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Polypropylene glycol is a selective binding inhibitor for LTA and other structurally related TLR2 agonists

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Polypropylene glycol (PPG) is commonly added to bacterial cultures to avoid foaming. However, lipoteichoic acid (LTA) from bacteria grown with PPG lacked cytokine-inducing potency in human blood. We tested the blocking efficacy of several glycols on the cytokine response to staphylococcal LTA in human blood. PPG 1200 was the most potent inhibitor tested, shown for TNF, IL-1β, IL-6, IL-8, IL-10 and TGF-β induction, and displayed no cytotoxic effects. TNF induction by *Staphylococcus aureus* or by Toll-like receptor (TLR)2 agonists (di- and triacylated lipopeptides and LTA) was also inhibited by PPG 1200, but not that induced by *Escherichia coli* or TLR4 agonists. In flow cytometric studies, PPG-carrying nanobeads bound more rhodamine-labeled LTA than those with glycerol. Additionally, the methyl group peak in the ^{1}H-NMR of LTA shifted after incubation with increasing PPG 1200 concentrations. Sequential incubation of polystyrene plates with LTA, then PPG 1200 and then blood, with washing steps in between, showed that LTA-induced TNF release was inhibited. But when PPG 1200 was pre-incubated with blood that was washed before LTA was added, TNF induction was not repressed, demonstrating that PPG binds LTA and not cellular structures. In summary, PPG 1200 is a novel inhibitor of cytokine induction by TLR2 agonists, which interferes directly with the ligands.

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Introduction

Pathogen-associated molecular patterns are recognized by the cells of the innate immune system *via* specialized host receptors, also called pathogen recognition receptors, which alert the immune system to the presence of microbial invasion. In this context, the Toll-like receptors (TLR) are the key molecules. To date, ten TLR, which bind to bacterial cell wall components like lipopolysaccharides (LPS), lipoteichoic acid (LTA) or flagellin and putative endogenous ligands like heat shock proteins [1, 2], have been identified in humans.

LPS are the major constituents of the outer leaflet of the outer membrane of gram-negative bacteria. These are amphiphilic molecules that consist of highly variable carbohydrate chains (O-antigen), core polysaccharides, and a lipid A anchor with five to seven fatty acids that is highly conserved and represents the immunostimulatory part of LPS. Hirschfeld *et al.* could show that highly purified LPS from many different bacterial species signals *via* TLR4 [3, 4]. LPS is bound by the LPS-binding

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Abbreviations: **LBP:** LPS binding protein **LTA:** lipoteichoic acid **PBG:** polybutylene glycol **PEG:** polyethylene glycol **PPG:** polypropylene glycol

protein (LBP), and this complex is recognized by TLR4 and the cofactors CD14 and MD-2 [5, 6].

Gram-positive bacteria carry LTA, which is anchored in the cytoplasmic membrane and which is also amphiphilic. The LTA from *Staphylococcus aureus* consists of a glycolipid anchor and a hydrophilic backbone with repetitive units of glycerophosphate and D-alanyl esters, free hydroxyl and glycosyl substituents [7, 8]. Most bacterial species have LTA with a fairly similar structure to that of staphylococcal LTA, except for LTA from *Streptococcus pneumoniae* [9]. LTA employs TLR2, CD14, and partially LBP [10–12], as well as CD36 [13].

Beside LTA there are other well-known TLR2 agonists. The cytokine pattern induced by LPS from *Porphyromonas gingivalis* resembles that typically induced by LTA rather than LPS, with induction of low amounts of TNF and high amounts of IL-8 [14]. It is also TLR2 dependent and requires CD14 as coreceptor [14–16]. The synthetic triacylated lipopeptide $Pam_3Cys\text{-}SK_4$, an analogue of the unique N-terminal part of lipoproteins, signals *via* a heterodimer consisting of TLR1 and TLR2 [17, 18]. However, diacylated lipopeptides like MALP-2 from *Mycoplasma fermentans* require TLR6 beside TLR2 to stimulate cytokine release [19, 20]. Although zymosan is a crude cell wall preparation from *Saccharomyces cerevisiae*, TLR2 and TLR6 dependence is also described with CD14 as coreceptor and no influence of serum proteins [21–23].

For the TLR4 agonist LPS, several inhibitors have been described. They have been used as research tools to study the role of LPS in a broad variety of experimental models. The cationic polymyxin B is a cyclic polypeptide that neutralizes LPS by binding to the negative charges of the phosphoryl and carboxyl groups of its lipid A moiety [24]. The LPS-specific binding protein LAL-F inhibits endotoxin-induced signals more specifically than polymyxin B by binding to the lipid A portion [25]. Neutrophils secrete the bactericidal/permeability-increasing protein which has a strong binding affinity to LPS and inhibits LPS-induced cytokine release [26]. High-density lipoproteins are able to bind and neutralize LPS but also LTA [27–31]. However, no specific inhibitors for LTA and other TLR2 agonists are known so far.

Polypropylene glycol (PPG) is a synthetic polymer that consists of 1,2-propane oxide including structural isomers derived from primary and secondary alcohols. Since PPG contains an asymmetric carbon, it has various optical isomers. It can be straight or has a branched-chain structure [32]. PPG with molecular weights greater than approximately 1000 Da are usually water insoluble, in contrast to polyethylene glycol (PEG) which is completely water soluble, even with a molecular weight of up to a few million Dalton. In the context of microbiology, PPG is commonly added at low concentrations to bacterial growth media to avoid foaming. We cultivated *S. aureus* in the presence and absence of PPG and isolated LTA from these cultures. Only the LTA from bacteria whose media contained no PPG was immunostimulatory in human whole blood. In NMR spectra of such inactive LTA preparations, we identified PPG which had obviously co-eluted in different separation steps, suggesting binding.

Therefore, we investigated the inhibitory properties of glycols on the cytokine release induced by LTA from *S. aureus*. PPG 1200 turned out to be the best inhibitory structure of those tested and so we focused our interest especially on this glycol. We investigated whether it is cytotoxic and its influence on the release of different cytokines. Moreover, we were interested in whether the cytokine induction by different TLR2 and TLR4 agonists and, additionally, of whole bacteria could be inhibited by PPG 1200. The interference of PPG 1200 with ligand or receptor was also investigated by NMR, flow cytometry and functional studies. The identification of a novel inhibitor might help find a versatile tool to characterize the interaction between bacterial ligands and the immune system.

Results

PPG 1200 is a potent inhibitor of LTA-induced cytokine release

To prevent foaming, bacteria are often cultured in the presence of PPG. However, we observed that LTA isolated from bacteria grown in the presence of PPG had lost its ability to stimulate cytokine release in human whole blood. Therefore, we investigated the inhibitory properties of different glycols like PPG of different molecular weights, PEG and polybutylene glycol (PBG) on TNF induction by 1 μg/mL LTA from *S. aureus*. None of the tested glycols induced detectable cytokine release, even at the highest concentrations tested. PPG of different molecular masses all inhibited TNF induction by LTA, whereas neither PEG nor PBG showed this property (Table 1). The lowest IC_{50} value (38 ng/mL) was obtained with PPG 1200, which was used in all subsequent experiments.

We measured further cytokines to determine whether PPG 1200 also affects their release after LTA stimulation. In addition to the low IC_{50} value for TNF induction, we calculated the following IC_{50} values (n 8, all $p<0.001$): for IL-1β induction 85 ng/mL, for IL-6 induction 201 ng/mL, for IL-8 induction 136 ng/mL, for IL-10 induction 261 ng/mL, and for TGF-β induction 329 ng/mL, indicating that PPG 1200 generally inhibits cytokine induction by LTA. PPG 1200 was not cytotoxic

Table 1. Inhibition of LTA induced TNF α release in human whole blood by different glycols[a)]

Substance + 1 μg/mL LTA	IC_{50} ng/mL	*p*
PPG 400	1 200	********
PPG 1200	38	***
PPG 2000	77	***
PPG 2150	126	***
PPG 3000	135	n.s.
PPG 3250	524	n.s.
PEG 600	>30 000	n.s.
PEG 1000	>30 000	n.s.
PEG 2000	>30 000	n.s.
PEG 3000	>30 000	n.s.
PEG 6000	>30 000	n.s.
PBG 640	>10 000	
PBG 1240	>10 000	

[a)] LTA from *S. aureus* (1 μg/mL) was incubated with increasing concentrations of glycols in 20% human whole blood from eight different donors, and TNF α release was measured by ELISA. IC_{50} values were determined by non linear regres sion; significance was determined by Kruskal Wallis test. A *p* value <0.05 was considered significant; ** and *** represent *p* values <0.01 and <0.001, respectively; ns, not significant.

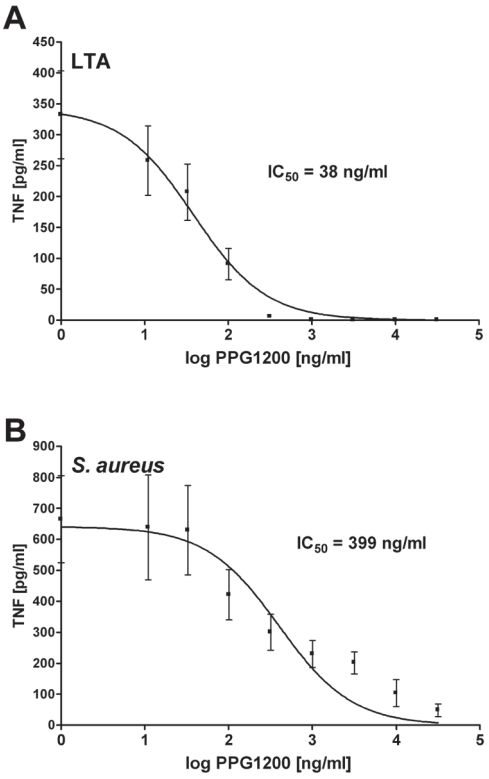

Figure 1. PPG 1200 inhibits LTA and *S. aureus* induced TNF release. Human whole blood from eight healthy volunteers was stimulated with 1 μg/mL LTA (A) or 10^6 *S. aureus*/mL (B), and PPG 1200 was added in increasing concentrations for 22 h. TNF release was measured in the cell free supernatants by ELISA. The detection limit for the TNF ELISA was 10 pg/mL. The cytokine release by the unstimulated controls was below this detection limit. Data are means ± SEM. IC_{50} values were determined by non linear regression.

at concentrations up to 30 μg/mL, as measured by Alamar blue metabolism in blood cells after incubation (data not shown). Furthermore, cytotoxicity for monocytes was assessed by vital staining with propidium iodide and CD14 counterstaining for monocytes. The vital staining showed that 5% of the unstimulated monocytes were dead, and this value did not change upon incubation with LTA, PPG 1200 or LTA and PPG 1200 together, indicating that PPG 1200 is not cytotoxic for monocytes (data not shown).

PPG 1200 effectively inhibits TNF release induced by UV-inactivated *S. aureus* in whole blood

Knowing that the cell wall of *S. aureus* contains LTA, we were interested in whether and to what extent PPG 1200 can inhibit TNF induction by UV-inactivated whole bacteria in human whole blood. UV-inactivated bacteria (10^6/mL) were incubated with increasing concentrations of PPG 1200 in human whole blood and TNF was measured in the supernatant. The cytokine release induced by *S. aureus* was inhibited by PPG 1200 with an IC_{50} value of 399 ng/mL, which is tenfold higher than that for 1 μg/mL LTA (Fig. 1A, B). These data indicate that whole *S. aureus* are blocked with regard to their immunostimulatory activity similar to their isolated membrane component LTA. This suggests that LTA is the major immunostimulatory agent of *S. aureus*.

PPG 1200 affects TNF release induced by LPS or by UV-inactivated *Escherichia coli* only at very high concentrations

We investigated whether the inhibitory effects of PPG 1200 on LTA and whole gram-positive bacteria are also observed in gram-negative bacteria and their main immunostimulatory component LPS. Since lower LPS concentrations are required to induce cytokine release in human whole blood in comparison to LTA, we used 100 pg/mL LPS from *Salmonella abortus equi* and added increasing concentrations of PPG 1200. TNF release was only inhibited at very high concentrations, resulting in an IC_{50} value of 8400 ng/mL (Fig. 2A). We obtained similar results after stimulation of human whole blood with 10^6 UV-inactivated *E. coli*/mL and addition of PPG 1200 (Fig. 2B). These findings indicate the selectivity of the inhibitor for gram-positive stimulation.

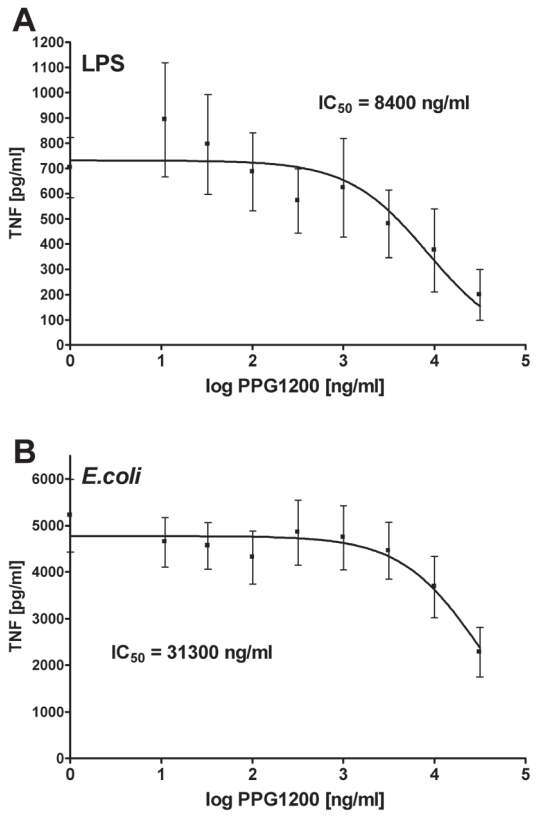

Figure 2. PPG 1200 does not inhibit LPS and *E. coli* induced TNF release. Human whole blood from eight healthy volunteers was stimulated with 100 pg/mL LPS (A) or 10^6 *E. coli* (B), and PPG 1200 was added in increasing concentrations for 22 h. TNF release was measured in the cell free supernatants by ELISA. The detection limit for the TNF ELISA was 10 pg/mL. The cytokine release by the unstimulated controls was below this detection limit. Data are means ± SEM. IC_{50} values were determined by non linear regression.

PPG 1200 selectively inhibits TLR2 agonist- but not TLR4 agonist-induced TNF release in human whole blood

To answer whether the inhibitory activity of PPG 1200 (LTA: IC_{50} 38 ng/mL; LPS: IC_{50} 8400 ng/mL) is limited to LTA or also extends to other TLR2 agonists, we stimulated human whole blood with several TLR2 and TLR4 agonists (Table 2). TNF induction by LTA from the 12 different bacterial species tested was inhibited by the addition of PPG 1200, resulting in low IC_{50} values between 10 and 320 ng/mL. Only for the LTA from *Lactobacillus plantarum* and *Bifidobacterium b* the IC_{50} values were higher compared to the other LTA, with 1225 and 2275 ng/mL, respectively. All tested LPS from seven different bacterial strains showed more than 100-fold higher IC_{50} values than those obtained for LTA from *S. aureus*. Further TLR2 agonists like MALP-2, Pam_3Cys-SK_4 as well as LPS from *P. gingivalis* were similarly inhibited by PPG 1200 to the LTA, resulting in very low IC_{50} values (Fig. 3, Table 2). However, zymosan was only inhibited at high PPG 1200 concentrations. TLR2 dependence was tested and affirmed for all TLR2 ligands by stimulation of murine bone marrow cells from $TLR2^{+/+}$ and $TLR2^{-/-}$ cells with 10 μg/mL of each stimulus and measurement of TNF (Table 3).

Beside TLR2 and TLR4 ligands, we also tested non-lipid TLR ligands like poly I:C as TLR3 ligand, CL097 and *E. coli* RNA as TLR7/8 ligands, R837 as TLR7 ligand and CpG 2216 as TLR9 ligand. We isolated PBMC from eight healthy volunteers, stimulated them with these stimuli in combination with increasing concentrations of

Table 2. PPG 1200 selectively inhibits TLR2 agonist induced but not TLR4 agonist induced TNF α release in human whole blood[a)]

LTA (1 μg/mL) (TLR2 agonist) + PPG 1200	IC_{50} ng/mL	*n*	*p*	Substance (1 μg/mL) + PPG 1200	IC_{50} ng/mL	*n*	*p*	LPS (10 ng/mL) (TLR4 agonist) + PPG 1200	IC_{50} ng/mL	*n*	*p*
B. subtilis	140	12	***	MALP 2				*E. coli* (011:B4)	>10 000	4	n.s.
Bifidobacterium a	138	7	***	(10 μg/mL)	65	7	***	*E. coli* (026:B6)	>10 000	4	n.s.
Bifidobacterium b	2275	7	*	Pam_3Cys SK_4	145	8	**	*Klebsiella*			
E. faecium	119	4	***	*Porphyromonas*				*pneumoniae*	>10 000	4	n.s.
L. delbrueckii	13	4	***	*gingivalis* (LPS)	275	8	**	*S. abortus equi*	>30 000	12	n.s.
L. plantarum	1225	7	***	Zymosan A				*Serratia*			
L. monocytogenes 1	318	4	*	*S. cerevisiae*	>3000	8	*	*marcesens*	>10 000	4	n.s.
L. monocytogenes 2	52	4	***					*Shigella flexneri*	>10 000	4	n.s.
S. aureus	38	8	***					*Vibrio cholerae*	>3 000	4	n.s.
S. epidermidis	40	4	***								
S. agalacticae	164	4	*								
S. pneumoniae											
Fp23	148	8	***								
R6	101	8	***								
S. suis	322	4	*								

[a)] Different concentrations of PPG 1200 and fixed concentrations of LTA, other TLR2 agonists or LPS were incubated with 20% human whole blood of different donors (indicated in the table with *n*), and TNF α release was measured by ELISA. IC_{50} values were determined by non linear regression; significance was determined by Kruskal Wallis test. A *p* value <0.05 was considered significant; *, ** and *** represent *p* values <0.05, <0.01 and <0.001, respectively; ns, not significant.

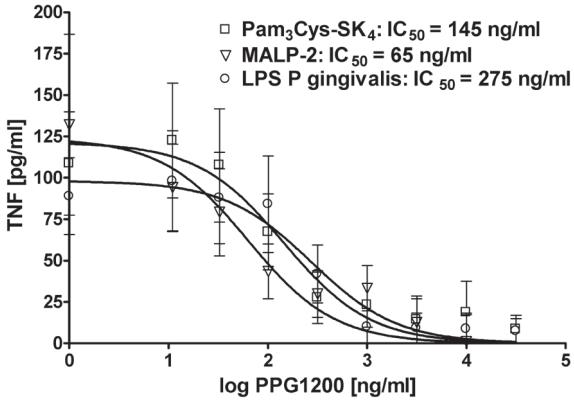

Figure 3. PPG 1200 inhibits TNF release induced by Pam_3Cys, MALP 2 and LPS from *P. gingivalis*. Human whole blood from eight healthy volunteers was stimulated with 1 μg/mL Pam_3Cys, LPS from *P. gingivalis* or 10 μg/mL MALP 2, and PPG 1200 was added in increasing concentrations. TNF release after 22 h was measured in the cell free supernatants by ELISA. The detection limit for the TNF ELISA was 10 pg/mL. The cytokine release by the unstimulated controls was below this detection limit. Data are means ± SEM. IC_{50} values were determined by non linear regression.

PPG 1200 and measured the released IL-6. For all these stimuli, like for LPS, the IC_{50} values were higher than 10 000 ng/mL (data not shown). In conclusion, all TLR2 agonists were antagonized, suggesting an effect towards structures shared between them or the TLR2 signal transduction pathway.

PPG 1200 binds to LTA

To clarify whether PPG 1200 interacts with the TLR2 ligands or the receptor, we performed flow cytometry using nanobeads carrying PPG 1200 or glycerol, and rhodamine-labeled LTA. Labeled LTA retained its full ability to induce TNF and IL-8 formation in whole blood (for 5 μg/mL stimulus: rhodamine-labeled LTA induced 470 ± 80 pg/mL TNF and 70 ± 6.2 ng/mL IL-8, while unlabeled LTA induced 570 ± 150 pg/mL TNF and 76 ± 6 ng/mL IL-8, n 4, both n.s.), while rhodamine alone did not induce any cytokine release. The PPG-carrying nanobeads bound more rhodamine-labeled LTA than those with glycerol (Fig. 4), suggesting that PPG 1200 binds directly to LTA.

We also investigated the binding of PPG 1200 to staphylococcal LTA by NMR analysis. The ^{1}H-NMR spectrum of staphylococcal LTA has been previously published [7]. Keeping the LTA concentration constant, we added increasing concentrations of PPG 1200. The addition of PPG 1200 to the LTA led to shifted peaks in the ^{1}H-NMR spectrum (Table 4). The peak at δ_H 0.8845 ppm, which showed a maximal shift of

Table 3. TLR2 dependence of LTA and other TLR2 ligands[a)]

Substance (10 μg/mL) (TLR2 agonist)	TNF (pg/mL)						
	$TLR2^{+/+}$	± SEM	n	$TLR2^{-/-}$	± SEM	n	p
co	7.3	1.5	29	6.375	1.5	26	ns
LTA *B. subtilis*	192.7	47.0	8	4.0	1.9	8	***
LTA *Bifidobacterium a*	33.6	3.0	8	3.3	0.5	8	***
LTA *Bifidobacterium b*	399.1	45.7	8	4.1	1.9	8	***
LTA *E. faecium*	368.5	59.9	8	12.7	4.9	8	***
LTA *L. delbrueckii*	38.2	4.4	5	3.1	0.7	5	*
LTA *L. plantarum*	206.1	32.0	8	20.2	4.4	8	***
LTA *L. monocytogenes 1*	235.7	35.3	8	3.2	0.6	5	**
LTA *L. monocytogenes 2*	573.1	49.8	8	10.2	6.3	5	**
LTA *S. aureus*	315.1	31.9	8	23.2	2.9	8	***
LTA *S. epidermidis*	447.3	77.5	8	30.6	2.3	8	***
LTA *S. agalacticae*	103.8	17.3	8	20.7	5.2	5	**
LTA *S. pneumoniae*							
Fp23	173.2	28.8	13	1.8	2.9	13	***
R6	217.7	36.3	13	10.5	2.3	13	***
LTA *S. suis*	231.5	34.0	8	41.1	4.9	8	***
MALP 2	351.3	40.1	7	16.3	5.5	4	**
$Pam_3Cys\ SK_4$	532.7	52.7	8	66.1	12.2	8	***
LPS *P. gingivalis*	367.0	44.3	12	12.8	4.1	6	***
LPS *S. a. e.* (10 ng/mL) (TLR4 agonist)	183.7	13.9	29	159.4	24.4	26	ns

[a)] TNF response of bone marrow cells derived from $TLR2^{+/+}$ and $TLR2^{-/-}$ mice to stimulation with 10 ng/mL LPS from *S. abortus equi* (*S. a. e.*) and 10 μg/mL TLR2 agonist, respectively. Data are means ± SEM. A p value <0.05 was considered significant; *, ** and *** represent p values <0.05, <0.01 and <0.001, respectively; ns, not significant.

Table 4. Shifts in the LTA ^{1}H NMR spectrum in the presence of increasing concentrations of PPG 1200[a)]

PPG + LTA (molar ratio)	Peak at δ_H (ppm)				
0 : 1	0.8845	2.0961		3.9517	4.2999
0.1 : 1	0.8830	2.0966		3.9527	4.2999
1 : 1	0.8899	2.0966	(3.6955)	3.9532	4.2994
10 : 1	0.8938	2.0961	3.7288	3.9605	4.2984
100 : 1	0.8948	2.0908	3.7302	3.9634	4.2945
1 : 0			3.7315		

a) LTA from *S. aureus* (1 mg) was pre incubated with increasing concentrations of PPG 1200 for 1 h at 37°C. Spectra were measured in D_2O using sodium 3 trimethylsilyl 3,3,2,2 tetradeutero propanoate as an internal standard for ^{1}H NMR (δ_H 0.00 ppm). NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer at 300 K.

0.0103 ppm, is the terminal methyl group of the fatty acids, indicating that PPG 1200 may bind to the lipid part of LTA. We also found a shift at δ_H 3.9517 ppm (maximal shift of 0.0117 ppm), representing groups of the backbone. Other typical peaks like for D-*N*-acetylglucosamine (δ_H 2.0961 ppm) or D-alanine (δ_H 4.2999 ppm) showed no shift. Shifts in the ^{1}H-NMR spectra of LTA were observed to be below 0.006 ppm after addition of increasing concentrations of PEG 1000 (data not shown), affirming the results that the observed shift by PPG 1200 at 0.8845 ppm is caused by direct binding of PPG 1200 to the fatty acids of LTA. Beside LTA, Pam_3Cys was used as a TLR2 ligand in this experimental setting. The incubation with PPG 1200 resulted in a maximal shift of the fatty acid of about 0.03 ppm. However, the maximal shift of LPS signals or of lipid signals like cholesterol, sodium cholesteryl sulfate and glycocholic acid in the presence of PPG 1200 in the NMR spectra was below 0.006 ppm, indicating that only TLR2-active ligands are bound by PPG 1200.

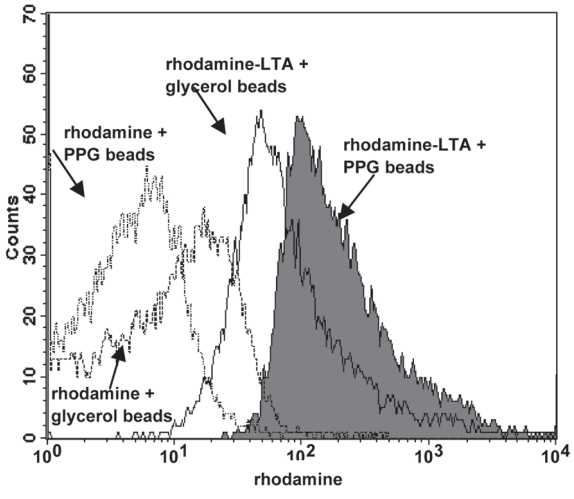

Figure 4. Fluorescent LTA binds PPG ended silica spheres. Rhodamine labeled LTA was incubated with PPG ended or glycerol ended spheres, washed and measured by flow cytometry (representative of three independent experiments).

These observations were supported by functional assays. To determine whether PPG 1200 binds to LTA, we allowed LTA to bind to polystyrene plates at 37°C for 2 h. After washing the unbound LTA away and adding PPG 1200 and blood, the TNF release induced by the bound

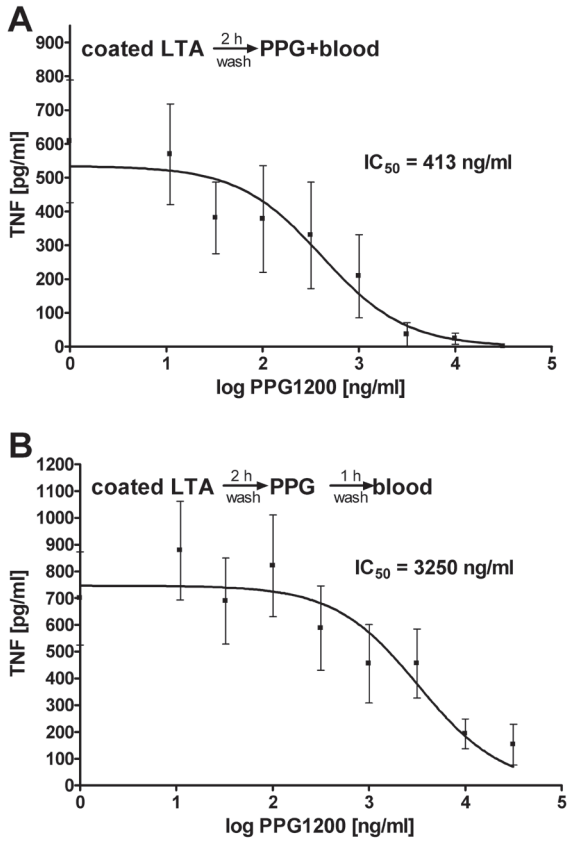

Figure 5. PPG 1200 directly binds to LTA. Human whole blood from 12 healthy volunteers was stimulated with 1 μg/mL LTA coated to polystyrene plates together with increasing concentrations of PPG 1200 (A) or with 1 μg/mL LTA coated to polystyrene plates and pre incubated with increasing concentrations of PPG 1200 before blood was added (B). After 22 h, TNF release was measured in the cell free supernatants by ELISA. The detection limit for the TNF ELISA was 10 pg/mL. The cytokine release by the unstimulated controls was below this detection limit. Data are means ± SEM. IC_{50} values were determined by non linear regression.

LTA was again inhibited by PPG 1200 (IC_{50} 413 ng/mL; Fig. 5A). If after the washing step PPG 1200 was added alone for 1 h and the unbound PPG 1200 was then washed away before the addition of whole blood, an inhibition of LTA-induced cytokine release still took place, albeit with a higher IC_{50} value (Fig. 5B). This indicates that the PPG 1200 can bind to the bound LTA and exert its inhibitory action.

The alternative possibility would be that PPG 1200 binds to the TLR2 receptor. We checked this by pre-incubating PPG 1200 (10 μg/mL) with human whole blood. After 1 h, the blood was washed three times with saline and reconstituted with autologous plasma before LTA was added. The TNF release of the PPG 1200-pre-incubated blood stimulated with LTA was not different (black bar) from the TNF release induced by LTA without PPG (clear bar; Fig. 6), indicating that the PPG 1200 had not bound to the immune cells. When LTA and PPG 1200 where added simultaneously to the reconstituted blood, the TNF induction was completely inhibited, as expected (hatched bar; Fig. 6). To exclude that other blood components interact with PPG 1200, we purified PBMC from human whole blood of eight healthy volunteers and stimulated these cells in the absence of serum with LTA in the same manner as whole blood. The IC_{50} value of 66 ng/mL was comparable to the results obtained with human whole blood, indicating no influence of other blood components on the inhibitory effect of PPG 1200.

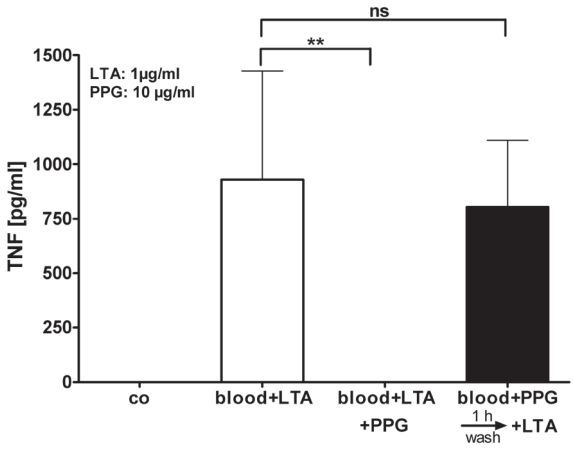

Figure 6. PPG 1200 does not bind to immune cells. Human whole blood from eight healthy volunteers was washed and reconstituted with autologous plasma before stimulation with LTA alone (clear bar) or LTA and PPG 1200 together (below detection limit), or pre incubated with 10 μg/mL PPG 1200 for 1 h, washed and reconstituted with plasma before stimulation with 1 μg/mL LTA (black bar). After 22 h, TNF release was measured in the cell free supernatants by ELISA. The detection limit for the TNF ELISA was 10 pg/mL. The cytokine release by the unstimulated controls was below this detection limit. Data are means ± SEM. A p value <0.05 was considered significant. In the figure, ** represents a p value <0.01; ns, not significant.

Discussion

After successfully establishing and optimizing the isolation and purification of immunostimulatory LTA from different gram-positive bacterial species [7, 9, 11, 33], we aimed to establish a mass culture procedure to up-scale LTA production. In this, we used PPG to avoid foaming. However, the LTA isolated from *S. aureus* grown under these conditions was not able to stimulate cytokine induction. In NMR spectra, we identified PPG that had obviously been extracted together with LTA, despite considerable different physicochemical properties, suggesting binding. This indicated that PPG might be an inhibitor of LTA-induced cytokine release.

We tested the blocking efficacy of several glycol compounds such as PPG, PEG and PBG of different molecular weights by stimulating human whole blood with LTA from *S. aureus* and measuring cytokine release. Only PPG efficiently blocked LTA-induced cytokine release and PPG 1200 showed the most potent inhibitory action for all measured cytokines. Therefore, the length of the PPG chain and the lipophilic character of PPG seem to determine the blocking efficacy.

PPG alone was not able to induce cytokine release in human whole blood. To exclude that PPG has a cytotoxic effect on the immune cells, resulting in the decrease of the cytokine induction, we performed an Alamar blue metabolism test on the blood cells after the incubation. The vitality of the blood cells was not influenced in the presence of PPG 1200 up to concentrations of 30 μg/mL. Additionally, we performed vital staining of CD14-positive cells with propidium iodide, also showing that PPG 1200 had no cytotoxic effects on monocytes. Interestingly, several studies have reported that PPG and closely related glycols have bactericidal or bacteriostatic properties [34–37]. Especially at high concentrations, PPG delayed or inhibited growth of *Mycobacterium avium* cultured in Isolator-BACTEC bottles compared with growth on the solid medium that was inoculated simultaneously [38]. It has even been investigated whether glycols like PPG are suitable as air-disinfecting agents. PPG inhibit the growth of several bacterial species only at concentrations higher than 10% [39].

Then, we focused our interest on the specificity of PPG 1200 inhibition. While cytokine release by TLR4 agonists such as various LPS and whole *E. coli*, by the TLR3 agonist poly I:C, by the TLR7/8 agonists CL097, *E. coli* RNA and R837, and by the TLR9 agonist CpG 2216 was only affected at very high concentrations of PPG 1200, all TLR2 agonists tested, *i.e.* LTA from numerous gram-positive bacterial strains, MALP-2, $Pam_3Cys\text{-}SK_4$ and LPS from *P. gingivalis* and also whole *S. aureus*, were inhibited by PPG 1200. The near complete suppression of cytokine induction by *S. aureus* in the presence of PPG indicates the importance of LTA for the recognition of

gram-positive pathogens by the immune system. It was not surprising that cytokine induction by zymosan from *S. cerevisiae*, which is described as a TLR2 agonist, was only inhibited at high PPG concentrations, because we used commercially available zymosan which was shown to be contaminated with high amounts of endotoxins [40]. Additionally, zymosan is a crude mixture derived from the cell wall of *S. cerevisiae* consisting of approximately 73% polysaccharides, 15% proteins, 7% lipids and inorganic compounds [41]. One component is mannan (a polymer of [1,4]-glycosidic linked α-D-mannose), which has an immunostimulatory activity that requires the presence of TLR4, LBP and CD14 [42, 43].

The question whether PPG 1200 interacts with the TLR2 ligands or the receptor was approached from different angles, firstly by demonstrating direct binding of labeled LTA to PPG-coated microspheres. The results of these FACS experiments showed that microspheres carrying PPG bound more rhodamine-labeled LTA than beads carrying glycerol. Secondly, functional assays, *i.e.* pre-incubating LTA and PPG 1200 and blood in different sequences and determining cytokine release, revealed that PPG 1200 did not bind to cells and their receptors, but directly to LTA. There are two possible explanations for the IC_{50} after pre-incubation of LTA with PPG alone being higher than after incubation of LTA with PPG and blood together: (i) The pre-incubation of PPG with LTA for only 1 h was too short for complete binding or (ii) some of the bound PPG may have been washed away by the additional washing step. Finally, NMR analyses were used to characterize the binding of LTA to PPG 1200. The peak for the terminal methyl group of the fatty acids of LTA shifted in the presence of PPG 1200, reaching a maximal shift of 0.0103 ppm at the highest PPG 1200 concentration. With Pam_3Cys, a maximal shift of 0.03 ppm of the fatty acid peak was detected in the presence of PPG 1200; however, when LPS was incubated with PPG 1200, the maximal shift was less than 0.006 ppm and similar results were obtained upon incubation of different lipid structures like cholesterol, sodium cholesteryl sulfate and glycocholic acid with PPG 1200. The incubation of LTA with increasing concentrations of PEG 1000, which did not inhibit cytokine induction, resulted in a maximal shift of less than 0.006 ppm. These results together indicate that PPG 1200 directly binds to LTA and other TLR2 ligands *via* their fatty acids, which are common features of all TLR2 ligands. Until now it is unclear how the TLR2 ligands interact with the receptor on a molecular level, but it is likely that they do this *via* a common structure, *i.e.* fatty acids.

All approaches suggest direct binding of PPG 1200 to LTA and not to the receptors on the immune cells. Also, the influence of other blood components on the inhibition by PPG 1200 could be excluded since the cytokine release by PBMC in the absence of serum was also inhibited by PPG 1200. These data are strengthened by the observation that LTA isolated from *S. aureus* grown in medium containing PPG 1200 is biologically inactive. Others have shown that PPG binds irreversibly to bacteria, because after incubation of 5×10^9 *S. pneumoniae* with PPG, followed by three washing steps, only 10^3 bacteria could be recovered [39]. Interestingly, a special PEG-PPG polymer for covering biomaterials appears to reduce various blood-biomaterial interactions by preventing coagulation, cell and protein adhesion [44–47]. This indicates no interference between PPG and immune cells or their receptors.

Taken together, in this study, we describe and characterize PPG 1200 as a novel inhibitor of TLR2-induced cytokine release that does not interfere with other immune functions. This effect appears to be mediated by direct binding of PPG to the ligand. This inhibitor of TLR2 agonistic activity may aid in the identification of TLR2-mediated activation pathways, as a form of quality control of preparations of bacterial components, and, being a non-toxic compound, may have potential for clinical application as an anti-inflammatory agent in gram-positive bacterial disease.

Materials and methods

Materials

LTA were isolated by butanol extraction and hydrophobic interaction chromatography as described [7, 9] from *S. aureus* (DSM 20233); *Bacillus subtilis* (DSM 1087); *Listeria monocytogenes* (ATCC 43251); *Streptococcus agalacticae* (strain COH1), provided by P. Henneke, Albert Ludwigs University of Freiburg, Germany; *S. pneumoniae* R6 (serotype 2), provided by E. Tuomanen, St. Jude's Children's Research Hospital, Memphis, TN; *S. pneumoniae* strain Fp23 (serotype 4), provided by M. R. Oggioni, Università di Siena, Italy; *Bifidobacterium animalis* variant a and b (MB 254), both gifts from Dr. Reinscheid, University of Ulm, Germany; *L. plantarum* (NCIMB 8826), a gift from E. Palumbo, Université Catholique de Louvain, Belgium; *Streptococcus suis* (serotype 2, 3/S33), provided by M. Gottschalk, Université de Montréal, Canada; *Enterococcus faecium* (strain VISA), provided by J. Vranes, Zagreb Institute of Public Health, Croatia; *Staphylococcus epidermidis* O 47, from T. Biedermann, University Hospital, Tübingen, Germany; and *Lactobacillus delbrueckii* (ATCC 15808), provided by L. Räisänen, University of Oulu, Finland.

Other substances were LPS from *S. abortus equi, Serratia marcesens, Shigella flexneri, Klebsiella pneumoniae, Vibrio cholerae* and *E. coli* O26:B6 and O11:B4 (Sigma, Deisenhofen, Germany) and LPS from *P. gingivalis* (MCCM 03199, kindly provided by Alexander Dalpke, University of Heidelberg, Germany), which was purified in house in analogy to LTA;

$Pam_3Cys\ SK_4$ (EMC microcollections, Tübingen, Germany), MALP 2 from *M. fermentans* (kindly provided by Peter F. Mühlradt, Braunschweig, Germany), zymosan A from *S. cerevisiae* (Sigma), CpG2216 (MWG Biotech AG, Ebersberg, Germany), poly I:C, CL097, R837 and *E. coli* RNA (all from Invivogen, San Diego, USA); PEG and PPG (Fluka, Buchs, Switzerland) and PBG (a kind gift from H. Mach, BASF, Ludwigshafen, Germany), which were sonicated for 30 min before use; cholesterol (highly purified, Calbiochem, Darmstadt, Germany), sodium cholesteryl sulfate and glycocholic acid (both from Sigma).

All non endotoxins except zymosan A (not possible to test) were tested by kinetic Limulus amoebocyte lysate assay (Charles River, Charleston, USA) and contained $<$1.0 EU/mg LTA, *i.e.* less than 100 pg LPS equivalents per mg LTA. Only *E. coli* RNA revealed a higher value of $<$5 EU/mg.

Bacteria were cultured aerobically in Luria Bertani broth (MP Biomedicals, Heidelberg, Germany) for 16 h at 37°C and 5% CO_2. They were harvested and washed three times in PBS. For UV inactivation and subsequent whole blood stimulation, bacteria were adjusted to 10^8 bacteria/mL, and 1 mL per well was irradiated on ice (UV Stratalinker 1800; Stratagene, La Jolla, CA) with an energy density of 1 kN/cm^2 (3 mWatt/cm^2 $\times$ 300 s) for 5 min in a 6 well cell culture plate (Greiner Bio One, Frickenhausen, Germany). No colonies grew on blood agar plates (Columbia blood agar; Heipa Diagnostika, Eppelheim, Germany) after 24 h at 37°C and 5% CO_2.

Whole blood incubations

Human whole blood incubations were performed as described previously [48]. Briefly, human blood was drawn from healthy volunteers into heparinized S monovettes® (Sarstedt, Nümbrecht, Germany) and diluted fivefold in RPMI 1640 medium (Biochrom, Berlin, Germany). The final volume was adjusted to 500 μL, and the incubations were carried out in open polypropylene vials overnight for 22 h at 37°C and 5% CO_2.

When PPG was pre incubated with blood for 1 h at 37°C and 5% CO_2, the blood was subsequently washed three times with saline and reconstituted with autologous plasma before the LTA was added. Polystyrene cell culture plates (96 well; Greiner Bio One) were used to coat LTA on a surface. Of LTA, 1 μg/well was pre incubated for 2 h at 37°C and 5% CO_2, followed by two washing steps with physiological saline. Either PPG 1200 was pre incubated for 1 h followed by two washing steps and the addition of human whole blood, or PPG 1200 and blood were added together. The final volume was adjusted to 200 μL by adding RPMI 1640, and the samples were incubated for 22 h at 37°C and 5% CO_2.

The pelleted blood cells were then resuspended by gentle shaking and were centrifuged at 400 $\times$ g for 2 min. The cell free supernatants were stored at −80°C until cytokine measurement by ELISA.

Preparation of human PBMC

Human PBMC of healthy volunteers were prepared with CPT™ Cell Preparation Tubes (BD Biosciences). After centrifugation at 1600 $\times$ g for 20 min, PBMC were collected and washed at 300 $\times$ g for 5 min with RPMI 1640 and 2.5 IU/mL Liquemin (Hoffmann La Roche, Mannheim, Germany). The cells were plated at a density of 5 $\times$ 10^5/tube in the absence of any serum supplement. PBMC were stimulated in the presence of 5% CO_2 at 37°C for 22 h. The cell free supernatants were stored at −80°C until cytokine measurement by ELISA.

Isolation of murine bone marrow cells

TLR2 deficient mice kindly provided by Tularik (South San Francisco, CA, USA) and the corresponding wild type mice (129Sv/C57BL/6) were bred in the animal facilities of the University of Konstanz and genotyped. Mice were killed by terminal pentobarbital anesthesia (Narcoren; Merial, Halbergmoos, Germany). The humeri and femurs of the mice were lavaged with 10 mL ice cold sterile PBS (Life Technologies, Karlsruhe, Germany). The lavages were transferred to siliconized glass tubes (Vacutainer; Bioscience, Heidelberg, Germany) and bone debris was removed. After one centrifugation step, the cell counts were determined. The cells were diluted to 5 $\times$ 10^6/mL with RPMI 1640 supplemented with 10% heat inactivated FCS (Biochrom) and plated on 96 well culture plates for stimulation with different stimuli. After 22 h at 37°C and 5% CO_2, supernatants were frozen at −80°C until cytokine measurement by ELISA.

Cytotoxicity exclusion

After incubation, the vitality of the blood pellet (70 μL) was measured after adding 150 μL of 10% Alamar blue in RPMI 1640 (Biosource, Nivelles, Belgium) for 2 h. Quantification was done by measurement at 560/580 nm (Victor^2 D fluorometer; Wallac, Turku, Finland). As controls, we used freshly drawn blood and blood that was treated with 10% dimethyl sulfoxide.

Vital staining of monocytes was carried out after stimulation of human whole blood for 22 h without or with LTA (1 μg/mL), PPG 1200 (31.6 μg/mL) or both. Erythrocytes were lysed three times with distilled water. The white blood cells were stained with anti CD14 APC antibody (BD Biosciences) for 25 min at room temperature. After a washing step, propidium iodide solution (500 ng/mL; Sigma) was added 5 min before measuring the fluorescence of the samples in a FACSCalibur (BD Biosciences).

Cytokine measurement

Cytokines released by human whole blood were measured by in house sandwich ELISA using commercially available antibody pairs and recombinant standards. Monoclonal antibody pairs against human TNF and IL 8 were purchased from Endogen (Perbio Science, Bonn, Germany), against human IL 1β and IL 6 from R&D Systems (Wiesbaden, Germany), and against human IL 10 from BD Biosciences (Pharmingen, Heidelberg, Germany). Recombinant standards for TNF, IL 1β and IL 6 were kind gifts from S. Poole (NIBSC, Herts, UK); rIL 8 was from PeproTech (Tebu, Frankfurt, Germany) and rIL 10 from BD Biosciences. The release of human TGF β and murine TNF was measured with the DuoSet kit from R&D Systems. Assays were carried out in flat bottom, ultrasorbant 96 well plates (MaxiSorp; Nunc, Wiesbaden, Germany). The

secondary biotinylated antibodies were detected by horseradish peroxidase conjugated streptavidin (Biosource, Camarillo, CA), and 3,3′,5,5′ tetramethyl benzidine (Sigma) was used as substrate. The reaction was stopped with 1 M H_2SO_4, and the absorption was measured in an ELISA reader at 450 nm with a reference wavelength of 690 nm.

LTA labeling with rhodamine

Rhodamine labeled LTA was prepared by sonifying 3 mg LTA from *S. aureus*, 4.5 mg sulforhodamine Q 5 acid fluoride (Fluka), 2.5 mL dimethylsulfoxide (Wak Chemie Medical GmbH, Steinbach, Germany) and 25 μL trimethylamine (Acros Organics, Loughborough, UK) for 10 min. The mixture was shaken overnight at 37°C, followed by centrifugation (four times) for 90 min at 7000 × g in pyrogen free centrifugal ultrafiltration tubes (Microsep 3K; Centricons, Pall, MI) to remove the remaining unbound dye. The labeled LTA was filtered through a pyrogen free Sepharose column (PD 10 desalting column; Amersham Biosciences, Freiburg, Germany). The yield of labeled LTA was determined by phosphate content [9] and the fluorescence was measured (Victor^2 Fluorometer; Wallac). The labeling efficiency, calculated as fluorescence (560 nm/620 nm) per phosphate content, was one to two rhodamine molecules per LTA. The rhodamine labeled LTA was negative in the kinetic Limulus amoebocyte lysate assay (Charles River), and its biological activity was not affected by the dye, as tested by measuring TNF and IL 8 release in human whole blood in comparison to unlabeled LTA.

Preparation of PPG- and glycerol-ended silica spheres

PPG 1200 and glycerol surface ended amorphous silica spheres were prepared by modification of the standard alkoxide precipitation method [49]. We took advantage of the self adsorption of the organic PPG or glycerol chains to the hydrolyzed alkoxide at alkaline pH to obtain silica spheres with surfaces saturated with the organic precursors. Two batches were prepared, one with PPG 1200 and another with glycerol. The silica spheres were synthesized by adding into the reaction beaker an aqueous solution of NH_3, PPG 1200 or glycerol (Sigma), absolute ethanol and tetraethoxysilane (Fluka), respecting the addition order. The volumes were calculated to obtain the following final molar concentrations: NH_3 0.2 M, PPG 1200 (or glycerol) 0.02 M, and tetraethoxysilane 0.2 M. The precipitation reaction was carried out at 50°C under continuous stirring for 24 h. The spheres were then centrifuged in 1.5 mL tubes at 8000 × g and 20°C, and the remnant non reacted liquid was discarded. The spheres were then washed twice in PBS.

Interaction of LTA and PPG

PPG 1200 or glycerol containing spheres were incubated with rhodamine labeled LTA under agitation. Blood erythrocytes were lysed with FACS lysing solution (BD Biosciences). Cells were then incubated with PPG 1200 or saline at 4°C and washed twice before incubation with rhodamine labeled LTA. In another experiment, rhodamine labeled LTA was pre incubated with PPG 1200, then washed and added to the blood cells. The fluorescence of spheres or leukocytes was measured in a FACSCalibur (BD Biosciences).

NMR spectroscopy

NMR spectra were recorded on a Bruker Avance DRX 600 spectrometer equipped with an inverse TXI H/C/N triple resonance probe at 300 K using 3 mm Bruker Match^{TM} sample tubes. Spectra were measured in D_2O using sodium 3 trimethylsilyl 3,3,2,2 tetradeutero propanoate as an internal standard for ^{1}H NMR (δ_H 0.00 ppm). PPG 1200, PEG 1000 and LTA from *S. aureus* were separately measured, and after pre incubation of increasing concentrations of PPG 1200 or PEG 1000 with 1 mg LTA at molar ratios of 0.1 : 1, 1 : 1, 10 : 1 and 100 : 1. Similar experiments were carried out with 1 mg Pam_3Cys or LPS from *S. abortus equi*. For cholesterol, sodium cholesteryl sulfate and glycocholic acid, methanol d_4 was used as solvent.

Statistics

Statistical analysis was performed using the Graph Pad Prism program (Graph Pad Software, San Diego, CA). Repeated measure analysis of variance followed by Dunnett's Multiple Comparison test was assessed. IC_{50} values were obtained by non linear regression (sigmoidal dose response) and significance was determined by Kruskal Wallis test. For statistical analysis of two groups of nonparametric data, the Mann Whitney test was used. Data are given as means ± SEM. A p value <0.05 was considered significant; *, ** and *** represent p values of <0.05, <0.01 and <0.001, respectively; ns, not significant. Cytokine levels are given per milliliter blood, *i.e.* corrected for the dilution factor 5 in the 20% blood incubation.

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