Agonistic and antagonistic properties of a *Rhizobium sin*-1 lipid A modified by an ether-linked lipid†

Mahalakshmi Vasan, Margreet A. Wolfert* and Geert-Jan Boons*

Received 23rd March 2007, Accepted 17th May 2007
First published as an Advance Article on the web 29th May 2007
DOI: 10.1039/b704427e

LPS from *Rhizobium sin*-1 (*R. sin*-1) can antagonize the production of tumor necrosis factor alpha (TNF-α) by *E. coli* LPS in human monocytic cells. Therefore these compounds provide interesting leads for the development of therapeutics for the prevention or treatment of septic shock. Detailed structure activity relationship studies have, however, been hampered by the propensity of these compounds to undergo β-elimination to give biological inactive enone derivatives. To address this problem, we have chemically synthesized in a convergent manner a *R. sin*-1 lipid A derivative in which the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether linked moiety. As expected, this derivative exhibited a much-improved chemical stability. Furthermore, its ability to antagonize TNF-α production induced by enteric LPS was only slightly smaller than that of the parent ester modified derivative demonstrating that the ether-linked lipids affect biological activities only marginally. Furthermore, it has been shown for the first time that *R. sin*-1 LPS and the ether modified lipid A are also able to antagonize the production of the cytokine interferon-inducible protein 10, which arises from the TRIF-dependent pathway. The latter pathway was somewhat more potently inhibited than the MyD88-dependent pathway. Furthermore, it was observed that the natural LPS possesses much greater activity than the synthetic and isolated lipid As, which indicates that di-KDO moiety is important for optimal biological activity. It has also been found that isolated *R. sin*-1 LPS and lipid A agonize a mouse macrophage cell line to induce the production of TNF-α and interferon beta in a Toll-like receptor 4-dependent manner demonstrating species specific properties.

Introduction

The innate immune system is an evolutionary ancient system of defense against microbial infections.[1] It responds rapidly to highly conserved families of structural patterns, called pathogen associated molecular patterns (PAMPs), which are integral parts of pathogens and are perceived as danger signals by the host. Recognition of PAMPs is mediated by sets of highly conserved receptors,[2] each of which binds to a variety of PAMPs. Cellular activation by these receptors results in acute inflammatory responses that include the production of a diverse set of cytokines and chemokines, direct local attack against the invading pathogen and initiation of responses that activate and regulate the adaptive component of the immune response.

Lipopolysaccharides (LPSs) are structural components of the outer surface membrane of Gram-negative bacteria that trigger innate immune responses through Toll-like receptor 4 (TLR4), a member of the TLR family that participates in pathogen recognition. TLRs are transmembrane glycoproteins having an extracellular domain that contains multiple leucine-rich repeating motifs, a transmembrane domain and an intracellular signaling domain.[3,4] The intracellular domain serves as a docking site for a number of adaptor proteins,[5] which in turn recruit kinases to initiate specific down-stream processes, such as activation of mitogen-activated protein (MAP) kinases and transcription factors (NF-κB, AP-1 and interferon regulatory factor 3 (IRF-3)). The end result is the up-regulation of hundreds of genes resulting in the production of a multitude of cytokines and chemokines.

TLR4 initiates cell-signaling by two cascades that involve recruitment of the intracellular TIR adaptor proteins MyD88 or TRIF.[3,4] Thus, the TIR domain of TLR4 can bind to the dimeric adapter protein MyD88, that then recruits and activates a number of kinases, subsequently leading to activation of the MAP kinases, such as p38, JNK and ERK1/2 and the transcription factor NF-κB. This *MyD88-dependent pathway* results in the synthesis of pro-inflammatory cytokines and chemokines including tumor necrosis factor alpha (TNF-α), interleukin 1beta (IL-1β) and IL-6. Another adaptor protein, called TRIF,[6] can also be recruited to the TIR domain leading to activation of the transcription factor IRF-3, NF-κB and the MAP kinase JNK. This *TRIF-dependent pathway* results in the synthesis of important inflammatory mediators, including interferon beta (IFN-β), interferon-inducible protein 10 (IP-10) and nitric oxide.

Although the initiation of acute inflammatory responses is important for the prevention of infections, over-activation of this response may lead to the clinical symptoms of septic shock. Septicemia is a serious world-wide health problem and is associated with mortality rates of 40–60%.[7,8] It has been estimated that 1% of hospital patients and 20–30% of ICU patients develop sepsis and that septic shock results in 100000 deaths annually in the United States.[7,8] A number of strategies for the prevention and

Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Road, Athens, GA, 30602, USA. E-mail: gjboons@ccrc.uga.edu, mwolfert@ccrc.uga.edu; Fax: +1 706-542-4412

† Electronic supplementary information (ESI) available: Fig. S1 and S2 and NMR spectra of synthesized compounds. See DOI: 10.1039/b704427e

treatment of sepsis have been directed against the lipid A region of LPS.[9] For example, structural analogs of lipid A have been examined for their ability to antagonize the effects of LPS. These antagonists include naturally occurring lipid A precursors,[10] as well as a number of synthetic analogs of these precursors.[11,12] The most widely studied analog is a synthetic analog based on the lipid A of *Rhodobacter sphaeroides* or *R. capsulatus*, two species having very similar lipid A structures.[13–16] Although the lipid As of *R. sphaeroides*/*R. capsulatus* and *E. coli* have the same bis-1,4′-phosphorylated glucosamine disaccharide backbone their fatty acyl patterns differ considerably. In this respect, *R. sphaeroides*/*R. capsulatus* lipid A consists of two 3-oxomyristic acid, two β-hydroxydecanoic acid and one dodecenoic acid residues.

Recently, we reported that LPS from *Rhizobium sin*-1 (*R. sin*-1), a nitrogen-fixing bacterial species, can prevent the induction of TNF-α by *E. coli* LPS in human monocytic cells.[17,18] Furthermore, another study showed that the biological properties of *R. sin*-1 LPS are species specific and most notably it was found that it can agonize mouse macrophages in a TLR2-dependent manner.[19,20] The lipid A of *R. sin*-1 LPS is a structurally unusual lipid A differing in almost every aspect from those known to contribute to the toxicity of enteric LPS (Fig. 1).[21] In particular, the disaccharide moiety of *Rhizobial* lipid A is devoid of phosphate and the glucosamine phosphate is replaced by 2-aminogluconolactone. It contains a very long chain fatty acid, 27-hydroxyoctacosanoic acid which, in turn, can be esterified by β-hydroxybutyrate. As is the case with other naturally occurring LPSs, the inherent microheterogeneity of the lipid A region of *R. sin*-1 makes it impossible to be developed as a therapeutic agent for Gram-negative septicemia. Furthermore, the inability to separate the different species limits identification of specific structural features that makes *R. sin*-1 lipid A an antagonist as opposed to an agonist. To address these problems, we have synthesized a range of well-defined lipid A derivatives based on the structure of *R. sin*-1 LPS for structure–activity relationship studies. We have already shown[18] that the synthetic compound **1** is able to antagonize *E. coli* LPS. In addition, we have demonstrated that the 2-aminogluconolactones can exist as a 2-aminogluconate.[22] The chemical synthesis and biological evaluation of a compound locked in the 2-aminogluconate established that this species possesses antagonistic properties.[23] Detailed biological evaluations of the synthetic *R. sin*-1 lipid As have been hampered by their chemical lability due to elimination to give the enone derivative **2**. To address this problem, we report here the chemical synthesis of compound **3**, which is derived from **1**, however, the β-hydroxy ester at C-3 of the proximal sugar unit has been replaced by an ether derivative. It was anticipated that this compound would be less prone to β-elimination due to the poor leaving group ability of the ether.[24–26] As a result, we have been able to investigate the ability of a *R-sin* 1 lipid A to antagonize cell-signaling events arising from the MyD88- and TRIF-dependent pathways. Furthermore, species-specific properties of **3** have been investigated by comparing biological properties of the compound exposed to human and mouse macrophages.

Results and discussion

Chemical synthesis

It was envisaged that coupling of glycosyl donor **4** with glycosyl acceptor **5** would give disaccharide **12** (Scheme 1), which is appropriately protected for the selective introduction of β-hydroxyl fatty acids and oxidation of the C-1 position to lactone. Glycosyl acceptor **5** is modified by an ether linked γ-benzyloxy fatty acid, because it was anticipated that the harsh conditions required for its introduction would affect functionalities present in the disaccharide. Another feature of **5** is that its anomeric center is protected as a thioglycoside.[27,28] This functionality is stable under a wide range of chemical conditions, however, it can be hydrolyzed at a late stage of the chemical synthesis to give a lactol, which can then be oxidized to a lactone. Furthermore, the selenoglycoside of **4** was expected to be significantly more reactive towards activation with NIS–TMSOTf than the thioglycoside of **5**, and thus it was expected that these compounds could be employed in a chemoselective glycosylation to give **12**. The phthalimido and azido functions of **12** offer an attractive set of orthogonal protecting groups that

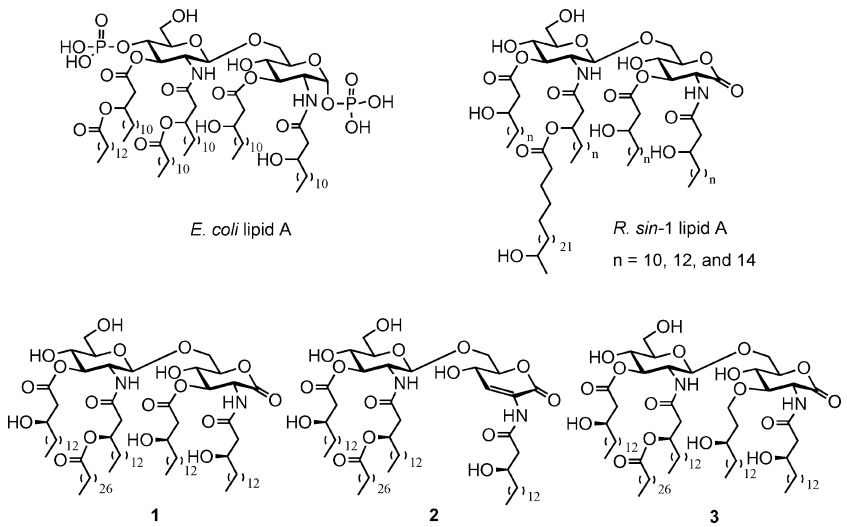
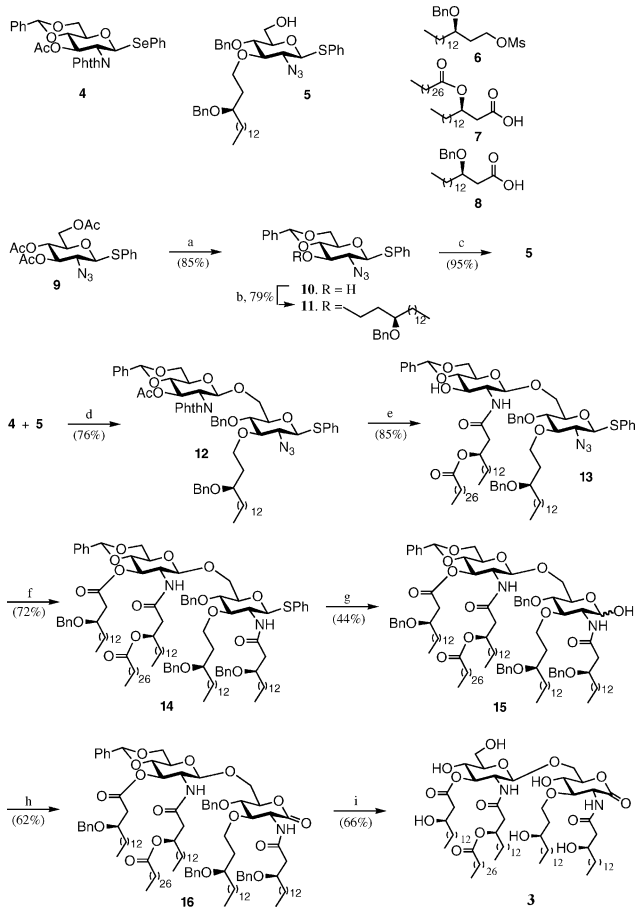


Fig. 1 Structures of *E. coli* and *R. sin*-1 lipid A and synthetic *R. sin*-1 lipid A derivatives **1–3**.



Scheme 1 *Reagents and conditions*: a) NaOMe, MeOH then $C_6H_5CH(OMe)_2$, CSA, CH_3CN; b) **6**, NaH, DMF, 0 °C; c) $BH_3{\cdot}NHMe_2$, $BF_3{\cdot}Et_2O$, toluene, −30 °C; d) NIS, TMSOTf, MS 4 Å, DCM, −35 °C; e) $NH_2NH_2{\cdot}H_2O$, EtOH, Δ, then **7**, DCC, DCM; f) Zn, AcOH then **8**, DCC, DMAP, DCM; g) NIS, TMSOTf, DCM, H_2O, 0 °C; h) PCC, 3 Å MS, DCM; i) Pd/C, H_2, *t*-BuOH, THF.

allow selective derivatization of the two amino groups. Removal of the phthalimido group will result in cleavage of the *O*-acetyl ester. However, by exploiting the higher nucleophilicity of primary amines compared to hydroxyls it is possible to selectively acylate the amine.

Glycosyl acceptor **5** was readily obtained from known derivative **9**.[29] Thus, the acetyl esters of **9** were cleaved by treatment with sodium methoxide in methanol and the resulting triol was selectively protected by reaction with benzylaldehyde dimethyl acetal in the presence of camphorsulfonic acid (CSA) in acetonitrile to give **10**. The C-3 hydroxyl of **10** was alkylated with sulfonate **6** by treatment with sodium hydride in DMF to give **11** in a good yield of 79%.[26] Next, the benzylidene acetal of **11** was regioselectively opened by reaction with $BH_3{\cdot}NHMe_2$ and $BF_3{\cdot}Et_2O$ in toluene at −30 °C to give **5** in an excellent yield. A number of other reaction conditions led to the formation of mixtures of regioisomeric benzyl ethers. For example, the use of $BH_3{\cdot}NHMe_2$ in DCM, which is the conventional solvent for this reagent,[30] gave a mixture of products. Glycosyl donor **4**[31] and fatty acids **6**,[26] **7** and **8**[32,33] were prepared by reported procedures.

Having glycosyl donor **4** and acceptor **5** at hand, attention was focused on the preparation of the disaccharide **12**, instalment of the β-hydroxyl fatty acids and oxidation of the anomeric center. Thus, a NIS–TMSOTf mediated coupling of the glycosyl donor **4** with acceptor **5** in dichloromethane at −35 °C gave disaccharide **12** in a yield of 76%.[31,34,35] Only the β-anomer was formed due to neighboring group participation of the phthalimido group. Next, the phthalimido moiety and acetyl ester of compound **12** were removed by treatment with hydrazine hydrate in refluxing ethanol[36] and the amine of the resulting compound was selectively acylated with alkanoyloxy fatty acid **7** in the presence DCC to give **13**. Reduction of the azido moiety of **13** was easily accomplished by reaction with activated Zn in acetic acid and the amine and hydroxyl of the resulting compound were immediately acylated with **8** using 1,3-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as the activation reagents to afford **14**. It is important to note that selective *N*-acylation could be accomplished by performing the reaction in the absence of DMAP, thereby making it possible to synthesize derivatives having different substituents at C-2 amine and C-3 and C-3′ hydroxyls. The thioglycoside of compound **14** was hydrolyzed by treatment with NIS–TMSOTf[37] in wet dichloromethane and the resulting lactol was oxidized with PCC in DCM to afford lactone **16**. Finally, the benzyl ethers and benzylidene acetal of **16** were removed by catalytic hydrogenation over Pd/C to give the target compound **3**. As expected, this derivative had an excellent shelf-life and after storage for three months at −20 °C no decomposition was observed. Under similar storage conditions compound **1** was decomposed.

It is important to note that alternative synthetic strategies, which employed either an allyl or TBDMS ether for protection of the anomeric center of the proximal sugar, led to failure. Thus, the anomeric TBDMS function was not compatible with the alkylation conditions required for the instalment of **6**. Furthermore, attempts to cleave an allyl glycoside at the final stage of the synthesis led either to recovery of starting material or decomposition. The use of a thioglycoside gave the best results for the preparation of the target compound.

Biological evaluation

Based on the results of recent studies,[1–5] it is clear that enteric LPS-induced cellular activation through TLR4 is complex as many signaling elements are involved. However, it appears that there are two distinct initiation points in the signaling process, one being a specific intracellular adaptor protein called MyD88 and the other an adaptor protein called TRIF, which operates independently of MyD88. It is well established that TNF-α secretion is a prototypical measure for activation of the MyD88-dependent pathway, whereas secretion of IFN-β and IP-10 are commonly used as an indicator of TRIF-dependent cellular activation.

Compound **3** was tested over a wide concentration range for its ability to activate a human monocytic cell line (Mono Mac 6; MM6) to produce TNF-α and IP-10 protein and the resulting values were compared with similar data obtained for *E. coli* LPS and lipid A and *R. sin*-1 LPS and lipid A. Thus, MM6 cells were exposed to the isolated and synthetic compounds and after 5.5 hours, the supernatants were harvested and examined for human TNF-α and IP-10 using capture ELISAs. Potencies (EC_{50}, concentration producing 50% activity) and efficacies (maximal level of production) were determined by fitting the dose–response

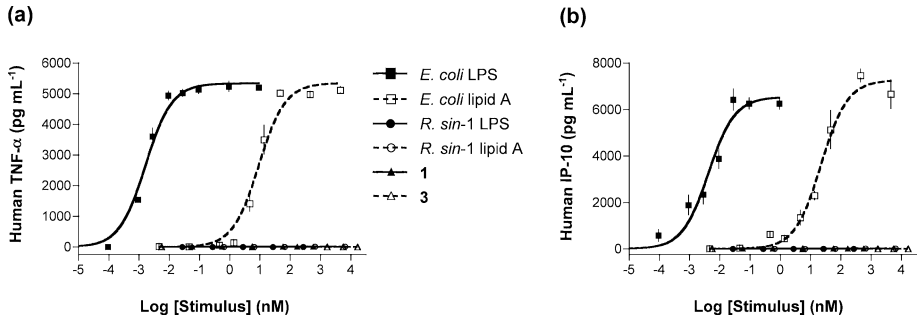


Fig. 2 Concentration–response curves of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** in human monocytic cells. MM6 cells were incubated for 5.5 h at 37 °C with increasing concentrations of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** as indicated. TNF-α (a) and IP-10 (b) proteins in cell supernatants were measured using ELISAs. (Please note that *R. sin-1* LPS, *R. sin*-1 lipid A, **1** and **3** show background values and therefore overlap in the figure.) Treatment with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A, **1** and **3** did not affect cell viability, as judged by cellular exclusion of trypan blue.

curves to a four parameter logistic equation using PRISM software. As can be seen in Fig. 2, *E. coli* LPS and lipid A yielded clear dose–response curves for TNF-α and IP-10, whereas *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compound **3** did not induce significant production of the cytokines. The EC_{50} values for *E. coli* 055:B5 LPS were significantly smaller than that of *E. coli* lipid A (Table 1) which is probably due to the di-KDO moiety of LPS, which is attached to the C-6′ position of lipid A. In this respect, recent studies[38] have shown that meningococcal lipid A expressed by a strain defect in KDO biosynthesis has significantly reduced bioactivity compared to KDO containing *Meningococcal* lipooligosaccharides. It has also been shown that removal of the KDO moieties by mild acidic treatment reduces cellular responses.[18]

It was observed that the EC_{50} values for TNF-α secretion were approximately three times smaller than that of IP-10 when *E. coli* LPS or *E. coli* lipid A was employed as an activator. Thus, it appears that the MyD88-dependent pathway is slightly more responsive than TRIF-mediated cellular activation.

Table 1 EC_{50} values[a] (nM) of *E. coli* LPS and lipid A in MM6 cells

	E. coli LPS	*E. coli* lipid A
TNF-α	0.0016 (0.0014–0.0019)	9.1 (7.3–11.4)
IP-10	0.0042 (0.0032–0.055)	22.2 (18.5–26.8)

[a] EC_{50} values are reported as best-fit values and as minimum–maximum range (best-fit value ± std. error).

Based on its lack of proinflammatory effects, compound **3** was tested over a wide concentration range for its ability to antagonize TNF-α and IP-10 production by MM6 cells incubated with *E. coli* LPS (1 ng mL^{-1}) (Fig. 3). An IC_{50} (concentration producing 50% inhibition) of 22 μM (38 μg mL^{-1}) was established for TNF-α inhibition by compound **3**. Similar inhibition experiments with *R. sin*-1 lipid A and compound **1** gave IC_{50} values of 2.0 μM (3.0 μg mL^{-1}) and 7.3 μM (13 μg mL^{-1}), respectively. As expected, *R. sin*-1 LPS was a much more potent inhibitor of TNF-α production than the corresponding lipid A and in this case an IC_{50} value of 6.5 nM (239 ng mL^{-1}) was determined. Thus, it is probable that the KDO moiety of LPS accounts for the higher inhibitory activity. Interestingly, for IP-10 secretion the IC_{50} values of *R. sin*-1 LPS and compound **3** were smaller than the corresponding values for TNF-α (1.4 nM; 51 ng mL^{-1}) and 3.7 μM (6.5 μg mL^{-1}), respectively) and at the highest concentration tested compound **3** abolished all IP-10 biosynthesis induced by enteric LPS. Similar inhibition results were obtained when *E. coli* lipid A was employed as the agonist (Supplementary data†).

The results of the cellular activation studies show that the inhibitory activity of compound **3** is only slightly smaller than that of the parent compound **1** demonstrating that the ether linked fatty acid at C-3 of the proximal monosaccharide unit has only marginal effect on the biological activity. However, compound **3**

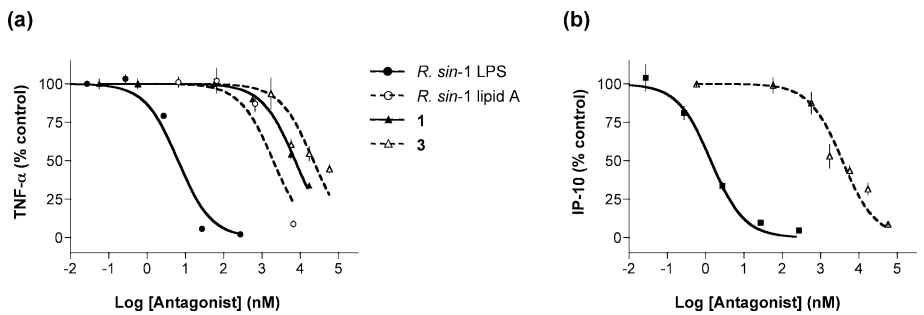


Fig. 3 Antagonism of *E. coli* LPS by *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compounds **1** and **3** in human monocytic cells. TNF-α (a) and IP-10 (b) concentrations were measured after preincubation of MM6 cells with increasing concentrations of *R. sin*-1 LPS, *R. sin*-1 lipid A, **1**, or **3** as indicated for 1 h at 37 °C, followed by 5.5 h of incubation with *E. coli* LPS (1 ng mL^{-1}). Results are expressed as percentage of cytokine concentration of control cells, which are incubated only with *E. coli* LPS.

has a much greater chemical stability than **1** making it a preferred compound for biological studies. Furthermore, the KDO moiety of *E. coli* and *R. sin*-1 LPS appears to significantly increase the agonistic and antagonistic properties, respectively. The data also reveal that the agonists and antagonists affect the MyD88 and TRIF pathways slightly differently. Thus, the agonists (*E. coli* LPS and lipid A) display somewhat higher potencies for TNF-α compared to IP-10, whereas for the antagonists (*R. sin*-1 LPS and lipid A), IP-10 was more potently inhibited at lower concentrations compared to TNF-α.

There are several reports that indicate that structurally different lipid As may differentially induce proinflammatory responses.[39–42] However the heterogeneity in the structure of lipid A within a particular bacterial strain and possible contamination with other inflammatory components of the bacterial cell-wall complicated the dissecting of the biological responses to specific lipid As. Synthetic compounds may address this important issue.

Next, attention was focused on cellular activation studies using a mouse macrophage cell line (RAW 264.7 γNO(−) cells). Thus, secretion of TNF-α and IFN-β protein was measured after exposure of the cells for 5 h to a wide concentration range *E. coli* LPS and lipid A, *R. sin*-1 LPS and lipid A and compound **3** (Fig. 4). Interestingly, *E. coli* and *R. sin*-1 LPS and lipid As activated the cells to produce TNF-α and IFN-β. No cytokine production was measured for compound **3** even when a very high concentration was employed (57 μM; 100 μg mL^{-1}). Furthermore, compound **3** was not able to antagonize the production of TNF-α or IFN-β induced by *E. coli* LPS.

For each agonist, the potency for TNF-α secretion was higher by 5- to 7-fold compared to that of IFN-β. (Table 2). Furthermore, for the *E. coli* derived compounds the EC_{50} values were significantly smaller than those derived from *R. sin*-1. As expected, the lipid As were less potent than their parent LPSs, however, the difference was much larger between *E. coli* LPS and lipid A (10000-fold) than between *R. sin*-1 LPS and lipid A (100-fold).

Recent reports indicate that LPS of non-enterobacterial species such as *Porphyromonas gingivalis*, *Leptospira interrogans* and *R. sin*-1 are capable of signaling independent of TLR4, instead utilizing TLR2-mediated signal transduction.[19,20,43,44] However, TLR2 can only recruit the adaptor protein MyD88 and as a result can only initiate the production of MyD88-dependent cytokines such as TNF-α, but not those TRIF-dependent cytokines such as IFN-β.[3,4] The fact that our results show that *R. sin*-1 can induce the production of IFN-β prompted us to investigate the TLR utilization of these compounds. Thus, *R. sin*-1 LPS and lipid A and *E. coli* LPS and lipid A were exposed at a range of concentrations to HEK 293T cells stably transfected with mouse TLR2/TLR6 or TLR4/MD2 and transiently transfected with a plasmid containing the reporter gene pELAM-Luc (NF-κB-dependent firefly luciferase reporter vector) and a plasmid containing the control gene pRL-TK (*Renilla* luciferase control reporter vector). As a negative control, wild type HEK 293T cells transiently transfected with plasmids containing the reporter gene pELAM-Luc and control gene pRL-TK were used. After an incubation time of 4 h, the activity was measured using a commercial dual-luciferase assay. *E. coli* LPS and the lipopeptide Pam_3CysSK_4[45] were employed as positive controls for cellular activation by TLR4 and TLR2/6, respectively. As can be seen in Fig. 5, *R. sin*-1 LPS and lipid A can induce cellular activation in a TLR4-dependent manner, whereas no activity was observed in cells transfected with TLR2/6. The TLR4-dependent NF-κB activation showed clear dose responses for *E. coli* LPS, *R. sin*-1 LPS and *R. sin*-1 lipid A (Supplementary data†).

Previously it was established that the lipid A region of several *Rhizobiaceae* can stimulate bone marrow granulocytes of TLR4-deficient mice to induce the expression of CD14.[19]

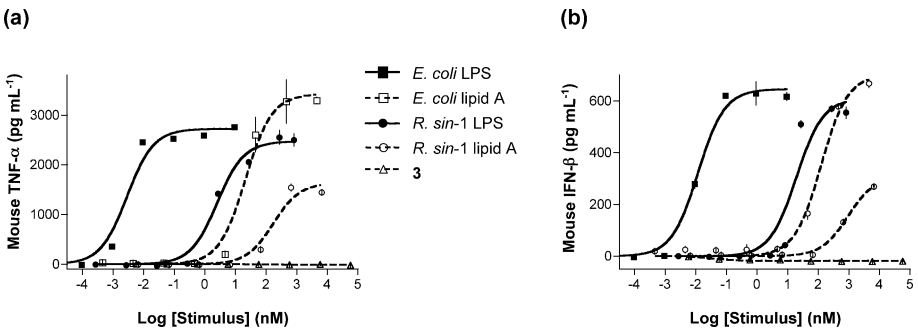


Fig. 4 TNF-α and IFN-β production by murine macrophages after stimulation with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and synthetic compound **3**. Murine RAW γNO(−) cells were incubated for 5.5 h with increasing concentrations of *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and **3** as indicated. TNF-α (a) and IFN-β (b) in cell supernatants were measured using ELISAs. Treatment with *E. coli* LPS, *E. coli* lipid A, *R. sin*-1 LPS, *R. sin*-1 lipid A and **3** did not affect cell viability, as judged by cellular exclusion of trypan blue.

Table 2 EC_{50} values[a] (nM) of *E. coli* and *R. sin*-1 LPS and lipid A in RAW cells.

	E. coli LPS	*E. coli* lipid A	*R. sin*-1 LPS	*R. sin*-1 lipid A
TNF-α	0.0028 (0.0020–0.0041)	21 (16–28)	2.5 (2.0–3.2)	171 (109–268)
IFN-β	0.0118 (0.0096–0.0145)	124 (105–147)	19.3 (11.3–32.8)	932 (816–1067)

[a] EC_{50} values are reported as best-fit values and as minimum–maximum range (best-fit value ± std. error).

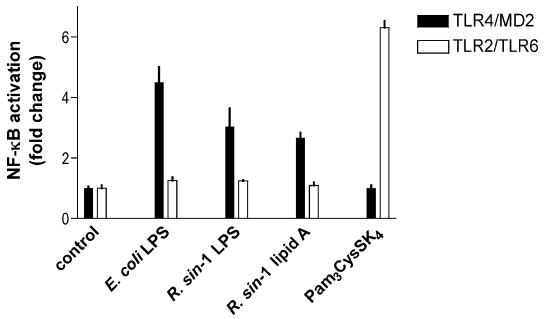


Fig. 5 Response of HEK 293T cells expressing murine TLRs to *R. sin*-1 LPS and *R. sin*-1 lipid A. Induction of NF-κB activation was determined in triplicate cultures of HEK 293T cells stably transfected with murine TLR4/MD2 or TLR2/TLR6 and transiently transfected with pELAM-Luc, pRL-TK and pcDNA3 plasmids. Forty-four h post-transfection, cells were treated with *E. coli* LPS (1 ng mL^{-1}), *R. sin*-1 LPS (1 μg mL^{-1}), *R. sin*-1 lipid A (1 μg mL^{-1}) and Pam_3CysSK_4 (1 μg mL^{-1}) or were left untreated (control). Forty-eight h post-transfection, NF-κB activation was determined by firefly luciferase activity relative to *Renilla* luciferase activity. In the transfection experiments shown, human TNF-α (10 ng mL^{-1}) induced 24.5 ± 0.6 and 21.8 ± 0.3-fold activation of NF-κB in HEK 293T cells stably transfected with TLR4/MD2 and TLR2/6, respectively.

Furthermore, no detectable levels of TNF-α were measured after mouse peritoneal macrophages were exposed to 100 ng mL^{-1} *R. sin*-1 LPS. Surprisingly, HEK cells transfected with TLR2/6 with an ELAM luciferase reporter plasmid showed activity at this concentration. Our results show clearly that at similar concentrations, *R. sin*-1 LPS and lipid A can induce the production of TNF-α and IFN-β in a TLR4-dependent manner.

The observation that synthetic compound **3** possessed no activity in the mouse cell line was surprising. LPS and lipid A isolated from *R. sin*-1 are composed of a complex mixture of compounds differing in fatty acid substitution. Probably, a compound with unique fatty acid composition is responsible for the TLR4 agonistic properties. A larger range of derivatives will need to be synthesized to establish which derivatives account for this activity. The synthetic approach reported here provides such an opportunity. Furthermore, the observation that TLR ligands exhibit species-specific properties should be considered when immuno modulators are being developed.

Conclusion

It has been shown that a derivative of *R. sin*-1 lipid A in which the C-3 fatty acid is replaced by an ether-linked moiety has a much improved chemical stability. Furthermore, this compound could antagonize cytokine production by a human monocytic cell line induced by enteric LPS with a similar potency to the natural ester-linked counter part. For the first time, it has been shown that such an antagonist can inhibit both MyD88- and TRIF-dependent cell signaling events. *R. sin*-1 LPS and lipid A agonized mouse macrophages to produce TNF-α and IFN-β demonstrating species specific properties. For the agonists examined, the potency for TNF-α secretion was higher by 3–7 fold compared to that of IFN-β or IP-10. For, the antagonists, the IC_{50} values for IP-10 were smaller than the corresponding values for TNF-α. These data indicate that the MyD88 and TRIF pathways are somewhat differently activated or inhibited by the examined compounds. Finally, the LPS agonist and antagonist were much more potent indicating that the KDO moiety of LPS is important for optimal biological properties.

Experimental

Chemical synthesis

General synthetic methods. Chemicals were purchased from commercial suppliers and used without further purification, unless otherwise noted. Dichloromethane (DCM) and toluene were distilled from calcium hydride under Argon. Tetrahydrofuran (THF) was distilled under argon from sodium directly prior to application. Dry *N*,*N*-dimethylamineformamide (DMF) was used without purification. Powdered molecular sieves (4 Å) were activated *in vacuo* at 390 °C for 8 h and cooled to room temperature *in vacuo* prior to application. Column chromatography was performed on silica gel 60 (EM Science, 70–230 mesh), size exclusion was performed on Sephadex LH-20 and eluted with a mixture of MeOH–CH_2Cl_2, (1 : 1, v/v). Reactions were monitored by thin-layer chromatography (TLC) on kieselgel 60 F_{254} (EM Science) and compounds were visualized by examination under UV light and by charring with cerium sulfate–ammonium molybdate solution. Organic solvents were removed under reduced pressure at <40 °C. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Merc 300, Varian Inova 500 or Inova 600 equipped with Sun Workstations. ^{1}H NMR were recorded in $CDCl_3$ and referenced to residual $CHCl_3$ at 7.24 ppm. ^{13}C NMR spectra were referenced to the central peak of $CDCl_3$ at 77.0 ppm. Assignments were made by standard gCOSY and gHSQC. High resolution mass spectra were obtained on a Bruker model Ultraflex MALDI-TOF-TOF mass spectrometer. Optical rotations were measured on a Jasco model P-1020 polarimeter. Signals marked with a subscript L belong to the ether-linked lipid at C-3, whereas signals marked with subscript LL belong to the lipid at C-2′. Signals marked with subscript LL′ refer to the C-28 side chain. Signals marked with subscript LA belong to lipids at C-2, C-3′.

Phenyl 3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-4,6-*O*-benzylidene-2-deoxy-2-azido-1-thio-β-D-glucopyranoside (11). NaH (55% oil dispersion, 0.12 g, 5.0 mmol) was added to a solution of compound **10** (0.34 g, 0.88 mmol) in DMF (6 mL) at 0 °C followed by addition of **6** (0.34 g, 0.79 mmol) dissolved in DMF (3 mL). The reaction mixture was allowed to reach room temperature and stirring was continued for 14 h. The reaction mixture was diluted with ethyl acetate (20 mL), quenched with water (1 mL) and subsequently washed with saturated aqueous $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL). The combined organic layers were dried ($MgSO_4$), filtered and the filtrate was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (10% ethyl acetate in hexane) to afford **9** as a colorless syrup (0.45 g, 79%, yield based on mesylate): R_f = 0.70 (20% ethyl acetate in hexane); $[\alpha]_D^{25}$ = −55.94 (c = 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.27–7.59 (m, 15H, aromatic), 5.52 (s, 1H, >C*H*Ph), 4.45–4.50 (m, 3H, H-1, H-3$_L$CH_2Ph), 4.38–4.03 (dd, 1H, $J_{5,6a}$ = 5.1 Hz, $J_{6a,6b}$ = 10.2 Hz, H-6a), 4.02–3.98 (m, 1H, H-1$_L$), 3.84–3.73 (m, 2H, H-1$_L$, H-6b), 3.58–3.39 (m, 4H, H-3$_L$, H-4, H-3, H-5), 3.30 (t, 1H, J = 10.2 Hz, J = 8.7 Hz, H-2), 1.82–1.80 (m, 2H, H-2$_L$), 1.50–1.46 (m, 2H, H-4$_L$), 1.31–1.24 [m, 22H, H-(5$_L$–15$_L$)], 0.89 (t, 3H,

H-16$_L$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 126.17–139.26 (aromatic), 101.46 (>*C*HPh), 86.91 (C-1), 82.07 (C-4), 81.42 (C-3), 76.19 (C-3$_L$), 71.16 (C-3$_L$ *C*H$_2$Ph), 70.73 (C-5), 70.47 (C-1$_L$), 68.70 (C-6), 65.08 (C-2), 35.02 (C-2$_L$), 34.24 (C-4$_L$), 32.1–22.93 [C-(5$_L$–15$_L$)], 14.37 (C-16$_L$); HRMS (*m*/*z*) for $C_{42}H_{57}N_3O_5S$[M + Na]$^+$: calcd 738.4019, found 738.4613.

Phenyl 3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-4-*O*-benzyl-2-deoxy-2-azido-1-thio-β-D-glucopyranoside (5). To a solution of compound **11** (0.26 g, 0.36 mmol) in toluene (10 mL) was added BH_3·$NHMe_2$ (0.11 g, 1.79 mmol). After cooling the reaction mixture (−30 °C), BF_3·OEt_2 (0.31 g, 2.15 mmol) was added dropwise. The temperature was allowed to reach 0 °C over a period of 1 h after which TLC analysis indicated completion of the reaction. The reaction mixture was then quenched by the very slow addition of methanol (3 mL) followed by evaporation of the organic solvents *in vacuo*. The crude product was purified by silica gel column chromatography (20% ethyl acetate in hexane) to obtain compound **5** (0.25 g, 95%) as a white solid. R_f = 0.40 (20% ethyl acetate in hexane); $[\alpha]_D^{25}$ = −33.56 (*c* = 1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.19–7.46 (m, 15H, aromatic), 4.71 (d, 2H, $J_{a,b}$ = 10.8 Hz, H-3$_L$ C*H*$_a$H$_b$Ph), 4.53 (d, 2H, $J_{b,a}$ = 10.8 Hz, H-3$_L$CH$_a$*H*$_b$Ph), 4.42 (bd, 2H, H-4 C*H*$_2$Ph), 4.35 (d, 1H, $J_{1,2}$ = 9.9 Hz, H-1), 3.91–3.76 (m, 3H, H-1$_L$, H-6a), 3.61–3.57 (m, 1H, H-6b), 3.47–3.43 (m, 1H, H-3$_L$), 3.39–3.33 (m, 1H, H-3), 3.28–3.13 (m, 3H, H-4, H-5, H-2), 2.63 (s, 1H, H-6 O*H*), 1.82–1.76 (m, 2H, H-2$_L$), 1.30–1.48 (m, 2H, H-4$_L$), 1.18 [m, 22H, H-(L$_5$–L$_{15}$)], 0.81 (t, 3H, H-16$_L$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 127.67–139.13 (aromatic), 86.28 (C-1), 85.56 (C-4), 79.76 (C-5), 77.50 (C-3), 76.46 (C-3$_L$), 75.26 (C-3$_L$ *C*H$_2$Ph), 71.11 (C-4 *C*H$_2$Ph, C-1$_L$, C-6), 65.48 (C-2), 35.10 (C-2$_L$), 34.33 (C-4$_L$), 32.17–29.93 [C-(5$_L$–15$_L$)], 14.37 (C-16$_L$); HRMS (*m*/*z*) for $C_{42}H_{59}N_3O_5S$[M + Na]$^+$: calcd 740.4175, found 740.4748.

Phenyl 3-*O*-acetyl-6-*O*-(3-*O*-acetyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-azido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-4-*O*-benzyl-2-deoxy-1-thio-β-D-glucopyranoside (12). A suspension of **4** (0.59 g, 1.03 mmol) and **5** (0.61 g, 0.86 mmol) and molecular sieves (4 Å, 0.5 g) in DCM (10 mL) was stirred under an argon atmosphere for 2 h. The mixture was cooled (−35 °C) followed by the addition of NIS (0.23 g, 1.04 mmol) and TMSOTf (0.01 g, 0.05 mmol). The reaction mixture was stirred for 45 min allowing it to slowly reach −10 °C after which TLC analysis showed complete consumption of the starting materials. The reaction mixture was quenched with pyridine (0.1 mL) and diluted with DCM (10 mL). The molecular sieves were removed by filtration through a pad of Celite. The filtrate was then washed with aqueous $Na_2S_2O_3$ (2 × 20 mL, 15%) followed by water (20 mL). The organic phase was dried ($MgSO_4$) and concentrated *in vacuo*. The crude mixture was purified by a silica gel chromatography (25% ethyl acetate in hexane) to afford **12** (0.74 g, 76%, yield based on acceptor) as a white solid. R_f = 0.55 (30% ethyl acetate in hexane); $[\alpha]_D^{25}$ = −19.28 (*c* = 1, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.70–7.07 (m, 24H, aromatic), 5.89 (t, 1H, $J_{3'2'}$ = 9.6 Hz, $J_{3'4'}$ = 10.2 Hz, H-3′), 5.57–5.56 (d, 2H, $J_{1'2'}$ = 9.0 Hz, H-1′, >C*H*Ph), 4.49–4.39 (m, 5H, H-3$_L$, H-4 C*H*$_2$Ph, H-6′a), 4.38 (t, 1H, $J_{2'3'}$ = 9.6 Hz, $J_{1'2'}$ = 9.0 Hz, H-2′), 4.27–4.24 (m, 2H, H-1, H-3$_L$ C*H*$_2$Ph), 4.06 (bd, 1H, H-6a), 3.87–3.79 (m, 4H, H-6′b, H-4′, H-1$_L$), 3.73–3.66 (m, 2H, H-5′, H-6b), 3.47–3.45 (m, 1H, H-3$_L$), 3.33 (m, 1H, H-5), 3.19–3.18 (m, 2H, H-4, H-3), 3.12 (m, 1H, H-2), 1.91 (s, 3H, COC*H*$_3$), 1.79–1.76 (m, 2H, H-2$_L$), 1.51–1.42 (m, 2H, H-4$_L$), 1.32–1.24 [m, 22H, H-(4$_L$–15$_L$)], 0.86 (t, 3H, H-16$_L$). ^{13}C NMR (300 MHz, $CDCl_3$): δ = 170.41 (*C*=O), 123.80–139.11 (aromatic), 101.92 (*C*HPh), 98.79 (C-1′), 85.79 (C-1), 85.39 (C-3), 79.58 (C-4′), 78.44 (C-4, C-5), 76.39 (C-3$_L$), 75.04 (C-3$_L$ *C*H$_2$Ph, C-4 *C*H$_2$Ph), 71.10 (C-6′), 71.05 (C-1$_L$), 70.09 (C-3′), 68.94 (C-6), 68.56 (C-5′), 66.56 (C-2), 55.49 (C-2′), 35.06 (C-2$_L$), 34.32 (C-4$_L$), 32.17–22.94 (C-5$_L$–15$_L$), 20.83 (CO*C*H$_3$), 14.38 (C-16$_L$). HRMS (*m*/*z*) for $C_{65}H_{78}N_4O_{12}S$[M + Na]$^+$: calcd 1161.5337, found 1161.741.

Phenyl 2-azido-4-*O*-benzyl-6-*O*-(4,6-*O*-benzylidene-2-deoxy-2′-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (13). To compound **12** (0.73 g, 0.64 mmol) in ethanol (20 mL) was added hydrazine hydrate (1.5 mL). The reaction mixture was heated under reflux at 90 °C for 5 h, after which TLC analysis showed complete consumption of starting material. The reaction mixture was cooled to room temperature and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (5% MeOH in DCM) to afford free amine (0.59 g, 95% yield) as a white solid. R_f = 0.25 (2% methanol in DCM); $[\alpha]_D^{25}$ = −23.76 (*c* = 1, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.57–7.30 (m, 20H, aromatic), 5.53 (s, 1H, >C*H*Ph), 4.81 (d, 1H, $J_{a,b}$ = 10.8 Hz, H-3$_L$ C*H*$_a$H$_b$Ph), 4.56 (d, 1H, $J_{a,b}$ = 11.4 Hz, H-3$_L$ CH$_a$*H*$_b$Ph), 4.51–4.46 (dd, 2H, *J* = 11.4 Hz, H-4 C*H*$_2$Ph), 4.44 (d, 1H, $J_{1',2'}$ = 10.2 Hz, H-1′), 4.32–4.30 (dd, 1H, $J_{5',6a'}$ = 4.8 Hz, $J_{6a',6b'}$ = 10.2 Hz, H-6a′), 4.26 (d, 1H, $J_{1,2}$ = 7.8 Hz, H-1), 4.09 (bd, 1H, H-6a), 3.96–3.91 (m, 2H, H-1$_L$), 3.76 (t, 1H, $J_{5',6b'}$ = 10.2 Hz, $J_{6b',6a'}$ = 10.2 Hz, H-6b′), 3.63–3.48 (m, 5H, H-6b, H-3, H-4, H-3$_L$, H-5), 3.39 (m, 1H, H-5′), 3.32–3.24 (m, 3H, H-4′, H-3′, H-2′), 2.76 (t, 1H, $J_{2,3}$ = $J_{1,2}$ = 8.4 Hz, H-2), 1.86 (m, 2H, H-2$_L$), 1.47–1.55 (m, 2H, H-4$_L$), 1.34–1.24 [m, 22H, H-(5$_L$–15$_L$)], 0.88 (t, 3H, H-16$_L$); ^{13}C NMR (300 MHz, $CDCl_3$): δ = 126.49–139.10 (aromatic), 105.12 (C-1′), 102.15 (>*C*HPh), 86.17 (C-1), 85.70 (C-4′), 81.58 (C-3), 79.05 (C-3$_L$), 78.16 (C-3′), 76.43 (C-5), 75.19 (C-3$_L$ *C*H$_2$Ph), 73.49 (C-4), 71.20 (C-1$_L$), 71.09 (C-4 *C*H$_2$Ph), 69.09 (C-6), 68.95 (C-6′), 66.66 (C-5′), 65.42 (C-2′), 58.22 (C-2), 35.09 (C-2$_L$), 34.31 (C-4$_L$), 32.15–22.92 (C 5$_L$–15$_L$), 14.36 (C-16$_L$); HRMS (*m*/*z*) for $C_{55}H_{74}N_4O_9S$[M + Na]$^+$: calcd 989.5177, found 989.6476. Lipid **7** (0.26 g, 0.39 mmol) was dissolved in DCM (10 mL) and DCC (92.5 mg, 0.45 mmol) was added. The mixture was stirred for 20 min and then the above free amine was added (0.28 g, 0.29 mmol). The reaction mixture was stirred for 16 h at room temperature after which TLC analysis indicated completion of the reaction. The urea was filtered off over a pad of Celite and the organic solvent was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (15% ethyl acetate in toluene) to afford compound **13** (0.43 g, 89%) as a white solid. R_f = 0.65 (30% ethyl acetate in toluene); $[\alpha]_D^{25}$ = −15.96 (*c* = 1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.57–7.26 (20H, m, aromatic), 6.05 (d, 1H, $J_{NH',2'}$ = 5.5 Hz, N*H*′), 5.57 (s, 1H, >C*H*Ph), 5.07–5.05 (m, 1H, H-3$_{LL}$), 4.79 (d, 1H, $J_{a,b}$ = 11.0 Hz, H-3$_L$ C*H*$_a$H$_b$Ph), 4.75 (d, 1H, $J_{1',2'}$ = 8.5 Hz, H-1′), 4.54–4.46 (m, 4H, H-3$_L$ CH$_a$*H*$_b$Ph, H-1, C-4 C*H*$_2$Ph), 4.36–4.33 (dd, 1H, $J_{5',6a'}$ = 5.0 Hz, $J_{6a',6b'}$ = 10.5 Hz, H-6a′), 4.13–4.11 (bd, 1H, H-6a), 4.08 (t, 1H, H-3′), 3.98–3.91 (m, 2H, H-1$_L$), 3.79 (t, 1H, $J_{5',6a'}$ = 10.5 Hz, $J_{6b',6a'}$ = 10.5 Hz, H-6b′), 3.63–3.56 (m, 3H, H-6b, H-5, H-4′), 3.53–3.44

(m, 3H, H-3_L, H-2′, H-5′), 3.36 (t, 1H, $J_{2,3} = J_{3,4} = 9.0$ Hz, H-3), 3.26–3.21 (m, 2H, H-4, H-2), 2.25–2.31 (m, 4H, H-2_{LL}, H-$2_{LL'}$), 1.89–1.85 (dd, H, H-2_L), 1.60–1.46 (m, 6H, H-4_L, H-4_{LL}, H-$3_{LL'}$), 1.41–1.15 [m, 92H, H-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$)], 0.89 (t, 9H, 16_L, 16_{LL}, $28_{LL'}$); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 174.39$, 172.03 (*C*=O), 126.60–139.08 (aromatic), 102.19 (*C*HPh), 101.41 (C-1′), 85.63 (C-1), 85.51 (C-3), 81.72 (C-4′), 79.09 (C-5), 78.45 (C-4), 76.41 (C-3_L), 75.24 (C-3_L *C*H_2Ph), 71.97 (C-3′), 71.62 (C-3_{LL}), 71.24 (C-1_L), 71.08 (C-4 *C*H_2Ph), 69.14 (C-6), 68.83 (C-6′), 66.67 (C-5), 65.09 (C-2), 59.48 (C-2′), 42.58 (C-2_{LL}, $2_{LL'}$), 35.08 (C-2_L), 34.30–34.79 (C-4_L, C-4_{LL}, C-$3_{LL'}$), 32.16–22.92 [C-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$], 14.36 (16_L, 16_{LL}, $28_{LL'}$); HRMS (*m*/*z*) for $C_{99}H_{158}N_4O_{12}S$[M + Na]$^+$: calcd 1650.1597, found 1650.2234.

Phenyl 4-*O*-benzyl-6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3′-benzyloxy-hexadecanoyl]-2-deoxy-2′-[(*R*)-3-octacosanoyloxy-hexadecan]-amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-1-thio-β-D-glucopyranoside (14). Compound **13** (0.26 g, 0.16 mmol) was dissolved in DCM (10 mL) and zinc dust (0.11 g, 1.67 mmol) was added followed by acetic acid (100 μL, 1.75 mmol). The reaction mixture was stirred for 2 h at room temperature after which TLC analysis showed completion of the reaction. The reaction mixture was washed with $NaHCO_3$ (10 mL), water (10 mL) and the organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (2% MeOH in DCM) to afford a free amine (0.22 g, 84%) as a white solid. $R_f = 0.45$ (2% MeOH in DCM); $[\alpha]_D^{25} = -9.24$ ($c = 1$, $CHCl_3$). ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.56$–7.28 (m, 20H, aromatic), 6.05 (d, 1H, $J_{NH',2'} = 6.6$ Hz, N*H*′), 5.57 (s, 1H, >C*H*Ph), 5.11–5.09 (m, 1H, H-3_{LL}), 4.78 (d, 1H, $J_{a,b} = 11.0$ Hz, H-3_L-CH_aH$_b$Ph), 4.74 (d, 1H, $J_{1',2'} = 8.5$ Hz, H-1′), 4.56–4.44 (m, 4H, H-3_L-CH$_a$*H*$_b$Ph, H-1, H-4 CH_2Ph), 4.35–4.32 (dd, 1H, $J_{6a',5'} = 5.4$ Hz, $J_{6a',6b'} = 12.6$ Hz, H-6a′), 4.12–4.14 (m, 1H, H-6a), 4.08 (t, 1H, $J = 10.8$ Hz, $J = 11.4$ Hz, H-3′), 4.00–3.97 (m, 1H, H-1_L-CH_1H), 3.87–3.85 (m, 1H, H-1_L-CHH_2), 3.79 (t, 1H, $J_{6b',5'} = 12.0$ Hz, $J_{6b',6a'} = 12.6$ Hz, H-6b′), 3.66–3.61 (m, 2H, H-4, H-3_L), 3.60–3.43 (m, 4H, H-6b, H-4′, H-2′, H-5′), 3.32–3.24 (m, 2H, H-3, H-5), 2.75 (t, 1H, $J = 10.8$ Hz, $J = 11.4$ Hz, H-2), 2.27–2.23 [m, 4H, H-(2_{LL}, $2_{LL'}$)], 1.86–1.83 (dd, 2H, H-2_L), 1.58–1.45 [m, 6H, H-(4_L, 4_{LL}, $3_{LL'}$)], 1.39–1.14 [m, 92H, H-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$)], 0.896 (t, 9H, H-16_L, 16_{LL}, $28_{L''}$). ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 139.02$–126.61 (aromatic), 102.17 (>*C*HPh), 101.48 (C-1′), 88.58 (C-1), 86.85 (C-4), 81.72 (C-4′), 79.31 (C-3), 78.98 (C-2), 76.36 (C-3_L), 75.07 (C-3_L *C*H_2Ph), 72.07 (C-3′), 71.49 (C-3_{LL}), 70.88 (C-1_L), 70.78 (C-4 *C*H_2Ph), 69.36 (C-6), 68.48 (C-6′), 66.67 (C-5′),59.52 (C-2′), 42.42 (C-2_{LL}, $2_{LL'}$), 35.04 (C-4_L), 34.13–34.78 (C-4_{LL}, $3_{LL'}$, 2_L), 22.92–32.16 (C-5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$), 14.35 (16_L, 16_{LL}, $28_{LL'}$). HRMS (*m*/*z*) for $C_{99}H_{160}N_2O_{12}S$[M + Na]$^+$: calcd 1624.1692, found 1624.3170. Lipid **8** (0.23 g, 0.62 mmol) was dissolved in DCM (13 mL) followed by the addition of DCC (0.16 g, 0.75 mmol) and DMAP (0.046 g, 0.38 mmol). The reaction mixture was stirred for 20 min followed by addition of the above amino compound (0.20 g, 0.13 mmol). The reaction mixture was stirred for 16 h at room temperature after which TLC analysis indicated completion of the reaction. The urea was filtered off over a pad of Celite and the organic solvent was concentrated *in vacuo*. The crude product was purified by silica gel chromatography (20% ethyl acetate in toluene) to afford compound **14** (0.25 g, 86% yield) as a white solid. $R_f = 0.35$ (20% ethyl acetate in toluene); $[\alpha]_D^{25} = -34.48$ ($c = 1$, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.23$–7.49 (m, 30H, aromatic), 6.66 (d, 1H, $J_{NH',2'} = 9.0$ Hz, N*H*′), 5.43 (s, 1H, >C*H*Ph), 5.37 (d, 1H, $J_{NH,2} = 9.0$ Hz, N*H*), 5.26 (t, 1H, $J = 9.5$ Hz, $J = 10$ Hz, H-3′), 5.02–4.96 (m, 1H, H-3_L), 4.75 (d, 1H, $J_{a,b} = 11.0$ Hz, H-3_L CH_aH$_b$Ph), 4.71 (d, 1H, $J_{1',2'} = 8.5$ Hz, H-1′), 4.57 (d, 1H, $J_{b,a} = 11.0$ Hz, H-3 $_L$ CH$_a$*H*$_b$Ph), 4.52–4.39 (m, 7H, H-1, H-4 CH_2Ph, H-3_{LA} × 2 CH_2Ph), 4.34–4.32 (dd, 1H, $J_{5',6a'} = 5.5$ Hz, $J_{6a',6b'} = 11.0$ Hz, H-6a′), 4.01 (d, 1H, $J_{a,b} = 11.0$ Hz, H-6a), 3.86–3.62 (m, 9H, H-2, H-6b′, H-3_L, H-3_{LA} C*H* × 2, H-1_L, H-6b, H-2′), 3.54–3.40 (m, 5H, H-3, H-4, H-5, H-4′, H-5′), 2.69–2.40 (m, 4H, H-2_{LA} × 2), 2.31–2.07 (m, 4H, 2_{LL}, $2_{LL'}$), 1.76–1.79 (m, 2H, 2_L), 1.68–1.39 [m, 10H, H-(4_L, 4_{LL}, $3_{LL'}$, 4_{LA}], 1.33–1.27 (m, 136H, 5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}–15_{LA} × 2), 0.90 (t, 15H, 16_L, 16_{LL}, $28_{L''}$, 16_{LA} × 2); ^{13}C NMR (300 MHz, $CDCl_3$): $\delta = 174.00$ (*C*=O), 171.44 (*C*=O), 171.32 (*C*=O), 169.70 (*C*=O), 139.22–126.33 (aromatic), 101.61 (>*C*HPh, C-1), 86.18 (C-1′), 83.68 (C-5), 79.79 (C-4′), 79.17 (C-4), 78.21 (C-5′), 76.35 (C-1_L), 75.82 (C-3), 74.84 (C-3_L *C*H_2Ph), 71.69 (C-3′), 71.42 (C-4 *C*H_2Ph), 71.32–70.65 (C-3_L, C-3_{LA} *C*H_2Ph), 70.25 (C-3_{LL}), 68.87 (C-6′), 68.56 (C-6), 55.21 (C-2), 55.02 (C-2′), 41.67, 41.53 (C-2_{LL}, $2_{LL'}$), 39.81 (C-2_{LA}), 35.09 (C-2_L), 34.80–34.29 [C-(4_{LL}, $3_{LL'}$, 4_L, 4_{LA} × 2)], 32.16–22.92 [C-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}–15_{LA})], 14.34 (C-16_L, 16_{LL}, $28_{LL'}$, 16_{LA}); HRMS (*m*/*z*) for $C_{99}H_{158}N_4O_{12}S$[M + Na]$^+$: calcd 2312.7020, found 2312.8816.

4-*O*-Benzyl 6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3′-benzyloxy-hexadecanoyl]-2-deoxy-2′-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucopyranose (15). Compound **14** (0.05 g, 0.02 mmol) was dissolved in a mixture of DCM and water (3.0 mL, 100 : 1 v/v) and the resulting solution was cooled to 0 °C. NIS (0.03 g, 0.13 mmol) and TMSOTf (0.5 μL, 0.28 μmol) were added and after stirring for 30 min at 0 °C, TLC analysis indicated completion of the reaction. It was then quenched with pyridine (0.1 mL) and washed with $Na_2S_2O_3$ (8 mL, 15%) and water (8 mL). The organic layer was dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography (1% MeOH in DCM) followed by size exclusion chromatography over LH-20 (MeOH–DCM, 1 : 1 v/v) to yield **15** (0.021 g, 44%) as a white solid. $R_f = 0.40$ (1% methanol–DCM); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.48$–7.24 (m, 25H, aromatic), 6.51 (d, 1H, $J_{NH,2} = 9.5$ Hz, N*H*), 5.91 (d, 1H, $J_{NH',2'} = 8.5$ Hz, N*H*′), 5.45 (s, 1H, >C*H*Ph), 5.43–5.39 (m, 1H, H-3′), 5.16 (d, 1H, $J_{1',2'} = 8.5$ Hz, H-1′), 5.09 (bs, 1H, H-1), 4.99–4.97 (m, 1H, H-3_{LL}), 4.81 (d, 1H, $J_{a,b} = 11$ Hz, H-3_L CH_aH$_b$Ph), 4.54–4.38 (m, 7H, H-4 CH_2Ph, H-3_L CH$_a$*H*$_b$Ph, H-3_{LA} CH_2Ph × 2), 4.38–4.34 (dd, 1H, $J_{5',6'} = 5.5$ Hz, $J_{6a',6b'} = 11$ Hz, H-6a′), 4.11–4.16 (m, 1H, H-2), 4.02–3.97 (m, 2H, H-1_L), 3.86–3.74 (m, 5H, H-6a, H-3_L, H-3_{LA} C*H* × 2, H-6b′), 3.68–3.62 (m, 5H, H-2′, H-6b, H-5′, H-4′, H-3), 3.58–3.42 (m, 3H, H-5, H-4, H-1 O*H*), 2.67–2.49 (m, 4H, H-2 $_{LA}$ CH_2 × 2), 2.37–2.20 (m, 4H, H-2_{LL}, $2_{LL'}$) 1.82–1.72 (m, 2H, H-2_L), 1.60–1.48 (10H, 4_L, 4_{LL}, $3_{LL'}$, 4_{LA}CH_2 × 2), 1.39–1.27 [m, 136H, H-(5_L–15_L, 5_{LL}–15_{LL}, $4_{LL'}$–$27_{LL'}$, 5_{LA}–15_{LA} × 2], 0.89 (t, 15H, H-16_L, 16_{LL}, $28_{L''}$, 16_{LA} × 2); HRMS (*m*/*z*) for $C_{139}H_{228}N_2O_{17}$[M + Na]$^+$: calcd 2220.6936, found 2220.9749.

4-*O*-Benzyl 6-*O*-(4,6-*O*-benzylidene-3-*O*-[(*R*)-3′-benzyloxy-hexadecanoyl]-2-deoxy-2′-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucono-1,5-lactone (16). Compound **15** (0.013 g, 5.92 μmol) was dissolved in DCM (2 mL) and molecular sieves (3 Å, 0.02 g) were added and, after stirring the resulting suspension for 2 h under an atmosphere of argon, PCC (0.063 g, 29.6 μmol) was added. The reaction mixture was stirred for 2 h at room temperature after which TLC analysis indicated completion of the reaction. After concentration *in vacuo*, the crude product was purified by iatrobead column chromatography (20% ethyl acetate in toluene) to afford **16** (0.008 g, 62%) as a white solid. $R_f = 0.60$ (20% ethyl acetate in toluene); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.48$–7.24 (m, 25H, aromatic), 6.99 (d, 1H, $J_{NH,2} = 8.5$ Hz, N*H*), 6.66 (d, 1H, $J_{NH',2'} = 7.5$ Hz, N*H*′), 5.67 (t, 1H, $J = 9.5$ Hz, $J = 9.0$ Hz, H-3′), 5.39 (s, 1H, >C*H*Ph), 5.12 (m, 1H, H-3$_{LL}$), 5.00 (d, 1H, $J_{1',2'} = 8.0$ Hz, H-1′), 4.74 (d, 1H, $J_{a,b} = 11.0$ Hz, H-3$_L$ CH_aH$_b$Ph), 4.62–4.38 (m, 8H, H-2, C-4 CH_2Ph, H-3 $_L$ CH$_a$$H_b$Ph, H-3′$_{LA}$ CH_2Ph, H-2$_{LA}$ CH_2Ph), 4.31–4.28 (dd, 1H, $J_{5',6a'} = 4.0$ Hz, $J_{6a',6b'} = 10.5$ Hz, H-6a′), 4.08 (d, 1H, $J = 10.5$ Hz, H-6a), 3.84–3.80 (m, 2H, H-1$_L$), 3.74–3.65 (m, 4H, H-6b′, H-3$_L$, H-3$_{LA}$ C*H* ×2), 3.63–3.47 (m, 7H, H-4′, H-5′, H-6b, H-2′, H-4, H-5, H-3), 2.69–2.46 (m, 4 H, H-2$_{LA}$, H-3′$_{LA}$), 2.42–2.24 (m, 4H, H-2$_{LL}$, 2$_{LL'}$), 2.07–1.44 (m, 12H, H-2$_L$, 4$_L$, 4$_{LL}$, 3$_{LL'}$, H-4$_{LA}$), 1.27–1.02 (m, 136H, 5$_L$–15$_L$, 5$_{LL}$–15$_{LL}$, 4$_{LL'}$–27$_{LL'}$, 5$_{LA}$–15$_{LA}$), 0.98–0.72 (t, 15H, 16$_L$, 16$_{LL}$, 28L″, 16$_{LA}$); HRMS (*m*/*z*) for $C_{139}H_{226}N_2O_{17}$[M + Na]$^+$: calcd 2218.6779, found 2218.8311.

2-Deoxy-6-*O*-(2′-deoxy-3-*O*-[(*R*)-3′-benzyloxy-hexadecanoyl]-2′-[(*R*)-3-octacosanoyloxy-hexadecan]amido-β-D-glucopyranosyl)-2-[(*R*)-3-benzyloxy-hexadecan]-amido-3-*O*-[(*R*)-3-benzyloxy-hexadecanoyl]-2-deoxy-α-D-glucono-1,5-lactone (3). Pd/C (10 mg, 10 wt%) was added to compound **16** (4.5 mg, 2.05 μmol) dissolved in a mixture of THF–*t*-BuOH (2 mL, 1 : 1, v/v). The flask was degassed and filled with H_2 gas and then stirred for 12 h. The reaction progress was monitored by MALDI. After completion of the reaction, the catalyst was filtered off through a PTFE filter and washed with THF (0.5 mL × 3) The combined filtrates were concentrated *in vacuo* and the residue was purified by LH-20 size exclusion column chromatography (*i*-PrOH–DCM, 1 : 1, v/v) to yield compound **3** (2.3 mg, 66%) as a white solid. ^{1}H NMR (500 MHz, THF-D : $(CD_3)_2$CDOD 1 : 1): $\delta = 5.12$ (m, 1H, 3$_{LL}$), 5.02 (t, 1H, $J = 10.0$ Hz, $J = 9.0$ Hz, H-3′), 4.67 (d, 1H, $J_{1',2'} = 9.0$ Hz, H-1′), 4.21 (m, 1H, H-2), 4.16 (dd, 1H, $J_{5'6'} = 2.0$ Hz, $J_{6a',6b'} = 11.5$ Hz, H-6a′), 4.05 (m, 1H, H-6a), 3.94 (m, 2H, H-1$_L$), 3.86–3.45 (m, 11H, H-6b′, H-2′, 3$_L$, H-3$_{LA}$ × 2 C*H*, H-6b, H-5′, H-4′, H-3, H-4, H-5), 2.46–2.18 (m, 14H, H-2$_{LA}$, 2$_{LL}$, 2$_{LL'}$, 2$_L$, 4$_L$), 2.06–1.94 (m, 6H, H-4$_{LL}$, 3$_{LL'}$, 4$_{LA}$), 1.84–1.27 (bm, 136H, 5$_L$–15$_L$, 5$_{LL}$–15$_{LL}$, 4$_{LL'}$–27$_{LL'}$, H-5$_{LA}$–15$_{LA}$), 1.65–0.67 (bm, 15H, 16$_L$, 16$_{LL}$, 28$_{L''}$, 16$_{LA}$); HRMS (*m*/*z*) for $C_{104}H_{198}N_2O_{17}$[M + Na]$^+$: calcd 1770.4588, found 1770.7673.

Biological experiments

Reagents for biological experiments. *E. coli* 055:B5 LPS was obtained from List Biologicals, Pam_3CysSK_4 was obtained from Calbiochem and *R. sin*-1 LPS and lipid A were kindly provided by Dr R. Carlson (CCRC, Athens, GA). All data presented in this study were generated using the same batches of *E. coli* 055:B5 LPS and *R. sin*-1 LPS. The synthesis of *E. coli* lipid A has been reported elsewhere.[46] The *E. coli* lipid A was reconstituted in PBS with DMSO (10%) and stored at −80 °C. Synthetic compounds **1** and **3** were stored lyophilized at −80 °C and reconstituted in dry THF on the day of the experiment; final concentrations of THF in the biological experiments never exceeded 0.5% to avoid toxic effects.

Cell maintenance. Mono Mac 6 (MM6) cells, provided by Dr H.W.L. Ziegler-Heitbrock (Institute for Inhalation Biology, Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (BioWhittaker) supplemented with penicillin (100 u mL^{-1})–streptomycin (100 μg mL^{-1}; Mediatech, OPI supplement (1%; Sigma; containing oxaloacetate, pyruvate and bovine insulin) and fetal calf serum (FCS; 10%; HyClone). New batches of frozen cell stock were grown up every 2 months and growth morphology evaluated. Before each experiment, MM6 cells were incubated with calcitriol (10 ng mL^{-1}; Sigma) for 2 days to differentiate into macrophage like cells. RAW 264.7 γNO(−) cells, derived from the RAW 264.7 mouse monocyte–macrophage cell line, were obtained from ATCC. The cells were maintained in RPMI 1640 medium (ATCC) with L-glutamine (2 mM), adjusted to contain sodium bicarbonate (1.5 g L^{-1}), glucose (4.5 g L^{-1}), HEPES (10 mM) and sodium pyruvate (1.0 mM) and supplemented with penicillin (100 u mL^{-1})–streptomycin (100 μg mL^{-1}) and FBS (10%). Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium (ATCC) with L-glutamine (4 mM), glucose (4.5 g L^{-1}) and sodium bicarbonate (1.5 g L^{-1}) supplemented with penicillin (100 u mL^{-1})–streptomycin (100 μg mL^{-1}), Normocin (100 μg mL^{-1}; InvivoGen) and FBS (10%). Stably transfected HEK 293T cells with murine TLR4/MD2 and murine TLR2/TLR6 were obtained from InvivoGen and grown in the same growth medium as for HEK 293T cells supplemented with the appropriate selective agents HygroGold (50 μg mL^{-1}; InvivoGen) and blasticidin (10 μg mL^{-1}; InvivoGen). All cells were maintained in a humid 5% CO_2 atmosphere at 37 °C.

Cytokine induction and ELISAs. On the day of the exposure assay differentiated MM6 cells were harvested by centrifugation and gently suspended (10^6 cells mL^{-1}) in prewarmed (37 °C) medium and RAW 264.7 γNO(−) cells were plated as 2×10^5 cells per well in 96-well tissue culture plates (Nunc). Cells were then incubated with different combinations of stimuli for 5.5 hours. Culture supernatants were then collected and stored frozen (−80 °C) until assayed for cytokine production. All cytokine ELISAs were performed in 96-well MaxiSorp plates (Nalge Nunc International). Concentrations of human TNF-α protein in culture supernatants were determined by a solid phase sandwich ELISA. Plates were coated with purified mouse anti-human TNF-α antibody (Pharmingen). TNF-α in standards and samples was allowed to bind to the immobilized antibody. Biotinylated mouse anti-human TNF-α antibody (Pharmingen) was then added, producing an antibody–antigen–antibody "sandwich". After addition of avidin–horseradish peroxidase conjugate (Pharmingen) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories), a green color was produced in direct proportion to the amount of TNF-α present in the sample. The reaction was stopped by adding peroxidase stop solution (Kirkegaard & Perry Laboratories) and the absorbance was measured at 405 nm using a microplate reader (BMG Labtech). Cytokine DuoSet ELISA Development Kits (R & D Systems) were used for the cytokine

quantification of human IP-10 and mouse TNF-α according to the manufacturer's instructions. The absorbance was measured at 450 nm with wavelength correction set to 540 nm. Concentrations of mouse IFN-β in culture supernatants were determined as follows. Plates were coated with rabbit polyclonal antibody against mouse IFN-β (PBL Biomedical Laboratories). IFN-β in standards and samples was allowed to bind to the immobilized antibody. Rat anti-mouse IFN-β antibody (USBiological) was then added, producing an antibody-antigen-antibody "sandwich". Next, horseradish peroxidase (HRP) conjugated goat anti-rat IgG (H + L) antibody (Pierce) and a chromogenic substrate for HRP 3,3′,5,5′-tetramethylbenzidine (TMB; Pierce) were added. After the reaction was stopped, the absorbance was measured at 450 nm with wavelength correction set to 540 nm. All cytokine values are presented as the means ± SD of triplicate measurements, with each experiment being repeated three times.

Transfection and NF-κB activation assay. The day before transfection, HEK 293T wild type cells and HEK 293T cells stably transfected with murine TLR2/TLR6 or murine TLR4/MD2 were plated in 96-well tissue culture plates (16000 cells per well). The next day, cells were transiently transfected using PolyFect Transfection Reagent (Qiagen) with expression plasmids pELAM-Luc (NF-κB-dependent firefly luciferase reporter plasmid, 50 ng per well)[47] and pRL-TK (*Renilla* luciferase control reporter vector, 1 ng per well; Promega) as an internal control to normalize experimental variations. The empty vector pcDNA3 (Invitrogen) was used as a control and to normalize the DNA concentration for all of the transfection reactions (total DNA 70 ng per well). Forty-four h post-transfection, cells were exposed to the stimuli in the presence of FCS to provide sCD14 at the indicated concentrations for 4 h, after which cell extracts were prepared. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and a combination luminometer–fluorometer microplate reader (BMG Labtech). Expression of the firefly luciferase reporter gene was normalized for transfection efficiency with expression of *Renilla* luciferase. The data are reported as the means ± SD of triplicate treatments. The transfection experiments were repeated at least twice.

Data analysis. Concentration–response and inhibition data were analyzed using nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). Concentration–response data were fit with the following four parameter logistic equation: $Y = E_{max}/(1 + (EC_{50}/X)^{Hill\ slope})$, where Y is the cytokine response, X is logarithm of the concentration of the stimulus, E_{max} is the maximum response and EC_{50} is the concentration of the stimulus producing 50% stimulation. The Hillslope was set at 1 to be able to compare the EC_{50} values of the different inducers. Inhibition data were fit with the following logistic equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(X - \log IC_{50})})$, where Y is the cytokine response, X is the logarithm of the concentration of the inhibitor and IC_{50} is the concentration of the inhibitor that reduces the response by half.

Acknowledgements

This research was supported by the Institute of General Medicine of the National Institutes of Health (GM061761).

References

1 B. Beutler, *Mol. Immunol.*, 2004, **40**, 845–859.
2 E. S. van Amersfoort, T. J. C. van Berkel and J. Kuiper, *Clin. Microbiol. Rev.*, 2003, **16**, 379.
3 S. Akira and K. Takeda, *Nat. Rev. Immunol.*, 2004, **4**, 499–511.
4 K. Takeda, T. Kaisho and S. Akira, *Annu. Rev. Immunol.*, 2003, **21**, 335–376.
5 A. F. McGettrick and L. A. J. O'Neill, *Mol. Immunol.*, 2004, **41**, 577–582.
6 M. Yamamoto, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda and S. Akira, *Science*, 2003, **301**, 640–643.
7 F. J. Roberts, I. W. Geere and A. Coldman, *Rev. Infect. Dis.*, 1991, **13**, 34–46.
8 D. Pittet, D. Tarara and R. P. Wenzel, *JAMA, J. Am. Med. Assoc.*, 1994, **271**, 1598–1601.
9 M. Pollack and C. A. Ohl, *Curr. Top. Microbiol. Immunol.*, 1996, **216**, 275–297.
10 D. T. Golenbock, R. Y. Hampton, N. Qureshi, K. Takayama and C. R. Raetz, *J. Biol. Chem.*, 1991, **266**, 19490–19498.
11 F. Peri, C. Marinzi, M. Barath, F. Granucci, M. Urbano and F. Nicotra, *Bioorg. Med. Chem.*, 2006, **14**, 190–199.
12 O. R. Martin, W. Zhou, X. F. Wu, S. Front-Deschamps, S. Moutel, K. Schindl, P. Jeandet, C. Zbaeren and J. A. Bauer, *J. Med. Chem.*, 2006, **49**, 6000–6014.
13 J. H. Krauss, U. Seydel, J. Weckesser and H. Mayer, *Eur. J. Biochem.*, 1989, **180**, 519–526.
14 W. Christ, P. McGuinness, O. Asano, Y. Wang, M. Mullarkey, M. Perez, L. Hawkins, T. Blythe, G. Dubuc and A. Robidoux, *J. Am. Chem. Soc.*, 1994, **116**, 3637–3638.
15 M. Shiozaki, Y. Watanabe, Y. Iwano, T. Kaneko, H. Doi, D. Tanaka, T. Shimozato and S. Kurakata, *Tetrahedron*, 2005, **61**, 5101–5122.
16 D. P. Rossignol, L. D. Hawkins, W. J. Christ, S. Kobayashi, T. Kawata, M. Lynn, I. Yamatsu and Y. Kishi, in *Endotoxin in Health and Disease*, ed. B. Helmut, S. M. Opal, S. N. Vogel and D. C. Morrison, Marcel Dekker, Inc., New York, 1999, vol. 1, pp. 699–717.
17 M. L. Vandenplas, R. W. Carlson, B. Jeyaretnam, B. McNeill, M. H. Barton, N. Norton, T. F. Murray and J. N. Moore, *J. Biol. Chem.*, 2002, **277**, 41811–41816.
18 A. V. Demchenko, M. A. Wolfert, B. Santhanam, J. N. Moore and G. J. Boons, *J. Am. Chem. Soc.*, 2003, **125**, 6103–6112.
19 R. Girard, T. Pedron, S. Uematsu, V. Balloy, M. Chignard, S. Akira and R. Chaby, *J. Cell Sci.*, 2003, **116**, 293–302.
20 T. Pedron, R. Girard, B. Jeyaretnam, R. W. Carlson and R. Chaby, *Immunology*, 2000, **101**, 262–270.
21 B. Jeyaretnam, J. Glushka, V. S. K. Kolli and R. W. Carlson, *J. Biol. Chem.*, 2002, **277**, 41802–41810.
22 B. Santhanam, M. A. Wolfert, J. N. Moore and G. J. Boons, *Chem.–Eur. J.*, 2004, **10**, 4798–4807.
23 H. S. Lee, M. A. Wolfert, Y. H. Zhang and G. J. Boons, *ChemBiochem*, 2006, **7**, 140–148.
24 Y. Watanabe, K. Miura, M. Shiozaki, S. Kanai, S. Kurakata and M. Nishijima, *Carbohydr. Res.*, 2003, **338**, 47–54.
25 M. Shiozaki, Y. Kobayashi, M. Arai, N. Ishida, T. Hiraoka, M. Nishijima, S. Kuge, T. Otsuka and Y. Akamatsu, *Carbohydr. Res.*, 1991, **222**, 69–82.
26 Y. Watanabe, K. Miura, M. Shiozaki, S. Kanai, S. Kurakata and M. Nishijima, *Carbohydr. Res.*, 2001, **332**, 257–277.
27 G. J. Boons, *Tetrahedron*, 1996, **52**, 1095–1121.
28 P. J. Garegg, *Adv. Carbohydr. Chem. Biochem.*, 1997, **52**, 179–205.
29 H. Weidmann and H. K. Zimmerman, *Justus Liebigs Ann. Chem.*, 1959, **628**, 255–256.
30 K. Fukase, Y. Fukase, M. Oikawa, W. C. Liu, Y. Suda and S. Kusumoto, *Tetrahedron*, 1998, **54**, 4033–4050.
31 S. Mehta and B. M. Pinto, *J. Org. Chem.*, 1993, **58**, 3269–3276.
32 S. Hatakeyama, H. Mori, K. Kitano, H. Yamada and M. Nishizawa, *Tetrahedron Lett.*, 1994, **35**, 4367–4370.
33 D. S. Keegan, S. R. Hagen and D. A. Johnson, *Tetrahedron: Asymmetry*, 1996, **7**, 3559–3564.
34 H. M. Zuurmond, G. A. van der Marel and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, 1991, **110**, 301–302.
35 S. Mehta and B. M. Pinto, *Tetrahedron Lett.*, 1991, **32**, 4435–4438.
36 S. Kurihara, T. Tsumuraya and I. Fujii, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 1179–1184.

37 G. H. Veeneman, S. H. van Leeuwen and J. H. van Boom, *Tetrahedron Lett.*, 1990, **31**, 1331–1334.
38 S. M. Zughaier, Y. L. Tzeng, S. M. Zimmer, A. Datta, R. W. Carlson and D. S. Stephens, *Infect. Immun.*, 2004, **72**, 371–380.
39 S. M. Zughaier, S. M. Zimmer, A. Datta, R. W. Carlson and D. S. Stephens, *Infect. Immun.*, 2005, **73**, 2940–2950.
40 M. G. Netea, B. J. Kullberg, L. A. Joosten, T. Sprong, I. Verschueren, O. C. Boerman, F. Amiot, W. B. van den Berg and J. W. van der Meer, *Eur. J. Immunol.*, 2001, **31**, 2529–2538.
41 G. Mathiak, K. Kabir, G. Grass, H. Keller, E. Steinringer, T. Minor, C. Rangger and L. F. Neville, *Int. J. Mol. Med.*, 2003, **11**, 41–44.
42 P. von der Ley, L. Steeghs, H. J. Hamstra, J. ten Hove, B. Zomer and L. van Alphen, *Infect. Immun.*, 2001, **69**, 5981–5990.
43 C. Werts, R. I. Tapping, J. C. Mathison, T. H. Chuang, V. Kravchenko, I. Saint Girons, D. A. Haake, P. J. Godowski, F. Hayashi, A. Ozinsky, D. M. Underhill, C. J. Kirschning, H. Wagner, A. Aderem, P. S. Tobias and R. J. Ulevitch, *Nat. Rev. Immunol.*, 2001, **2**, 346–352.
44 M. Hirschfeld, J. J. Weis, V. Toshchakov, C. A. Salkowski, M. J. Cody, D. C. Ward, N. Qureshi, S. M. Michalek and S. N. Vogel, *Infect. Immun.*, 2001, **69**, 1477–1482.
45 A. O. Aliprantis, R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski and A. Zychlinsky, *Science*, 1999, **285**, 736–739.
46 Y. Zhang, J. Gaekwad, M. A. Wolfert and G. J. Boons, *J. Am. Chem. Soc.*, 2007, **129**, 5200–5216.
47 J. C. Chow, D. W. Young, D. T. Golenbock, W. J. Christ and F. Gusovsky, *J. Biol. Chem.*, 1999, **274**, 10689–10692.