobic; aromatic versus long-chain aliphatic substituents), and L- and D- isomers of lysine. Earlier work from our laboratory had served to elucidate the pharmacophore necessary for small molecules to specifically bind and neutralize the lipid A moiety of bacterial lipopolysaccharides (LPS). Compounds with $C_{14}$-$C_{18}$ long-chain aliphatic substituents neutralize LPS with an $IC_{50}$ of ~ 1 ? M in an assay measuring NO production in murine J774 cells, as well as TNF-? in human whole blood. Administration of such compounds to D-galactosamine-sensitized mice challenged with supralethal doses of LPS provided significant protection against lethality. Potent anti-endotoxic activity, low toxicity, and ease of synthesis render this class of compounds promising candidate endotoxin-sequestering agents of potential significant therapeutic value.

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# Cytosolic NADP+-dependent isocitrate dehydogenase protects macrophages from LPS-induced nitric oxide and reactive oxygen species

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Macrophages activated by microbial lipopolysaccharides (LPS) produce bursts of nitric oxide and reactive oxygen species (ROS). Redox protection systems are essential for the survival of the macrophages since the nitric oxide and ROS can be toxic to them as well as to pathogens. Using suppression subtractive hybridization (SSH) we found that cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc) is strongly upregulated by nitric oxide in macrophages. The levels of IDPc mRNA and of the corresponding enzymatic activity were markedly increased by treatment of RAW264.7 cells or peritoneal macrophages with LPS or SNAP (a nitric oxide donor). Over-expression of IDPc reduced intracellular peroxide levels and enhanced the survival of $H_2O_2$- and SNAP-treated RAW264.7 macrophages. IDPc is known to generate NADPH, a cellular reducing agent, via oxidative decarboxylation of isocitrate. The expression of enzymes implicated in redox protection, superoxide dismutase (SOD) and catalase, was relatively unaffected by LPS and SNAP. We propose that the induction of IDPc is one of the main self-protection mechanisms of macrophages against LPS induced-oxidative stress.

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# Lipoteichoic acid is internalized by a Receptor mediated Mechanism and traffics to the Endoplasmatic Reticulum and Golgi before colocalizing with TLR2 in Lysosomes

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Toll-like receptor 2 (TLR2) is a signaling receptor for a variety of microbial products, including lipoteichoic acid (LTA), the lipopolysaccharide (LPS)- counterpart in Gram-positive bacteria. The aim of this study was to explore the uptake and trafficking pattern of LTA from *Staphylococcus aureus* in relation to TLR2 expression in HEK-293 cells expressing GFP- or YFP-TLR2 and in human monocytes. We found prominent expression of TLR2 in the plasma membrane and in endosomes and lysosomes. Uptake of fluorescently labeled LTA occurred rapidly in HEK cells independent of TLR2 or CD14, though CD14 enhanced binding of LTA. LTA uptake was inhibited in dynamin dominant negative transfectants suggesting receptor- mediated endocytosis of this ligand. LTA was first found in early endosomes before the ligand appeared in tubular structures that colocalized with markers for the endoplasmatic reticulum. Subsequently, LTA was observed in the Golgi apparatus, before trafficking to lysosomes where prominent co localization with TLR2 occurred. Blocking scavenger receptor CD36, and pretreatment with the TLR2/TLR6 ligand fibroblast stimulating lipopeptide -1 (FSL-1), strongly reduced LTA uptake in moncytes. Our results show that LTA has a uptake and trafficking pattern in cells that is unlike other TLR ligands such as LPS and CpG.

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# Nod1 mediated endothelial cell activation by *Chlamydophila pneumoniae*

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Seroepidemiological and animal studies as well as demonstrations of viable bacteria in atherosclerotic plaques have linked *Chlamydophila pneumoniae* infection and development of chronic vascular lesions and coronary heart disease. Inflammation and immune responses are dependent on host recognition of invading pathogens. The recently identified cytosolic Nod proteins are candidates for intracellular recognition of bacteria, such as the obligate intracellular chlamydia. In the study presented, the mechanism of endothelial cell activation by *C. pneumonaie* was examined. Viable but not heat-inactivated chlamydiae activated human endothelial cells, which expressed Nod1. Nod1 gene silencing by siRNA reduced the *C. pneumoniae*-induced IL-8 production markedly. Moreover, overexpressing experiments in HEK293 cells demonstrated that *C. pneumoniae* induced a Nod1- and Nod2-mediated NF-κB-activation. Interestingly, heat-inactivated bacteria were still able to induced a NF-κB-reporter gene activity –via Nod proteins when intracellularly transfected, but not when provided from the extracellular side. In contrast, TLR2 sensed extracellular heat-inactivated chlamydiae. In conclusion, we demonstrated that in HEK293 cells Nod1 and Nod2 mediated NF-κB activation induced by *C. pneumoniae*. Nod1 played a dominant role in triggering the inflammatory process by *C. pneumoniae* in endothelial cells.

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# Pathogen recognition by Toll like receptors: pathogens and commensals use different signal transduction pathways

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Mucosal surfaces form powerful barriers that protect internal tissues from microbial attack. The host must discriminate between potential pathogens and commensals, and recruit defense effector cells that eliminate the pathogens while allowing the normal flora to persist in the lumen. This functional dichotomy is unique for the mucosal compartment. The systemic anti-microbial defense, in contrast, is equipped to eliminate invading microbes, by chasing down and killing the prey at any cost. Urinary tract infections (UTIs) are among the most common bacterial infections in man, and an important cause of acute morbidity and chronic renal disease. Uro-pathogenic *E. coli* (UPEC) attack the epithelial cells from the lumen, and this interaction determines if the mucosa will remain inert, or mount a response to infection. When activated, epithelial cells produce inflammatory mediators that direct the recruitment of inflammatory cells, and the clearance of infection. Bacterial attachment is essential for epithelial cell activation and the epithelial response is controlled by *tlr4 in vitro* and *in vivo*. Adherence is not to TLR4 , but virulent UPEC strains express P fimbriae that attach the bacteria to glycosphingolipid GSLs) receptors on the human urinary tract mucosa. The molecular recognition is well understood, and the P fimbriae bind oligosaccharide receptor epitopes in epithelial GSLs and the papG adhesin at the fimbrial tip is the bacterial surface ligand. This study proposes ‘’pathogen recognition’’ as a discriminating step in TLR activation at mucosal surfaces. Specific adherence to epithelial cell receptors was shown to control TLR4 activation and to select the adapter proteins involved in downstream signalling. P fimbriae were needed to trigger a TLR4 dependent *in vivo* response to *Escherichia coli* urinary tract infection and TRIF/Lps-2, TRAM rather than the LPS related MyD88/TIRAP adaptors were involved. TRIF/TRAM KO mice developed transient symptomatic infection but MyD88 KO mice became asymptomatic carriers. The results offer a new mechanism to discriminate pathogen sensing by TLR4 that may reconcile the need for specificity with the convergence on a limited number of TLRs.

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# Significance of biofilm formation and polysaccharide intercellular adhesin (PIA) in protection of *Staphylococcus epidermidis* from innate immune defenses

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Staphylococcal biofilm formation is a two-step process: Initial attachment of the bacteria to an artificial surface is followed by PIA-mediated cell-cell-adhesion resulting in the development of multilayered slime-encased clusters. The ability of biofilm formation is considered to be the major virulence determinant of the nosocomial pathogen *S. epidermidis*, since biofilm-encased bacteria appear to be protected from antibiotics and the antibacterial action of human phagocytes. The mechanisms, which lead to the evasion of *S. epidermidis* from innate immune defences, are unknown. We asked whether the extracellular homoglycan PIA and/or biofilm protect bacteria from phagocyte killing by preventing activation of the human complement system. In bactericidal assays with human polymorphonuclear leukocytes (PMN), we showed that *S. epidermidis* wild-type (wt) was protected from killing, when it was cultured adherent and allowed to produce biofilm (33±13% killing); it was as sensitive (85±9%) as an isogenic PIA-deficient *ica* mutant (*ica*$^-$, 79±6%), when cultured in suspension. Active complement was found to be essential for an efficient inactivation of *S. epidermidis* by human PMN. We did not observe any differences in complement activation in human serum between planktonic *S. epidermidis* and PIA-negative *ica* mutant bacteria indicating that PIA per se does not significantly contribute to complement activation under these conditions. However, biofilm-encased *S. epidermidis* wt induced significantly more C3a than liquid-grown wild-type cells and *ica*$^-$. The *in vivo* relevance of the resistance against killing of biofilm-encased *S. epidermidis* by phagocytes was demonstrated in a murine catheter abscess model. *S. epidermidis* wt was precultured on the catheter and the *ica* mutant was added at a 1:1 ratio to the wt cells. 7 days after infection, *S. epidermidis* wt survived better in the catheter and in surrounding tissue than the *ica*$^-$ mutant, yet it did not elicit more inflammation. In conclusion, biofilm protects *S. epidermidis* from PMN killing, this does not occur by preventing complement activation, but possibly by preventing usage of complement components.

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# CD14 and TLR2 Pattern Recognition Receptors Differentially Modulate Host Response To *S. Pneumoniae* In Vivo And In Vitro

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CD14 binds to and TLR2 mediates phagocyte activation in response to Gram-positive cell wall components. The CD14- and /or TLR2-mediated effector functions, which are elicited by live Gram-positive bacteria, i.e. *S. pneumoniae* infection have not been defined. Therefore pathogenesis and outcome of and treatment response to *S. pneumoniae* meningitis was studied in wt, $CD14^{-/-}$, $TLR2^{-/-}$ and $CD14^{-/-}/TLR2^{-/-}$ mice. All Knockout mice died earlier than wt mice. Early after infection, brain bacterial numbers were increased in TLR2>CD14=wt=$CD14^{-/-}/TLR2^{-/-}$ mice. TNF release into cerebrospinal fluid (CSF) and in supernatant of in vitro infected macrophages was elevated in TLR2>CD14>wt=$CD14^{-/-}/TLR2^{-/-}$ mice. Early leukocyte immigration into the CSF was differentially regulated by the pattern recognition receptors. It was stronger ($p<0.05$) in $CD14^{-/-}$ than wt and delayed in $TLR2^{-/-}$ as compared to wt ($p<0.05$) mice. MIP-2 release in brain in vivo and in endothelial cells infected in vitro was higher in $CD14^{-/-}$ than wt mice. $CD14^{-/-}$ PMN demonstrated increased expression of CXCR2 after infection in vivo and stronger in vitro chemotaxis than wt PMN towards infected CSF from either wt or $CD14^{-/-}$ mice and towards MIP-2. Pretreatment with anti-CXCR2 antibodies abolished the earlier mortality in $CD14^{-/-}$mice. In conclusion, CD14 has a protective effect in pneumococcal meningitis by slowing PMN migration via MIP-2 and CXCR2 modulation. $TLR2^{-/-}/CD14^{-/-}$ mice behaved during meningitis like single $CD14^{-/-}$ mice.

In summary, the two pattern recognition receptors TLR2 and CD14 mediated differing functions in vivo: TLR2 deficiency affected bacterial load, CD14 deficiency chemokines and migration. In double KO mice, early infiltration linked to the lack of CD14 possibly compensated for the defect in bacterial killing of TLR2 deficiency. The differential host response of $TLR2^{-/-}$, $CD14^{-/-}$ and $CD14^{-/-}/TLR2^{-/-}$ double Knockout mice indicates that the two receptors are possibly not always coexpressed and affect different host functions.

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# Effect of neutrophil elastase inhibitor (sivelestat sodium hydrate) on ARDS in ICU

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Background/Aims: Neutrophil elastase(NE) is thought to play a central role in acute respiratory distress syndrome(ARDS). Sivelestat, a competitive inhibitor of NE, has been approved as a treatment for ARDS associated with systemic inflammatory response syndrome(SIRS) in Japan. Our studies was to determine whether sivelestat could increase $PaO_2/FiO_2$ and ventilator-free days, and whether sivelestat could reduce lung injury score and SOFA score. Methodology: sixty-eight patients with ARDS ($PaO_2/FiO_2$ <200) associated with SIRS were enrolled. The treatment group(the sivelestat group) included 34 patients receiving intravenous sivelestat infusion at a dose of 0.2mg・$kg^{-1}$・$hr^{-1}$. $PaO_2/FiO_2$ ,lung injury score and SOFA score were monitored intensively. Results : On day 7, $PaO_2/FiO_2$, lung injury score and SOFA score were improved with sivelestat, although there was no difference between the sivelestat group and the no sivelestat group on day 1-3. Furthermore, sivelestat increased the number of ventilator-free days. Conclusions : Intravenous sivelestat could be a novel therapeutic strategy to ARDS.