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Synthesis and In Vitro Activities of a Spacer-containing Glycophospholipid Ligand of a Lipopolysaccharide Receptor Involved in Endotoxin Tolerance

Daniel Charon,[a] Michelle Mondange,[a] Jean-François Pons,[b] Karine Le Blay[b] and Richard Chaby[b,*]

[a]*Equipe "Endotoxines", Centre National de la Recherche Scientifique, Université de Paris-Sud, Centre d'Etudes Pharmaceutiques, 92290 Châtenay-Malabry, France*

[b]*Equipe "Endotoxines", Centre National de la Recherche Scientifique, Université de Paris-Sud, Faculté des Sciences d'Orsay, 91405 Orsay, France*

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Abstract—A glycophospholipid consisting in a derivative of *N*,*N*′-acylated and bisphosphorylated 2,3-dideoxy-2,3-diamino-D-glucose, bearing a 6-aminocaproyl side chain as spacer arm at carbon 6 (PPDm2-B), has been synthesized and its effect on murine macrophages evaluated. The synthesis started from 2,3-diamino-D-glucose, which was best obtained from glucosamine essentially by known procedures, since attempts to use another known precursor (3-nitro-glycoside) led to unexpected results. Selective *N*-acylation was performed with the hydroxysuccinimide ester of (D)-3-benzyloxymyritic acid followed by esterification of the sole primary hydroxyl function by 6-azidocaproylchloride and phosphorylation of the resulting 1,4-diol by treatment with tetrabenzyl pyrophosphate. Hydrogenation on a Pd on carbon catalyst permitted the isolation of 6-(6-aminohexanoyl)-2,3-dideoxy-2,3-di-[(*R*)-3-hydroxy-tetradecanamido]-α-D-glucopyranose 1,4-diphosphate (PPDm2-B). In mouse macrophages, PPDm2-B enhanced the lipopolysaccharide (LPS)-dependent secretion of tumor necrosis factor alpha (TNF-α), and inhibited the LPS-induced desensitization of these cells. The data suggest that PPDm2-B interacts in a serum-independent way with an LPS receptor different from CD14, and involved in endotoxin tolerance. Binding studies of a fluorescent derivative of PPDm2-B indicated that the expression of this unknown receptor is down-regulated during in vitro culture of the cells. Owing to its spacer arm, PPDm2-B could thus be a promising tool for future studies of this receptor.

Introduction

The endotoxin of Gram-negative bacteria, chemically characterized as a lipopolysaccharide (LPS), produces an extraordinary array of pathophysiological disorders (fever, inflammation, disseminated intravascular coagulation, adult respiratory distress syndrome, multiple organ failure, and even death) in infected hosts.[1] It is now generally recognized that these pathophysiologic effects are elicited in the host by various mediators, and particularly by tumor necrosis factor α (TNF-α).[2] However, a development of a tolerant state to the pathophysiologic effects of LPS (endotoxin tolerance) can be induced by pre-exposure to sublethal doses of LPS.[3] A similar effect can also be induced in vitro: pre-exposure of macrophages to LPS desensitizes the cells.[4,5] Little is known about the mechanism of this desensitization. In a search for an understanding of the cellular and molecular basis of LPS effects (activation or desensitization) it is reasonable to assume that cellular responses to LPS require preliminary interactions with LPS receptors. Among a number of cell surface LPS-binding sites that have been detected[6] CD14 is the most

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*Corresponding author: Mailing address: Equipe "Endotoxines", ERS-0571 du C.N.R.S., Bâtiment 432, Université de Paris-Sud, 91405 Orsay, France. Tel: 33-169154830; Fax: 33-169853715; E-mail: rchaby@mailhost.pasteur.fr

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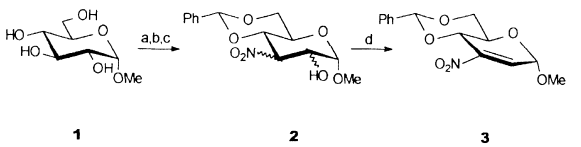
extensively studied since it has clearly been shown to play an important role in mediating LPS effects,[7] although its functional activity requires the presence of a serum constituent, the LPS-binding protein (LBP).[8]

However, several recent observations[9,10] pointed to the possible existence of other, still undiscovered, LPS receptors. In this regard we reported previously that a signaling LPS receptor, different from CD14, is constitutively present on mouse[11] and human[12] granulocytic bone marrow cells (BMC). We also showed that a synthetic glycophospholipid, consisting of *N*,*N*′-acylated and bisphosphorylated 2,3-dideoxy-2,3-diamino-D-glucose (compound SDZ 280746, also termed PPDm2) can block LPS-induced stimulation of pre-B cells[13] and BMC,[14] and inhibits the binding of LPS to the constitutive receptor of BMC.[9] Therefore, it appeared important to identify the receptor which interacts with this glycophospholipid, and to determine whether this type of LPS receptor is present on other cells of immunological interest. This requires the use of a labeled or reactive derivative of this lipidic ligand. To confer to this compound the ability of easy coupling to various molecules of interest (enzymes, fluorophores, radiolabelable residues, photoactivatable reagents, drugs), an analogue of PPDm2 bearing a spacer arm with amino functionality (6-aminocaproyl chain) was designed. We report here on the synthesis and biological evaluation of the spacer-containing glycophospholipid.

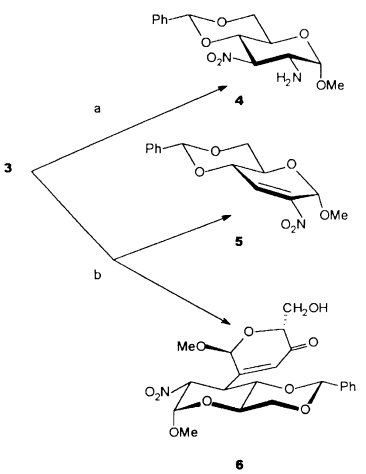
Results and Discussion

Chemistry

The target compound **15** is a highly functionalized derivative of 2,3-diaminoglucose, a monosaccharide whose *N*,*N*′-diacetate was found in lipid A of *Rhodospirillacae*.[15] Syntheses of diacetamido hexoses and hexosides have been performed using 2-acetamidoglycosides as starting material: for instance Meyer zu Reckendorf[16] described the obtention of 2,3-diacetamidoglucose from Benzyl 2-acetamido-2-deoxy-α-glucopyranoside following a sequence of reactions involving a double inversion at C3, first to a 3-*O*-acetylalloside, and then to a 3-azidoglucoside. Keeping in mind firstly that *N*-acetyl group might require harsh removal conditions, and secondly that a successful nucleophilic substitution of *N*-protected 2-amino-3-*O*-sulfonated glucoside (restricted by neighbouring group participation) is dependent on both the nucleophile and the protecting group,[17] we tried the displacement of the good leaving 3-*O*-triflate group on the -β-allyl 4,6-*O*-benzylidene-glucosaminide with either an acetate or a bromide ion: both results were disappointing. Consequently, we decided to obtain 2,3-diaminoglucose following essentially the method of Baer[18] who attained the 2,3-diaminoglucose structure upon addition of ammonia on a 3-nitro-2-hexenopyranoside **3** synthesized from methyl-α-glucopyranoside (Scheme 1). Indeed we were able to obtain a mixture of 4,6-*O*-benzylidene-3-deoxy-3-nitro-α-D-gluco and mannopyranoside on a mole scale with 40% yield (3 steps) and a gluco/manno ratio close to 1 (estimated by NMR). When this mixture was transformed into the 3-nitro olefin upon mesylation,[19] although the reaction appeared rather clean when monitored by TLC, the workup procedure failed several times to give the expected compound, and appeared unreliable. From the dark mixture that was shown by TLC to contain many slow moving products, we were able to identify three compounds. Isolation of a 1:1 mixture of 2-*O*-acetyl-4,6-*O*-benzylidene-3-deoxy-3-nitro-α-glucopyranoside and of the desired compound upon acetylation of the slow moving mixture established that rehydration of the nitro olefin occurred during work up. The known elimination of acetic acid from 2-*O*-acetyl-3-*O*-nitro glycoside under the conditions used to obtain the acetate likely explains the isolation of the nitro olefin. Two cristalline compounds (**5** and **6**) could be separated from the colored sirupy complex mixture by column chromatography (Scheme 2). Both showed in their IR spectrum the absorbtion of a nitro group. Elemental analysis of the first eluted compound **5** (R_f 0.66, EtOAc:cyclohexane,



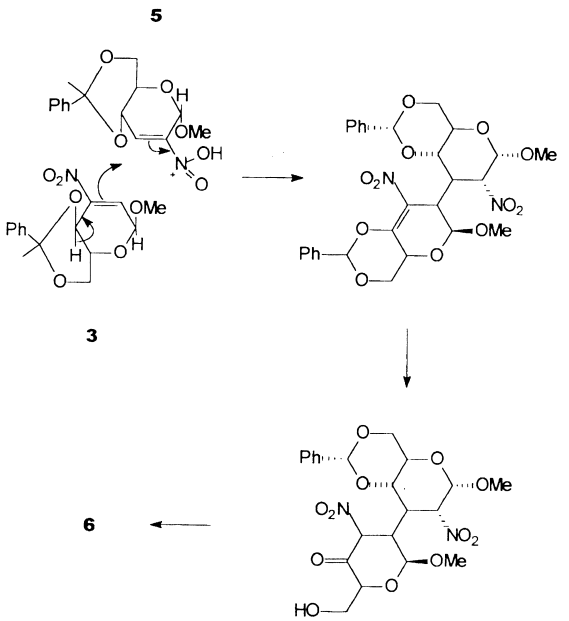
Scheme 1. Reagents (a) $NaIO_4$; (b) CH_3NO_2/MeO^-; (c) C_6H_5-$CH(OMe)_2$, TsOH; (d) CH_3SO_2Cl, Et_3N (2 equiv.).



Scheme 2. Reagents: (a) NH_3-toluene; (b) unexpected decomposition.

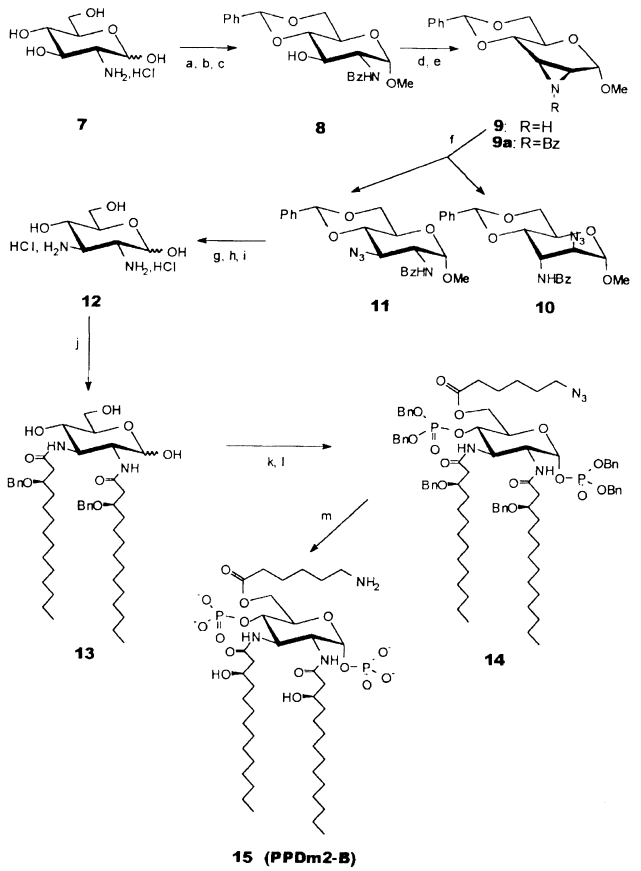
4:6) was consistent with a nitro enopyranoside. ^{1}H NMR indicated that it contains the methoxy and 4,6-benzylidene groups as well as 2 hydrogens whose systems agreed for H4 and H1, but unlike the 3-nitro-2-enopyranoside **3**, the olefinic H_2 could not be identified. Definitive assignment of the structure to the isomeric 2-nitro-hex-2-enopyranoside **5** resulted from X-ray cristallography, which clearly showed the position and substitution of the double bond. The IR spectrum of the compound eluted further displayed, beside the nitro band, the strong absorbtion of a keto group (1680 cm^{-1}). Its elemental analysis corresponded to $C_{21}H_{25}NO_{10}$ and was in agreement with a pic at *m/e* 469 in its chemically (NH_3) ionised mass spectrum. The structure of the second eluted compound, disaccharide **6**, was deduced from extensive NMR studies: the gluco structure of the 4,6-benzylidene methyl pyranoside residue was deduced from the large values of the H_2–H_3, H_3–H_4, and H_4–H_5, coupling constants. The position of the keto group on carbon 4 of the second residue was in agreement with its primary hydrogens being the only one to be consistently displaced downfield upon treatment with trichloroacetylisocyanate and with the A_2B system given by the hydrogens linked to C'_6 and C'_5. The C_3–C'_2 linkage between the two residues was deduced from direct and long range C-H correlations which indicate that C_3 ($\delta = 41$) and C_2 ($\delta = 80$) are respectively linked to a carbon and an heteroatom while $=C'_2$- and $=C'_3H$- have respectively $\delta = 155$ and 124. The mechanism by which these unexpected compounds were formed is not clearly understood. However, formation of the adduct **6** can be visualized as an acidic catalysed addition of the 3-nitro-2-enopyranoside **3** (with loss of hydrogen 4′) on the 2-nitro-2-enopyranoside **5** (Scheme 3). Subsequent easy hydrolysis of the enolic benzylidene group and 2′–3′ elimination of nitrous acid from the ketonic form would yield the isolated compound **6**. Keeping in mind that nitrite treatment of a 3-nitro alkene has been shown[19] to give a 2,3-dinitro alkane leading to the 2-nitro-alkene, and that isomerisation of 3-methyl-2-nitro butene to 3-methyl-1-nitro butene has been reported,[20] it appeared that the instability of the 3-nitro alkene **3** initially formed could lead to the 2-nitro alkene **5**, and adduct **6** (Scheme 2).

In view of the aforementioned results, it appeared more convenient to proceed directly to the addition of ammonia without isolating the 3-nitro olefin. However, the resulting 2-amino-3-nitro glucoside had to be purified by recrystallisation since it was observed that the crude product was a mixture of 2-amino gluco and manno glycosides. Actually, Michael addition of nucleophiles on 3-nitro alkene has been shown to give isomeric mixtures under acidic or neutral conditions.[21] All together, the nitromethane synthesis permitted the obtention of 2,3-diamino-α-methyl glucoside but, as it



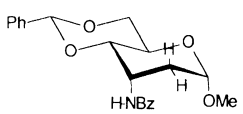
Scheme 3.

was not found easily reproducible, we preferred for routine syntheses the method of Guthrie[22] (Scheme 4) who obtained methyl 3-azido-2-benzamido-2,3-dideoxy-α-D-glucopyranoside by opening with sodium azide the methyl 2,3-benzoylepimino-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside, easily obtained from *N*-benzoyl-glucosamine in five high yielding steps. Contrary to the original report, the major compound resulting from the opening of the epimine had not the gluco but rather the altro configuration (diaxial opening). However despite the rather low yield (25%), the 3-azido glucoside which was highly crystalline was easily recovered from the reaction mixture. Furthermore we were able to obtain the epimine in one step from methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside **8** upon treatment of the latter in an heterogeneous mixture with tosylchloride and a solid base (KOH–K_2CO_3).[23] After quantitative benzoylation the *N*-benzoylated epimine **9a** was isolated (60%). Successive catalytic hydrogenation and acid hydrolysis of the 3-azido-glucoside led to 2,3-diamino-glucose hydrochloride **12** (72%) whose ^{1}H NMR showed that it formed in D_2O a 6:4 α:β equilibrium. It is noteworthy that the azido function of the 3-azido-2-benzamido-glucoside can be reduced to a deoxy function. Indeed, when we attempted *N*-debenzoylation by hydrazinolysis, we were able to isolate two compounds: the minor one **11a** was a 3-amino-2-benzamido-glucoside while the major crystalline one **11b** was methyl 2-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-ribopyranoside. The same reaction on the isomeric 2-azido-3-benzamido-altroside

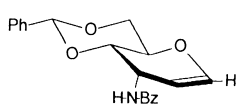


Scheme 4. Reagents: (a) MeONa (1 equiv), $(Bz)_2O$; (b) MeOH/H^+ reflux; (c) C_6H_5-CH $(OMe)_2$/TsOH, DMF, 50 °C; (d) TsCl, K_2CO_3-KOH, Tol/DMSO; (e) BzCl, Pyr; (f) NaN_3, NH_4Cl, DMF, 120 °C; (g) H_2, Pd/C, MeOH; (h) 80% AcOH, 100 °C; (i) 5 N HCl, 100 °C; (j) (*D*)-3-OBn-Myr-O-$C_4H_2NO_2$, $(iPr)_2NEt$ 2 equiv, DMF; (k) $N_3(CH)_5$-COCl 1 equiv, Pyr-$CHCl_3$; (l) LDA, TBPP, THF −70 °C; (m) H_2, $Pd(OH)_2$/C, MeOH.

gave the corresponding 3-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-ribopyranoside **10a** and the allal derivative **10b**.



10 a



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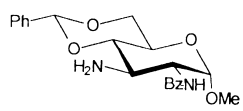
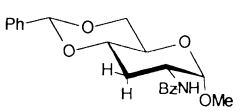


11 a



11 b

Bis-*N*,*N*′-acylation of 2,3-diamino-glucose was performed in good yield using *N*-3-(*R*)-benzyloxymyristoyloxysuccinimide.[24] It was best obtained upon acid catalysed benzylation of the stable 3-hydroxymyristoyloxysuccinimide with benzyltrichloroacetimidate.[25] Indeed, both basic benzylation of 3-hydroxy-myristate (BnBr-Ag_2O) and saponification of the resulting benzyloxy-ester are tedious. The bis-amidate was selectively 6-*O*-acylated with 6-azidohexanoylchloride (60%) in cold pyridine and phosphorylation of both the 1- and 4-position was accomplished by reacting the dilithium salt with tetrabenzylpyrophosphate according to the method of Watanabe.[26] The fully protected target compound **14** was characterized by ^1H and ^{31}P NMR spectroscopy which showed that the anomeric configuration was α(J H_1–H_2 = 3 Hz, J H_1–P = 6 Hz). Complete deprotection of **14** and concomitant reduction of the azido function to a primary amino group (H_2–$Pd(OH_2)$) allowed the isolation of the highly insoluble lithium salt **15**. Compound **15** (PPDm2-B), and its fluorescein-labeled

derivative (PPDm2B-FITC), were used in immunological studies.

Immunological studies

Because PPDm2-B has been designed as an analogue of PPDm2, a compound which exhibited in previous studies anti-LPS activities in bone marrow cells,[14] we examined in a preliminary experiment whether PPDm2-B can induce the same effect on these cells. We found that PPDm2-B inhibited the LPS-induced expression of LPS receptors in bone marrow cells as efficiently as PPDm2 (data not shown). However, since a large majority of the pathophysiological effects of LPS are due to its interaction with macrophages, we restricted our studies of the immunological activities of PPDm2-B to its effects in this cell type. All further experiments were thus performed with mouse peritoneal macrophages.

PPDm2-B enhances LPS-induced secretion of TNF-α in mouse macrophages. One of the most biologically important effect of LPS is its ability to induce the production of TNF-α by monocytes and macrophages.[2] Therefore, we first used TNF-α secretion by mouse macrophages as a test to determine if PPDm2-B can modify an LPS-induced effect. Since we were mainly interested in interactions with yet uncharacterized (non-CD14) LPS receptors, the experiment was carried out in the absence of serum. IFN-γ (50 U/mL), reported to enhance LPS-induced responses of macrophages,[27,28] was added in the culture medium used during exposure of the cells to LPS. The results in Figure 1 show that low doses of PPDm2-B alone did not induce TNF-α secretion. In conjunction with LPS, PPDm2-B did not block, but rather enhanced LPS activity. This observation can be explained either by a synergistic effect of the two agents, or by a blocking effect of PPDm2-B on a receptor involved in the down-regulation of LPS-induced TNF-α production.

PPDm2-B inhibits LPS-induced desensitization of macrophages. The second hypothesis mentioned above postulates the existence of positive and negative pathways concomitantly triggered by LPS. The latter is reminiscent to the mechanism of desensitization responsible for endotoxin tolerance. Therefore, to evaluate this hypothesis, we examined the influence of PPDm2-B on LPS-induced desensitization, as estimated by a reduced TNF-α production after a second exposure to LPS. A partial (50%) desensitization of the cells was observed (Figure 2) after incubation with 2 ng/mL of LPS alone, and almost complete desensitization occurred with higher concentrations of LPS (5 and 10 ng/mL). The results also showed that the desensitization induced by the lowest dose of LPS (2 ng/mL) was dose-dependently

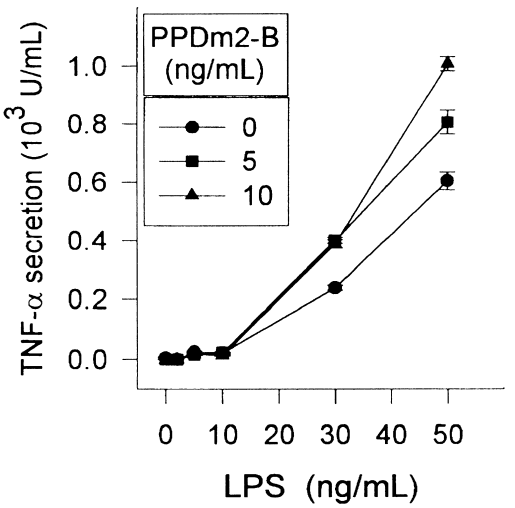


Figure 1. Influence of pretreatment with PPDm2-B on TNF-α responses to LPS. Thioglycolate-elicited mouse peritoneal macrophages (10^6 cells/well) were pre-incubated (30 min, 37 °C) in the absence (●) or in the presence of 5 ng/mL (■), or 10 ng/mL (▲) of PPDm2-B. LPS (50 ng/mL) and IFN-γ (50 U/mL) were then added, and the cells were re-incubated for 18 h at 37 °C in the presence of PPDm2-B. The amounts of TNF-α secreted were then determined. Results expressed as TNF-α units ± SD, are interpolated from data measured in seven serial dilutions of triplicate cell culture supernatants.

blocked by PPDm2-B. However, this antagonistic effect of the synthetic glycophospholipid was much lower when the desensitization was induced by a higher concentration of LPS (5 ng/mL), and almost absent with 10 ng/mL of LPS. This observation likely reflects a competitive interaction of LPS and PPDm2-B with the same receptor. Therefore, the results of Figure 2 sustain the second hypothesis mentioned above, and suggest that PPDm2-B is an antagonist of an LPS receptor involved in down-regulation of LPS-induced TNF-α secretion, and further desensitization of the cells.

Binding of PPDm2B-FITC to macrophages. Since compound PPDm2-B fulfills the conditions for a biological probe (blocking of at least one of the biological activities of LPS on macrophages), we then examined its potential use as a tool for the analysis of LPS receptors. Fluorescein isothiocyanate (FITC) was thus tethered to the amino group of its spacer arm. Macrophages were incubated with this fluorescent derivative (PPDm2B-FITC) in the presence of increasing concentrations of LPS, used as an unlabeled competitor. Incubations were carried out at 4 °C to avoid internalization of the fluorescent compound. The results (Figure 3) show that LPS significantly inhibits the binding of PPDm2B-FITC. In line with the con-

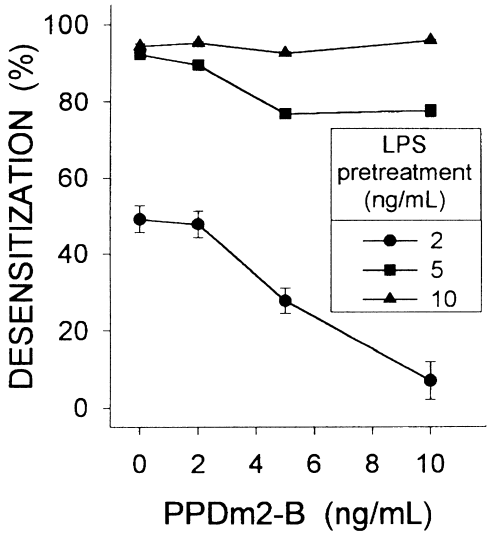


Figure 2. Influence of PPD-m2-B on LPS-induced desensitization of macrophages. Thioglycolate-elicited mouse peritoneal macrophages from Swiss mice (10^6 cells/well) were pre-incubated (30 min, 37 °C) with various concentrations of PPDm2-B. IFN-γ (50 U/mL) and LPS (2 ng/mL, ●; 5 ng/mL, ■; or 10 ng/mL, ▲) were then added, and the cells were re-incubated for 18 h at 37 °C in the presence of PPDm2-B. The cells were then washed three times (40 min incubations at 37 °C in 0.5 mL CM between each washing), and exposed again (18 h, 37 °C) to LPS (5 μg/mL). Results are expressed as desensitization for TNF-α responses to the second treatment with LPS.

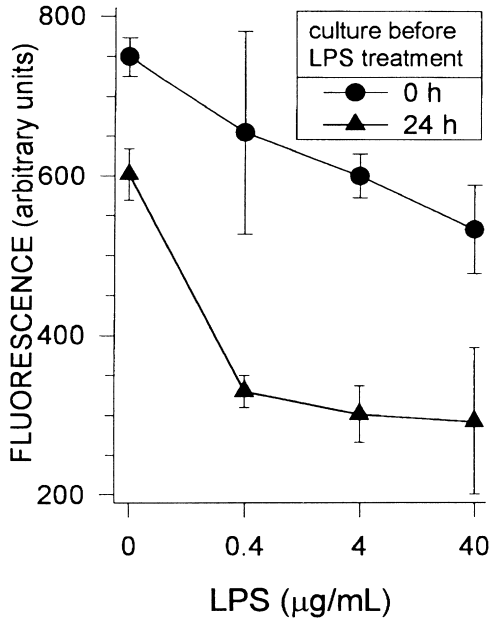


Figure 3. Binding of PPDm2B-FITC to mouse macrophages. After adhesion (2 h, 37 °C) to 96-well plastic plates, thioglycolate-elicited mouse peritoneal macrophages from Swiss mice (2×10^5 cells/well) were (▲) or were not (●) cultured in vitro for 24 h in CM. The cells were then incubated for 1 h at 37 °C in the presence of various concentrations of unlabeled LPS (used as a competitor ligand), equilibrated for 15 min at 4 °C, and exposed to the FITC-labeled ligand (18 h, 4 °C) in the presence of the inhibitor. After three washings with cold (4 °C) phosphate buffered saline, fluoresence intensities (arbitrary units) were determined. Results represent the mean ± SD of four determinations.

clusion of the preceding paragraph, this result confirms that PPDm2-B and LPS compete for the same receptor of mouse macrophages.

Since we observed in previous (unpublished) studies that responses of macrophages to LPS vary after in vitro culture of the cells, we wished to determine if a variation in the cell density of LPS receptors is detectable with PPDm2B-FITC. We found indeed that the specific (LPS-inhibitable) binding of PPDm2B-FITC was higher in macrophages cultured at 37 °C for 24 h (51 ± 15% inhibition of the binding with 40 μg/mL of LPS) than in uncultured cells (29 ± 7% inhibition). This can be taken as an indication that the expression of the receptor involved in endotoxin tolerance is down regulated during in vitro culture of the macrophages.

Conclusion

The overall results indicate that the spacer-containing glycophospholipid PPDm2-B interacts with, and blocks, an LPS receptor of macrophages involved in down-regulation of LPS-induced secretion of TNF-α which probably accounts for the phenomenon of endotoxin tolerance. The detection of this interaction in serum-free medium can be taken as an indication that the receptor involved is not the well-characterized and serum-dependent LPS receptor CD14. PPDm2-B can be considered as an efficient tool for the analysis of this unknown receptor since a fluorescent derivative of this glycophospholipid provided a new information on the modulation of its expression upon in vitro culture of the macrophages. The conjugation of PPDm2-B with photoactivatable reagents, and its use for photolabeling and identification of LPS receptors will be reported elsewhere.

Experimental

Synthesis

General methods. NMR spectra were determined on Bruker ACX 200 or ARX 400 spectrometers at ambient temperature. Chemical shifts (δ) are expressed in part

per million (ppm) relative to the signal of internal standards: Me_4Si, δ 0.0 (^1H in $CDCl_3$); δ 77.0 (^{13}C in $CDCl_3$); HDO, δ 4.75 (^1H in D_2O); PPh_3, δ 0.0 (^{31}P in $CDCl_3$). Assignments of signals was generally based on 2-D H-H and C-H COSY. Melting points (uncorrected) were measured in capillaries with an Electrothermal melting point apparatus. Infrared spectra were recorded on a Perkin–Elmer 841 spectrophotometer in KBr pellets. Column chromatography was performed on Kieselgel 60 (Merck, 230–400 mesh). Thin-layer chromatography (TLC) was performed on aluminum backed precoated silica gel plates (Merck 60 F_{254}), using detection with UV light and then charring with alcoholic 10% sulfuric acid. Reactions requiring anhydrous conditions were conducted under a dry argon atmosphere. Solvent removal was accomplished by a rotary evaporator operating at 45 °C (bath) under reduced pressure. Unless specified, all commercially available reagents were used without further purification.

Methyl 4,6-*O*-benzylidene-2,3-dideoxy-2,3-epimino-α-D-allopyranoside (9). Tosyl chloride (31.5 g, 165 mmol) was added to a stirred suspension of powdered potassium hydroxide:potassium carbonate (1:2.5, 130 g) in toluene:dimethylsulfoxide (1 L, 4:1) containing methyl 2-benzamido-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranoside (**8**) (58 g, 150 mmol).[29] After 30 min tetrabutylammonium hydrogen sulfate (0.5 g) was added and stirring was continued for 4 h. As TLC (EtOAc:cyclohexane, 6:4) showed the absence of starting material the mixture was diluted with toluene (1 L), the organic layer was decanted from insoluble salts, washed with water, dried (Na_2SO_4) and concentrated to a syrup crystallising from chloroform (27 g, 70% in 2 crops), mp 145 °C, identical with the product obtained from a 3-*O*-mesyl derivative by Richardson[30] (mp 141–145 °C); ^1H NMR (200 MHz, $CDCl_3$) δ 7.6–7.45 (m, 2H, Ar), 7.4–7.3 (m, 3H, Ar), 5.6 (s, 1H, PhCH), 4.9 (bs, 1H, H1), 4.2 (dd, $J=5$, 10 Hz, 1H, H_6), 4.1 (m, 1H, H_5), 3.9 (bd, $J=10$ Hz, 1H, H_4), 3.65 (t, $J=10$ Hz, 1H, $H_{6'}$), 3.45 (s, 3H, OMe), 2.7 (bd, 2H, H_2, H_3).

Methyl 4,6-*O*-benzylidene-2,3-benzoylepimino-2,3-dideoxy-α-D-allopyranoside (9a). Benzoic anhydride (19 g, 84 mmol) was added to a solution of **9** (17.65 g, 67 mmol) in pyridine (230 mL). After 30 min as TLC (EtOAc:cyclohexane) showed that benzoylation was complete pyridine was distilled under vacuo, last traces being removed by codistillation with toluene, and the residue was crystallized from EtOH-$CHCl_3$ affording 22.1 g (89.8%) of **9a** as crystals melting at 190–191 °C. Lit.[31,32,33] 190–191 °C, 194–195 °C, 194–198 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 8.3 (d, $J=7.5$ Hz, 2H, $=CH\text{-}CO_2$-), 7.7–7.2 (m, 8H, Ar), 5.6 (s, 1H, PhCH), 5 (d, $J=4$ Hz, 1H, H_1), 4.3 (m, 2H, H_5, H_6), 4 (dd, $J=2.3$, 8.4 Hz, 1H, H_4), 3.8 (t, $J=11.5$ Hz, 1H, $H_{6'}$), 3.5 (s, 3H, OMe), 3.45 (dd, $J=4$, 6.5 Hz, 1H, H_2), 3.1 (dd, $J=2.3$, 6.5 Hz, 1H, H_3).

Methyl 3-azido-2-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside (11) and methyl 2-azido-3-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-altropyranoside (10). Sodium azide treatment of 28 g (76.3 mmol) of the benzoylepiminoglycoside **9a** according to the original procedure of Guthrie[22] afforded 7.8 g (25%) of crystalline 3-azido-2-benzamido-glucoside (**11**) melting at 220 °C after crystallisation from isopropanol and recrystallisation from EtOH (Lit.[22] 233–235 °C from isopropanol), and 21.8 g (70%) of the 2-azido derivative (**10**) as a clear oil after flash chromatography eluting with EtOAc:cyclohexane, 6:4 (R_f 0.54, same solvent). (**11**) ^1H NMR (200 MHz, $CDCl_3$) δ 7.7–7.2 (m, 10H, Ar), 6.35 (d, $J=9.4$ Hz, 1H, NH), 5.6 (s, 1H, PhCH), 4.76 (d, $J=3.6$ Hz, 1H, H_1), 4.5 (dt, $J=3.6$, 10 Hz, 1H, H_2), 4.33 (m , 1H, H_6), 3.9 (t, $J=10$ Hz, 1H, H_3), 3.85 (m, 2H, H_5, $H_{6'}$), 3.7 (t, $J=10$ Hz, 1H, H_4), 3.35 (s, 3H, OMe); Anal. calcd for $C_{21}H_{22}N_4O_5$: C, 61.5; H, 5.4; N,13.65; found: C, 61.4; H, 5.2; N, 12.95. (**10**) ^1H NMR (200 MHz, $CDCl_3$) δ 7.8–7.2 (m, 10H, Ar), 7.15 (d, $J=9$ Hz, 1H, NH), 5.6 (s, 1H, PhCH), 4.94 (t d, $J=3$, 8.7 Hz, 1H, H_3), 4.75 (s, 1H, H_1), 4.33 (dd, $J=4.3$, 9.9 Hz, 1H, H_6), 4.14 (dd, $J=4$, 9.7 Hz, 1H, H_4), 4.02 (dt, $J=4$, 10 Hz, 1H, H_5), 4.01 (bd, $J=3$ Hz, 1H, H_2), 3.88 (t, $J=9.9$ Hz, 1H, H_6), 3.35 (s, 3H, OMe). The oily altroside (**10**) (1.5 g) was hydrogenated in methanolic hydrogen chloride (50 mL, 0.0125%) at 1 atm and at room temperature in the presence of 10% palladium on charcoal (200 mg). Purification by flash chromatography ($CHCl_3$:MeOH, 9:1) gave 1 g of 2-amino-3-benzamido-altroside as a yellow oil. ^1H NMR (200 MHz, DMSO-d_6) δ 7.8–7.2 (m, 10H, Ar), 5.65 (s, 1H, PhCH), 4.52 (s, 1H, H_1), 4.5 (dt, 1H, H_3), 4.35–4.05 (m, 3H, H_4, H_5, H_6), 3.75 (t, $J=10$ Hz, 1H, $H_{6'}$), 3.35 (s, 3H, OMe), 3 (d, $J=2.5$ Hz, 1H, H_2). Acetylation of the above amino-altropyranoside gave the 2-acetamido-3-benzamido derivative as an oil. ^1H NMR (200 MHz, $CDCl_3$) δ 7.8–7.1 (m, 11H, Ar, NHCOPh), 6.4 (d, $J=7.5$ Hz, 1H, NHAc), 5.45 (s, 1H, PhCH), 4.82 (t d, $J=9$, 2.5 Hz, 1H, H_3), 4.65 (s, 1H, H_1), 4.3 (bd, $J=7.5$ Hz, 1H, H_2), 4.2 (dd, $J=10$, 5 Hz, 1H, H_6), 3.95 (m, 2H, H_4, H_5), 3.75 (t, $J=10$ Hz, 1H, $H_{6'}$), 3.4 (s, 3H, OMe), 3.35 (s, 3H, OMe), 1.95 (s, 3H, CH_3CO−).

2,3-Diamino-2,3-dideoxy-D-glucopyranose (12). The azido-glucoside **11** (2.4 g) was hydrogenated in methanolic hydrogen chloride (150 mL, 0.0125%) overnight with $Pd(OH)_2$ on carbon (20%, 500 mg) at room temperature. The catalyst was removed by filtration, the filtrate was concentrated to dryness, and the residue taken up in 5 N aqueous hydrogen chloride (100 mL). After refluxing for 2.5 h as TLC (isopropanol: 20% ammonia: H_2O, 7:2:1) showed that hydrolysis was

complete (R_f 0.34) the solution was extracted with ether (3×100 mL), the aqueous layer was brought to dryness, the dark residue, triturated with a small volume of ethanol and dissolved with addition of water. After half an hour the cloudy solution was cleared of small amounts of floculent impurities by filtration, the solvents were evaporated in vacuo and the dihydrochloride of the diaminoglucose (**12**) was recovered by trituration with ethanol and centrifugation of the off-white solid (1.07 g, 68%), mp 195 °C with decomposition. Lit.[16]: 196 °C. ^{1}H NMR (200 MHz, D_2O) δ 5.45 (s, 0.71H, $H_{1\alpha}$), 4.95 (d, J=7.3 Hz, 0.28H, $H_{1\beta}$), 4–3.3 (m, 6H). Anal. calcd for $C_6H_{16}Cl_2N_2O_4$,1 H_2O: C, 26.8; H, 6.7; N, 10.4. Found: C, 26.9; H, 6.9; N, 10.4. *N*-acetylation in 50% aqueous methanol with acetic anhydride in presence of $NaHCO_3$ gave 2,3-diacetamido-2,3-dideoxy-glucose as a 4:3 α/β equilibrium. ^{1}H NMR (200 MHz, D_2O) δ 5.21 (d, J=3.4 Hz, $H_{1\alpha}$), 4.84 (d, J=8.7 Hz, $H_{1\beta}$), 4.15 (t, J=11 Hz, $H_{3\alpha}$), 4.0 (dd, J=3, 11 Hz, $H_{2\alpha}$), 3.7 (dd, J=11, 8.7 Hz, $H_{2\beta}$).

Methyl 2-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-ribopyranoside (11b). The azidoglycoside **11** (200 mg, 0.49 mmol) was heated in anhydrous hydrazine (10 mL) for 4 h at 100 °C. TLC indicated that starting material was transformed in a major product having R_f 0.75 and a minor one with R_f 0.25 ($CHCl_3$:MeOH, 95:5). After concentration of the mixture in vacuo and column chromatography (same solvent) 130 mg (72%) of crystalline **11b** was recovered. Recrystallised from dichloromethane-methanol, it had a mp of 275 °C. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.7–7.25 (m, 10H, Ar), 6.35 (d, J=9 Hz, 1H, NH), 5.5 (s, 1H, PhCH), 4.65 (d, J=3 Hz, 1H, H_1), 4.2 (m, 3H, H_4, H_5, $H_{6'}$), 3.35 (s, 3H, OMe), 2.2 (dt, J=11, 3 Hz, 1H, H_{3e}), 1.85 (q, J=11 Hz, 1H, H_{3ax}). Anal. calcd for $C_{21}H_{23}NO_5$: C, 68.3; H, 6.25; N, 3.8. Found: C, 68.1; H,6.4; N, 3.7. Isolation of the minor compound yielded 20 mg (10%) of Methyl 3-amino-2-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside (**11a**) as a solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.8–7.25 (m, 10H, Ar), 6.4 (d, J=9 Hz, 1H, $NHCOC_6H_5$), 5.5 (s, 1H, PhCH), 4.75 (d, J=3 Hz, 1H, H_1), 4.25 (m, 2H, H_2, H_6), 3.75 (m, 2H, H_5, $H_{6'}$), 3.35 (s and t J=9 Hz, 4H, H_4, OMe), 3.17 (t, J=9Hz, 1H, H_3). This spectrum was identical to that of the product obtained by catalytic (Pd/C) hydrogenation of methyl 3-azido-2-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside (**11**), mp 250 °C. Anal. calcd for $C_{21}H_{24}N_2O_5$, 1H_2O: C, 62.7; H, 6.5; N, 7.0. Found: C, 62.7; H, 6.5; N, 7.0.

Methyl 3-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-ribopyranoside (10a). Similar treatment with hydrazine of methyl 2-azido-3-benzamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-altropyranoside (**10**) gave after chromatography on a column eluted with EtOAc:cyclohexane (4:6) 55% of the 2-deoxy-glycoside **10a** as a clear oil. R_f 0.44 (same solvent); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.8–7.2 (m, 11H, Ar, NH-CO), 5.6 (s, 1H, PhCH), 4.9 (m, 1H, H_3), 4.8 (dd, J=2, 3 Hz, 1H, H_1), 4.3 (dd, J=4.7, 10 Hz, 1H, H_6), 4 (m, 1H, H_5), 3.8 (m, 2H, $H_{6'}$, H_4), 3.45 (s, 3H, OMe), 2.15 (m, 2H, H_{3e}, H_{3ax}) and 20% of 1,5-anhydro-3-benzamido-4,6-benzylidene-2,3-dideoxy-D-ribo-hex-1-enitol (**10b**); mp 183.5 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.8–7.2 (m, 10H, Ar), 6.45 (d, J=6 Hz, 1H, H_1), 6.35 (bd, J=6 Hz, 1H, NH-CO), 5.7 (s, 1H, PhCH), 5.2, (t, J=6 Hz, 1H, H_2), 4.8 (q, J=6 Hz, 1H, H_3), 4.5 (dd, J=3.4, 9.2 Hz, 1H, H_6), 4.1 (dd, J=6.2, 9.8 Hz, 1H, H_4), 3.9 (m, 2H, H_5, $H_{6'}$); Anal. calcd for $C_{20}H_{19}NO_4$: C, 71.2; H, 5.6; N, 4.15. Found: C, 71.1; H, 5.8; N, 4.1.

Methyl 4,6-*O*-benzylidene-2,3-dideoxy-2-nitro-α-D-erythro-hex-2-enopyranoside (5) and methyl 4,6-*O*-benzylidene-2,3-dideoxy-3-(methyl-2,3-dideoxy-4-keto-α-D-glycero-hex-2-enopyranosid-2-yl)-2-nitro-α-D-erythro-hex-2-enopyranoside (6). Methanesulfonyl chloride (40 mL, 517 mol) and triethylamine (140 mL, 1 mol) were added dropwise, under an atmosphere of argon to a cooled (−50 °C) solution of methyl 4,6-*O*- benzylidene-3-nitro-α-D-gluco and manno-hexopyranoside (**2**) (155 g, 500 mmol) in dry dichloromethane (500 mL). After 30 min, when addition was terminated, the cooling bath was removed and the reaction mixture was let to reach room temperature (1 h). Since TLC showed that the great majority of organic material was the expected 3-nitro olefin, the mixture was diluted with toluene (500 mL) and percolated through a pad of silica gel (12×8 cm) rinsed with EtOAc:cyclohexane (1:1). Concentration of the effluent gave a dark-brownish syrup whose TLC indicated that decomposition of the nitro olefin gave rise to many tailing spots. Careful chromatography (EtOAc:cyclohexane, 3:7 to 5:5) allowed the isolation of two crystallising compounds; the first eluted one, R_f 0.67 (EtOAc:cyclohexane, 4:6) was methyl 4,6-*O*-benzylidene-2,3-dideoxy-2-nitro-α-D-erythro-hex-2-enopyranoside (**5**) (1.5 g). Isolated as white crystals from EtOAc- petroleum ether, it had mp 185–190 °C with decomposition. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.6–7.4 (m, 6H, Ar, C_3H=), 5.6 (s, 1H, PhCH), 5.4 (s, 1H, H_1), 4.4 (dd and d, 2H, H_6, H_4), 4.1 (dt, J=4, 9.9 Hz, 1H, H_5), 3.85 (t, J=9.9 Hz, 1H, $H_{6'}$), 3.55 (s, 3H, OMe). ^{13}C NMR ($CDCl_3$) δ 147.1 (C_2), 136.6 (C arom. subst.), 134.13 (C_3), 129.5 (C arom. p), 128.4, 126.2 (C arom. o, m), 102.7 (Ph-CH), 94.1 (C_1), 74.5 (C_4), 69.0 (C_6), 63.0 (C_5), 57.2 (OMe). IR ν_{max} 1532 cm^{-1}. Anal. calcd for $C_{14}H_{15}NO_6$: C, 57.3; H, 5.1; N, 4.8. Found: C, 57.1; H, 5.3; N, 4.6. The compound eluted next was methyl 4,6-*O*-benzylidene-2,3-dideoxy-3-(methyl-2,3-dideoxy-4-keto-α-D-glycero-hex-2-enopyranosid-2-yl)-2-nitro-α-D-erythro-hex-2-enopyranoside (**6**) (4.5 g), mp 177 °C (from EtOAc), R_f 0.37 (EtOAc:cyclohexane, 5:5). ^{1}H NMR (400 MHz,

$CDCl_3$) δ 7.4–7.3 (m, 5H, Ar), 6.0 (s,1H, $H_{3'}$), 5.5 (s, 1H, PhCH), 5.28 (d, $J=4$ Hz, 1H, H_1), 5.19 (s, 1H, $H_{1'}$), 4.83 (dd, $J=4$, 11.5 Hz, 1H, H_2), 4.4 (t, $J=4.5$ Hz, 1H, $H_{5'}$), 4.32 (dd, $J=4.8$, 10.5 Hz, 1H, H_{6a}), 3.95 (m, 3H, H5, $H_{6'a}$, $H_{6'b}$), 3.75 (t, $J=10.5$ Hz, 1H, H_{6b}), 3.71 (t, $J=11.4$ Hz,1H, H_3), 3.56 (t, $J=11$ Hz, 1H, H_4), 3.55 (s, 3H, OMe), 3.45 (s, 3H, OMe). ^{13}C NMR ($CDCl_3$) δ 195 (C=O, $C_{4'}$), 155 (=C-, $C_{2'}$), 136.5 (C arom. subst.), 129.3, 128.4, 125.8 (C arom. p, o, m), 124.3 (=CH, $C_{3'}$), 101.8 (Ph-CH), 96.9 ($C_{1'}$), 96.6 (C_1), 82.8 (CNO_2, C_2), 79.7 (C_4), 73.1 ($C_{5'}$), 68.6 (C_6), 63.8 (C_5), 61.5 ($C_{6'}$), 57.2 (OMe), 57 (OMe), 40.7 (C_3) IR ν_{max}: NO_2, 1540 cm^{-1}, C=O, 1680 cm^{-1}; MS (CI, NH_3) m/z 452 ($M+H^+$), 469 ($M+NH_4^+$). Anal. calcd for $C_{21}H_{25}NO_{10}$: C, 55.9; H, 5.5; N, 3.1. Found: C, 55.9; H, 5.7; N, 3.0.

2,3-di-[(*R*)-3-Benzyloxytetradecanamido]-2,3-dideoxy-D-glucose (13). Diisopropylamine (2.2 mL, 20 mmol) was added to a stirred solution of 2,3-diamino-glucose dihydrochloride (1 g, 4 mmol) in DMF (70 mL) containing *N*-(*R*)-3-benzyloxytetradecanoyloxy-succinimide (6.3 g, 14.5 mmol). After 48 h at room temperature the mixture was evaporated under vacuo (1 mm Hg) to a residue which gave 1.4 g (43%) of the title compound as a waxy solid upon column chromatography with $CHCl_3$:MeOH (95:5) as eluent. Washed with petroleum ether it had a mp of 161 °C. ^1H NMR (200 MHz, $CDCl_3$:MeOH-d_4 3:2) δ 7.65 (d, $J=8$ Hz, 1H, NH), 7.3–7.1 (m, 10 H, Ar), 7.1 (d, $J=8$ Hz 1H, NH), 4.95 (d, $J=3.9$ Hz, 1H, $H_{1\alpha}$), 4.45 (b s, 4H, 2×CH_2-Ph), 4.1 (t, $J=9.9$ Hz, 1H, H_3), 3.9–3.5 (m, H_2, H_5, H_6, $H_{6'}$, OH), 3.6 (m, 2H, 2×CH-OBn), 3.2 (t, $J=9.9$ Hz, 1H, H_4), 2.25 (m, 4H, 2×CH_2-CON), 1.4 (bm, 4H, 2×-CH_2-COBn-), 1.15 (bs, 36H, 2×-$(CH_2)_9$-Me), 0.8 (bt, 6 H, 2×CH_3-). Upon acetylation (Ac_2O-pyridine) $H_{1\alpha}$ was deshielded to δ 6.05, trace of $H_{1\beta}$ was detected at δ 5.4 ($J=7.7$ Hz) and three acetoxy groups gave singlets at δ 1.8, 1.85, and 2.0.

6-(6-Azidohexanoyl)-2,3-di-[(*R*)-3-benzyloxytetradecanamido]-2,3-dideoxy-D-glucose (13a). An anhydrous chloroformic solution (1 mL) of 6-azidohexanoylchloride (116 mg, 0.66 mmol) was added with a seringe to a cooled (−50 °C) solution, protected from moisture, of the diamido derivative **13** (450 mg, 0.55 mmol) in a mixture of chloroform and pyridine (2:1, 9 mL). The temperature was allowed to reach slowly 20 °C (2 h) and the mixture was abandoned overnight. It was then diluted with chloroform, washed successively with solutions of sodium hydrogen sulfate, sodium hydrogenocarbonate and water. The dried (Na_2SO_4) organic phase was concentrated to a glass (500 mg), which was purified by chromatography (CCl_4:acetone, 25:5) to give pure **13a** (400 mg, 76%) as a colorless varnish melting at 140 °C. ^1H NMR (200 MHz, $CDCl_3$) δ 7.3 (m, 10H, Ar), 7.22, 6.75 (2d, 2H, 2×NHCO), 5.05 (d, $J=3$ Hz, 1H, $H_{1\alpha}$), 4.6, 4.52, 4.42 (d, s, d 1H, 2H, 1H, 2×CH_2-Ph), 4.2 (dd, $J=12$, 5.5 Hz, 1H, H_6), 3.8 (m, 2H, 2×CH-OBn), 3.25 (t, $J=6.6$ Hz, 1H, CH_2N_3), 2.35 (m, 6H, 3×CH_2-CO-), 1.75–1.15 (m, 46 H, 23×-CH_2-), 0.8 (t, $J=6.3$ Hz, 6 H, 2×CH_3-).

6-(6-Azidohexanoyl)-2,3-di-[(*R*)-3-benzyloxytetradecanamido]-4-*O*-dibenzylphosphoryl-2,3-dideoxy-α-D-glucopyranosyl-dibenzylphosphate (14). A 2 M solution of lithium diisopropylamide in heptane (0.75 mL, 1.5 mmol) was added, under an atmosphere of argon to a cooled (−70 °C) solution of the dried azido derivative **13a** (500 mg, 5.3 mmol) in tetrahydrofuran freshly distilled from sodium benzophenone ketyl. After 10 min, 10 mL of anhydrous tetrahydrofuran containing tetrabenzylpyrophosphate (1 g) was added, after one more hour the cooling bath was discarded and the reaction mixture was abandoned overnight at room temperature. The heterogeneous white mixture was diluted with chloroform (20 mL) and filtered through a pad of silica gel (1×3 cm). The filtrate was concentrated to give an oil (1 g). The product was purified by chromatography using EtOAc:-cyclohexane (1:1.5) as eluent. The title compound (450 mg) was a colorless syrup. ^1H NMR (400 MHz, $CDCl_3$) δ 7.4–7.2 (m, 30H, Ar), 6.97 (d, $J=7.8$ Hz, 1H, C_3NHCO), 6.76 (d, $J=7.6$ Hz, 1H, C_2NHCO), 5.76 (dd, $J=3$, 5.6 Hz, 1H, H_1), 4.97 (m, 7 H, P-O-CH-Ph), 4.75 (dd, $J=7.8$, 11.5 Hz, 1H, P-O-CH-Ph), 4.55 (m, 3H, -C-*O*-CH_2-Ph, H_3), 4.39 (t, $J=11$ Hz, 2H, C-O-CH_2-Ph), 4.31 (m 1H, H_4), 4.24 (dd, $J=4$, 10.4 Hz, 1H, H_6), 4.03 (m, 3H, H_2, H_5, $H_{6'}$), 3.8 and 3.74 (2 quintet, 2H, 2×CH-OBn), 3.22 (t, $J=7$ Hz, 2H, CH_2N_3), 2.4–2.10 (m, 6H, 3×-CH_2-CO-), 1.75–1.0 (m, 23×-CH_2-), 0.87 (t, $J=6$ Hz, 6H, 2×CH_3-). ^{31}P NMR ($CDCl_3$) δ −3 (q, $J=8$ Hz, P-O-C_4), −5.4 (m, P-O-C_1).

6-(6-Aminohexanoyl)-2,3-dideoxy-2,3-di-[(*R*)-3-hydroxytetradecanamido]-α-D-glucopyranose 1,4-diphosphate (15). A methanolic solution (100 mL) of the bis dibenzylphosphate **14** (425 mg) was stirred, in presence of palladium hydroxide (20%) on carbon (Pearlman's catalyst) under a slight overpressure of hydrogen. After 2 h at room temperature, as white precipitate started to deposit, the mixture was diluted with tetrahydrofuran (100 mL) and brought to a pH of roughly 5 with a 0.004 M lithium hydroxide solution and hydrogenation was continued overnight. The catalyst was then removed by filtration and extensively washed with chloroform: isopropanol (50:50). The filtrate and washing were brought to pH 6 (pH paper) and concentrated to give a waxy solid (200 mg) melting with decomposition at 219 °C. Anal. calcd for $C_{40}H_{77}Li_2N_3O_{15}P_2$, 2 H_2O: C, 50.5; H, 8.5; N, 4.4; P, 6.5. Found: C, 50.1; H, 8.6; N, 4.0; P, 6.5.

***N*-(*R*)-3-Hydroxytetradecanoyloxysuccinimide.** Equimolar amounts of (*R*)-3-hydroxy myristic acid and hydroxysuccinimide, in ethyl acetate were treated with

dicyclocabodiimide at 0 °C. After 16 h, the dicyclohexyl urea formed was discarded by filtration, the filtrate concentrated and the product crystallised from methanol at 4 °C. (80%) mp 109.5 °C. ^{1}H NMR (200 MHz, $CDCl_3$-) δ 4.05 (m, 1H, CH-O), 2.9–2.5 (m, 6H, CH_2-CO-), 1.7–1 (m, 20H, 10×-CH_2-), 0.8 (t, J = 6 Hz, 3H, CH_3). Anal. calcd for $C_{18}H_{31}NO_5$: C, 63.3; H, 9.1; N, 4.1. Found: C, 63.2; H, 9.0; N, 4.0.

***N*-(*R*)-3-Benzyloxytetradecanoyloxysuccinimide.** The hydroxysuccinimide ester of 3-hydroxymyristic acid (10 g) was suspended in 100 mL of anhydrous toluene and treated with benzyltrichloroacetimidate (15 g) in the presence of a catalytic amount of trifluoromethane sulfonic acid (0.7 mL). After 3 h at room temperature the mixture containing the product (R_f 0.42 in toluene: EtOAc, 9:1) was directly purified by chromatography (same solvent) to give the title compound (6.2 g, 50%) identical (mp 42 °C) to the product obtained by Macher upon esterification of the 3-benzyl ether of 3-hydroxymyristic acid.[24]

6-Azidohexanoylchloride. 6-Bromohexanoic acid (9.8 g, 50 mmol) was heated in 2-methoxyethanol, at 120 °C with sodium azide (6.5 g, 100 mmol) for 12 h. After dilution with dichloromethane the solution was washed with water, aqueous sodium hydrogen sulfate, and brine. Concentration of the dried organic layer gave an oil, which was distilled (160 °C, 14 mm Hg) to give 6 g (64%) of 6-azidohexanoic acid. The azido acid (1.5 g, 9.55 mmol) was treated, at 40 °C, with thionyl chloride (3.5 mL) for 1 h with a drop of dimethylformamide. Concentration to a syrup and distillation (130 °C) under vacuum (14 mm Hg) gave 1.3 g (72%) of the 6-azidohexanoylchloride which was stored under argon. ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.15 (t, J = 6 Hz, 2H, CH_2N_3), 2.8 (t, J = 7 Hz, 2H, CH_2-CO-), 1.8–1.2 (m, 6H, 3×-CH_2-).

Coupling of fluorescein isothiocyanate (FITC). FITC (50 μL, 1 mg/mL in DMSO) was added to a suspension of **15** (PPDm2B) in a triethylamine buffer (200 μL, 55 μM, pH 8.6). After magnetic stirring at room temperature for 150 min, in a plastic tube protected from light, the mixture was exhaustively dialyzed at 4 °C, first against PBS, and further against distilled water. TLC (5:3, isobutyric acid: 1 M ammonium hydroxide) showed the disappearance of compound **15** (R_f 0.63, visualized by charring), and the appearance of a single fluorescent conjugate (PPDm2B-FITC, R_f 0.71).

Immunology

Media and reagents. Culture medium (CM) was RPMI-1640 (Gibco, Grand Island, NY, USA) containing 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2-mercaptoethanol (5×10^{-5} M). Heparin, actinomycin D, crystal violet, and neutral red were purchased from Sigma Chemical Co. (St. Louis, MO, USA). A solution of trypsin in EDTA was purchased from Biochrom KG (Berlin, Germany). The lipopolysaccharide from *Salmonella typhimurium* LT2 was prepared in our laboratory, by the phenol-water extraction procedure, as described previously.[34] Thioglycolate broth and MEM were from Diagnostic Pasteur (Ville d'Avray, France), and fetal calf serum (FCS) was from Boehringer (Mannheim, Germany). Mouse recombinant interferon gamma (iFN-γ was purchased from Gibco (Gaithersburg, MD, USA).

Cell culture. The murine fibroblast cell line L929-α was from Dr M. Parant (Paris, France). Suspensions of L929 cells were prepared by treatment (1 min) of confluent cultures (75 cm^2) with trypsin (2 mL, 0.05% in 0.02% EDTA), followed by addition of heat-inactivated horse serum (1 mL) to stop the reaction. Swiss mice (4–8 weeks old, from R. Janvier, Le Genest Saint-Isle, France), were used as sources of peritoneal macrophages. Peritoneal exudates were harvested five days after ip injection of 1.7 mL of thioglycolate broth, by peritoneal washes with MEM containing 10 U/mL of sodium heparinate. Suspensions of peritoneal exudate cells (10^6 neutral red-positive cells/well in RPMI buffered with 25 mM HEPES), were incubated (2 h, 37 °C) in plates with 24 wells. Nonadherent cells were removed by four washings with MEM (500 μL/well). The adherent cell population contained 92 to 96% neutral red-positive macrophages.

Estimation of TNF-α secretion and macrophage desensitization. The macrophage culture supernatants were recovered, centrifuged (10 min, 900 *g*), and rapidly assayed (without storage) for TNF-α activity, by determination of the reciprocal of the dilution that gave 50% killing of actinomycin-D sensitized L-929 cells. The estimation of the target cell viability was performed by staining viable cells with crystal violet as described previously.[35] Macrophage desensitization was defined as the percentage of inhibition of LPS-induced production of TNF-α by macrophages desensitized by a preliminary treatment with LPS was computed by the formula:

$$\%\ \text{desensitization} = (1 - (C_y^x/C_y^0)) \times 100$$

Where terms of the C_y^x type represent the measured mean of the amount of TNF-α produced in response to y μg/mL of LPS, by macrophages which have been pretreated with x ng/mL of the same agent. A computer program written in our laboratory (and successfully tested on artificial sets of variables) was used to compute the standard deviation (SD) of the values obtained by the above formula.

Binding studies. Adherent cells (2×10^5 cells per well in 96-well plates) were preincubated for 1 h at 37 °C in CM, in the presence or absence of unlabeled LPS used as inhibitor. The cells were then equilibrated for 15 min at 4 °C, and exposed to the FITC-labeled ligand (18 h, 4 °C) in the presence of the inhibitor. After three washings with cold (4 °C) phosphate buffered saline, the plates were excited at 485 nm and scanned at 530 nm in a microplate fluorometer (Model 7600, Cambridge Technology, Watertown, MA, USA), and fluorescence intensities (arbitrary units) were determined.

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