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A mono-acyl phospholipid (20:1 lyso-PS) activates Toll-Like Receptor 2/6 hetero-dimer

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ABSTRACT

Toll-like receptor 2 (TLR2) is an important pattern recognition receptor on the surface of host immune cells that binds a variety of ligands that are released by microorganisms as well as by damaged or dying host cells. According to the current concept, TLR2/1 and TLR2/6 heterodimers are activated by tri- or di-acylated ligands, respectively. However, also mono-acyl phospholipid containing lipid fractions derived from parasites, were reported to be able to activate TLR2. In order to provide conclusive evidence for the TLR2 activating capacity of mono-acyl phospholipids derived from pathogens, we developed a biosynthetic method to enzymatically convert commercially available phospholipids into several mono-acyl-phospholipid variants that were examined for their TLR2 activating capacity. These investigations demonstrated that 1-(11Z-eicosenoyl)-glycero-3-phospho-serine 20:1 (20:1 lyso-PS) is a true agonist of the TLR2/6 heterodimer and that its polar headgroup as well as the length of the acyl chain are crucial for TLR2 activation. *In silico* modelling further confirmed 20:1 mono-acyl PS as a ligand for TLR2/6 heterodimer, as this predicted that multiple hydrogen bonds are formed between the polar headgroup of 20:1 mono-acyl PS and amino acid residues of both TLR2 and TLR6. Future studies can now be performed to further assess the functions of 20:1 lyso-PS as an immunological mediator, because this enzymatic method enables its preparation in larger quantities than is possible by isolation from the parasite that naturally produces this compound, *Schistosoma mansoni*, the source of the original discovery (Van der Kleij et al., 2002).

1. Introduction

Pattern recognition receptors on the surface of host immune cells fulfil a crucial function in the innate immune response, as these receptors sense the presence of distinct pathogen associated molecular patterns (PAMPs) that are released by micro-organisms as well as by damaged or dying host cells (Uematsu and Akira, 2006; Beutler, 2009). Among these pattern recognition receptors, Toll-like Receptors (TLR) are an important class that comprises multiple analogues in varying numbers in distinct organisms. The transmembrane TLRs, TLR1 to TLR6 and TLR11, consist of an extracellular domain containing leucine-rich repeats that recognize distinct PAMPs and an intracellular toll-interleukin1 (IL-1) receptor (TIR) domain required for downstream signaling that regulates the expression of pro-inflammatory cytokines (Uematsu and Akira, 2006; Beutler, 2009). TLR2 is probably the best studied TLR and its importance in the host immune response to many infectious diseases has been shown by various TLR2 knock-out mice studies (van Bergenhenegouwen et al., 2013). In addition, TLR2 is of interest because it is a unique TLR as it needs, unlike other TLRs, to form heterodimers with TLR1 or TLR6 to initiate downstream signaling (Ozinsky et al., 2000).

Over the years a wide variety of TLR2 ligands has been identified from many distinct microorganisms (van Bergenhenegouwen et al., 2013; Oliveira-Nascimento et al., 2012). Most identified TLR2 ligands are lipoproteins or polysaccharides to which two or three fatty acyl chains are attached. Further analysis showed that ligands that bind and activate TLR2/1 heterodimers are often tri-acylated ligands, whereas those that activate TLR2/6 are often di-acylated (Uematsu and Akira, 2006; van Bergenhenegouwen et al., 2013; Oliveira-Nascimento et al., 2012; Beutler et al., 2006). In contrast to this general concept, only very few pathogen-derived TLR2-ligands have been reported that contain a single hydrophobic side chain. The lipid fraction of the blood-dwelling

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fluke *Schistosoma mansoni* containing 1-(eicosenoyl)-glycero-3-phosphoserine (20:1 lyso-PS), was the first mono-acyl phospholipid shown to be able to activate TLR2 (Van der Kleij et al., 2002). Next, indirect evidence obtained by *in vivo* studies using TLR2 knock-out mice, suggested that purified lipid fractions of *S. mansoni* containing mono-acyl phosphatidylcholine (PC) species might be an agonist for TLR2 as well (Magalhaes et al., 2010). Later on, it was reported that mono-acyl PC species could trigger TLR2 as well as TLR4 mediated signalling in TLR-transfected HEK-293 cells, but in that study contradictory results were found as mono-acyl PC also counteracted the lipopolysaccharide-induced signalling via TLR4 in isolated macrophages (Carneiro et al., 2013). These three reports, which were mainly based on studies using purified lipid fractions from *S. mansoni*, are so far the only published evidence for mono-acylated molecules derived from pathogens that can activate TLR2. This raises the question whether mono-acylated phospholipids are true activating ligands of TLR2, or that the observed TLR2 activation was induced by other minor components inevitably present in purified fractions of biological origin?

To further investigate the activation of TLR2 by mono-acyl PS, we developed a biosynthetic method to enzymatically convert commercially available di-acyl PC to 20:1 mono-acyl PS, as well as other mono-acyl-phospholipid variants with either a different polar headgroup or different acyl-chains varying in number of carbon atoms. The products from this easy-to-use biosynthetic method allowed us to study the activating effect of specific mono-acyl PS species on TLR2, instead of using mixtures of mono-acyl PS species present in isolated fractions of schistosomes. Furthermore, the yield of the procedure can be substantially larger compared to isolating lyso-phospholipids from adult *S. mansoni* worms, which have to be isolated from infected laboratory animals.

For binding to and activation by bacterial lipopeptides, TLR2 has to form heterodimers with TLR1 or TLR6 (Uematsu and Akira, 2006; van Bergenhenegouwen et al., 2013; Oberg et al., 2011). Therefore, we also investigated which TLR2 heterodimer combination is activated by mono-acyl PS. Finally, we have generated an *in silico* model to study the interaction between mono-acyl PS and the involved TLR 2/6 heterodimer, which allowed structural comparison with FSL-1, a synthetic di-acyl lipoprotein and known activator of the TLR2/6 heterodimer.

2. Experimental procedures

2.1. Synthesis of mono-acyl phospholipids

Specific mono-acyl phospholipids were produced from commercially available di-acyl-phospholipid precursors by the following procedure. Different glycero-phosphocholines (PC) species were purchased from Avanti (Avanti Polar Lipids, Alabaster, Al, USA), which included 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC 18:1/18:1), 1,2-dieicosenoyl-sn-glycero-3-phosphocholine (PC 20:1/20:1), 1,2-dierucoyl-sn-glycero-3-phosphocholine (PC 22:1/22:1) and 1,2-dinervonoyl-sn-glycero-3-phosphocholine (PC 24:1/24:1). Unless otherwise specified, all other reagents were purchased from Sigma Aldrich, St. Louis, USA.

For the conversion of PC into glycero-phosphoserine (PS), headgroup substitution of PC was performed by a procedure based on the method of Comfurius and Zwaal (1977). In short, PC was dissolved in diethyl ether at a concentration of 5 mg/ml after which phospholipase D (10 U/ml) was added in an L-serine saturated acetate buffer (1 ml, 100 mM, pH 5.6) containing 100 mM $CaCl_2$. After continuous stirring for 3 hours at 37 °C, the reaction was stopped by acidification by addition of 50 µl 6 M HCl. Samples were spun down at 500*g* for 5 min, after which the ether phase was transferred to a new tube and dried under N_2. From the dried residue, lipids were extracted according to the method of Bligh and Dyer (1959). Subsequently, distinct classes of phospholipids (PS, PC and PA) were separated by column chromatography over a carboxymethyl cellulose CM-52 column (Serva, Heidelberg, Germany), essentially as described by Comfurius and Zwaal (1977).

Purified di-acyl-phospholipids were converted into mono-acyl phospholipids by enzymatic hydrolysis using phospholipase A_2 (20 U) in a two-phase system, consisting of 2 ml diethyl ether and 1 ml 100 mM TRIS-HCl, 4 mM $CaCl_2$, pH 7. After 90 min incubation the enzymatic conversion was stopped by acidification of the reaction medium by addition of 40 µl 1 M HCl, which also induced the translocation of the mono-acyl phospholipids from the aqueous phase to the organic diethyl-ether phase of the incubation. After centrifugation for 5 min at 500*g* the organic phase was collected and transferred to a sterile tube containing a physiological salt solution (NaCl, 0.9% w/v). After continuous stirring for 1 min at 37 °C followed by centrifugation for 5 min at 500*g*, the organic phase was slowly evaporated at 55 °C, eventually yielding solubilized mono-acyl phospholipids in a physiological salt solution. Phospholipid concentrations were quantified by a phosphate assay as described by Rouser et al. (1970).

The synthesized phospholipids were characterized by two methods. To monitor the efficiency of the synthesis process by a quick and simple method, the phospholipid composition during the synthesis process was analyzed by thin layer chromatography (TLC) as described before (Skipski et al., 1962). The identity of the synthesized endproducts was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS). For this analysis phospholipids were dissolved in MeOH: $CHCl_3$ (2:1); 0.5 µl of this solution was spotted on a stainless steel surface and overlaid with 0.5 µl of a matrix (2 mg *alpha*-cyano-4-hydroxycinnamic acid acid in 1 ml acetonitrile). MALDI-TOF spectra were recorded on an Ultraflex III MS (Bruker Daltonics) using the reflectron in both positive and negative ion modes as described in Ruttink et al. (2012). A laser repetition rate of 50 Hz was used and 1,000 shots (50 shots per raster spot) were accumulated. The laser power was chosen such that no signal saturation occurred after 50 shots at each raster spot.

2.2. Activation of Toll-Like Receptor 2 by synthesized phospholipids

To investigate whether the synthesized mono-acyl phospholipids were capable of activating TLR2, HEK-TLR2-Blue reporter cells were used according to the manufacturer's instructions (InvivoGen, San Diego, USA). These cells over-express TLR2, while having endogenous expression of TLR1 and TLR6. In these reporter cells, activation of TLR2 finally leads to Nf-κB dependent expression of secreted embryonic alkaline phosphatase, which can subsequently be quantified by measuring the alkaline phosphatase activity in the supernatant of the cell culture medium. For all assays 50,000 HEK-TLR2-Blue reporter cells per well were incubated in 280 µl culture medium with either the synthesized mono-acyl phospholipids in a range of 0-75 µM or the positive control compounds Pam2CGDPKHPKSF (FSL-1, InvivoGen) (2.2 pM) or Pam3CysSerLys4 (Pam3CSK4, InvivoGen) (23 pM) for activation of the TLR2/6 and TLR2/1 heterodimers, respectively. After overnight incubation at 37 °C the alkaline phosphatase activity in the culture medium was measured at 37 °C using the HEK Blue detection kit according to the manufacturer's instructions (absorbance measured at 620 nm). Negative controls were prepared by performing the complete biosynthesis process as described above, but without addition of PC in the first step of the biosynthesis process.

To determine the TLR2-heterodimer composition that is activated by the synthesized mono-acyl phospholipids, specific blocking-antibodies against TLR 1, TLR2 and/or TLR6 (InvivoGen, San Diego, USA) were added one hour before stimulation of reporter cells with the biosynthesized mono-acyl phospholipids or positive controls for TLR2/1 (Pam3CSK4) and TLR2/6 (FSL-1) activation. Blocking antibodies were used in equal volumes at a concentration of 1.5 mg/ml (TLR1 and TLR6) and 0.75 mg/ml (TLR2).

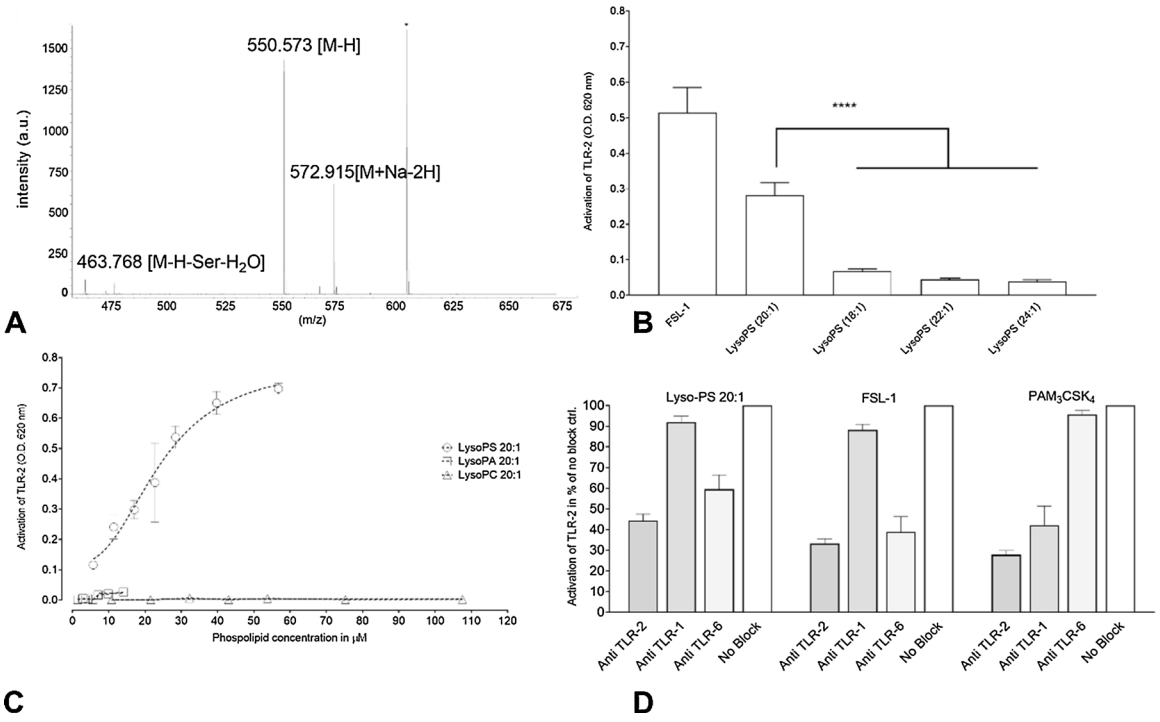

Fig. 1. TLR2 activating properties of biosynthesized mono-acyl phospholipids. Panel A shows the negative mode MALDI-TOF/MS spectrum of the synthesized 20:1 mono-acyl PS (predicted theoretical mass 551.3 amu). Peaks at 550.573 and 572.915 m/z correspond with 20:1 mono-acyl PS [M-H] and its sodium adduct [M + Na-2 H], respectively. The peak at 463 m/z corresponds with the mass of mono-acyl PS after neutral loss of the serine headgroup [M-H-Ser-H_2O]. A matrix peak is indicated by *. Panel B shows the TLR2 activation of 20:1 mono-acyl PS (Lyso-PS) in comparison to other mono-acyl PS molecules with a different acyl-chain configuration. TLR2 activation was quantified by measuring TLR2-induced expression of Nf-κB-SEAP after overnight incubation of HEK-TLR2-Blue with 25 µM synthesized mono-acyl PS 18:1, 20:1 mono-acyl PS, mono-acyl PS 22:1 or mono-acyl PS 24:1.FSL-1, 2.1 pM was added as positive control. Shown is the mean and standard deviation of the activity of the induced alkaline phosphatase (Nf-κB-SEAP) optical density (O.D.) at 620 nm of 3 independent incubations. Subtracted from all measurements in Fig. 1B is the medium control, with an absorbance value of 0.12. Non-parametric T-tests were performed to examine significant differences (**** indicates $p < 0.0001$). Panel C shows the TLR2 activation of 20:1 mono-acyl PS in comparison to mono-acyl PA 20:1 and mono-acyl PC 20:1 at the indicated concentrations. Shown is the mean and standard deviation of 3 independent incubations, as in panel B. Panel D. Selective blocking of TLR1, TLR2 or TLR6 reveals that mono-acyl PS is an agonist of the TLR2/6 heterodimer. HEK-Blue TLR2 cells were incubated with blocking antibodies against TLR1, TLR2 or TLR6 before 20:1 mono-acyl PS or the known agonists for TLR2/1 (PAM3CSK4, 23 pM) or TLR2/6 (FSL-1, 2.1 pM) were added. The TLR2 activation is expressed as the percentage of control incubations (no blocking antibodies). Shown is the mean and SD of 2 independent experiments, each performed in triplicate. The absorbance values at 620 nm of the no block controls were 0.67, 0.95 and 1.25, respectively.

2.3. In silico modeling of 20:1 mono-acyl PS in the TLR2/6 heterodimer complex

Ligand binding of 20:1 mono-acyl PS to the TLR2/6 heterodimer complex was modeled using AutoDock Vina (Trott and Olson, 2010) and subsequently analyzed with Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). SMILES models (Weininger, 1988) of 20:1 mono-acyl PS were created (see supplemental Fig. 1A) and converted to a PDB-file. Subsequently, a model of the crystal structure of TLR2/6 with its ligand FSL-1 (Jin et al., 2007) was downloaded from rcsb.org using identifier 3A79. The FSL-1 ligand was removed, and the software was queried to calculate a possible fit for the earlier drawn SMILES model of 20:1 mono-acyl PS.

3. Results

3.1. Synthesis of Lyso-PS

In order to provide conclusive evidence for the TLR2 activating capacity of mono-acyl phospholipids, which was so far based on the TLR2 activating capacity of purified lipid fractions derived from *S. mansoni* (Van der Kleij et al., 2002; Magalhaes et al., 2010; Carneiro et al., 2013), we developed a biosynthetic method to enzymatically prepare mono-acyl PS species with various acyl chains, as well as the related mono-acyl phospholipids: phosphatidic acid (mono-acyl PA) and mono-acyl phosphatidylcholine (mono-acyl PC).

We used two consecutive enzymatic reactions to produce these compounds. First, commercially available PC was transphosphatidylated with phospholipase D in the presence of serine, which results in the formation of PS (Comfurius and Zwaal, 1977). After purification on CM-cellulose, this di-acyl PS was then treated with phospholipase A2 to remove the acyl chain attached to the sn-2 position and to obtain a mono-acyl PS molecule with an acyl chain in the 1-position. The specificity of PLA2 for removal of the acyl chain at the 2 position is high, but not absolute. However, treatment of PS with PLA2 will result in the formation of lyso-PS with an acyl chain at position 1 exclusively, because if the acyl-chain at position 1 is hydrolysed by side-activity of PLA2, the remaining acyl chain (at position 2) will migrate by spontaneous isomerization to the more energetically favourable sn-1 position, resulting in lyso-PS with the acyl chain in position 1 (Plueckthun and Dennis, 1982). This means that the only 2 possible products of PLA2 treatment of PS, are lyso-PS with an acyl chain at the 1 position and glycerophosphoserine. This is indeed what we observed. To test this isomerization effect we treated our purified PS fraction with PLA1. In that case the spontaneous isomerization of the acyl chain to position 1 should result in formation of glycerophosphoserine exclusively, because after removal of the acyl chain at position 1, the chain in position 2 should move to position 1 and be subsequently hydrolysed. We investigated this effect of PLA1 on PS under the conditions of our method and the result was indeed the formation of

glycerophosphoserine exclusively (not shown). Starting with commercially available PC containing two identical fatty acid residues ensures that all formed lyso-PS molecules have one and the same fatty acid residue. As demonstrated by the MALDI-TOF/MS spectra (Fig. 1A), this method successfully yielded mono-acyl phospholipids from diacyl precursors.

The first step, the transphosphatidylation, was highly effective, leaving no detectable amounts of residual PC, as determined by TLC. Around 60 % of the PC was converted into PS, while the remainder was hydrolysed to PA. In the CM-52 column chromatography step, the first fractions containing PS also contain small amounts of PA. These fractions were discarded, and approximately 50% of the PS produced from PC, was used for the second step, the production of lyso-PS from PS. In this step, the activity of phospholipase A2 used to obtain lyso-PS was enhanced by the use of a two-phase system of diethyl ether and water containing Ca^{++} ions (Misiorowski and Wells, 1974). After conversion of PS to lyso-PS by PLA2 no residual PS remained after 90 minutes, which implies that exclusively lyso-PS with the acyl chain in position 1 was formed (reasoning see above). Isolation of the resulting lysophospholipids from the reaction mixture by extraction from the diethyl ether phase, followed by transfer to an aqueous solution, had an efficiency of around 60%. The final yield of the complete procedure was around 18%. The above described procedure for the complete synthesis and purification of lyso-phospholipids can be completed within 6 h.

3.2. 20:1 mono-acyl PS activates TLR2

Of the mono-acyl PS species that were examined for their TLR2 activating capacity at a concentration of 40 μM, 20:1 mono-acyl PS was found to be most effective in activating TLR2, whereas 18:1 mono-acyl PS, 22:1 mono-acyl PS and 24:1 mono-acyl PS hardly activated TLR2. (Fig. 1B). These results demonstrated that it is indeed 20:1 mono-acyl PS that activates TLR2 with a calculated ED50 of 24 μM, which is comparable the ED50 of the purified lyso-PS containing fractions of the parasite *S. mansoni* (Van der Kleij et al., 2002). In addition, these results show that the length of the acyl chain in the mono-acyl PS molecule is critical for TLR2 activation. Next, we examined the influence of the polar headgroup in 20:1 mono-acyl PS by comparing its TLR2 activating capacity to that of synthesized 20:1 mono-acyl PC and 20:1 mono-acyl PA. These experiments showed that 20:1 mono-acyl PS activated TLR2 in a dose-dependent manner, whereas 20:1 mono-acyl PC and 20:1 mono-acyl PA did not activate TLR2 (Fig. 1C). The range of concentrations in which these mono-acyl phospholipids were examined for their TLR2 activating capacity differed as the maximal concentrations that could be used were either limited by the maximal solubility in aqueous solutions as mono-acyl PA was very poorly soluble in aqueous solutions, or because higher concentrations (20:1 mono-acyl PS) lead to death of HEK-TLR2-Blue reporter-cells, a feature that has been reported for mono-acyl phospholipids before (Arouri and Mouritsen, 2013). Taken together, these results are in agreement with the earlier observations by Van der Kleij et al. (Van der Kleij et al., 2002) confirming 20:1 mono-acyl PS as TLR2 agonist. The fact that this synthetic 20:1 mono-acyl PS activates TLR2, rules out the possibility that minor other compounds present in the schistosomal lipid fractions were the TLR2 activating ligands.

3.3. Mono-acyl PS activates the TLR-2/6 heterodimer

In contrast to most other activating ligands for TLR2 heterodimers, 20:1 mono-acyl PS only comprises a single acyl chain. For this reason we determined the TLR2 heterodimer composition that is activated by 20:1 mono-acyl PS. By addition of blocking antibodies against TLR1, TLR2 or TLR6 before the TLR agonist is added, it can be determined which TLR heterodimer composition is activated by which agonist. Blocking of TLR2 before addition of 20:1 mono-acyl PS reduced the TLR2 activation by 60%, which was similar to the observed reduction by blocking TLR2 before addition of the positive control ligands Pam3CSK4 for TLR2/1 and FSL-1 for TLR2/6 heterodimer activation (Fig. 1D). Blocking of TLR1 by blocking antibodies before addition of 20:1 mono-acyl PS did not reduce the TLR2 activation, whereas it reduced the TLR2 activation induced by the well-known TLR2/1 heterodimer agonist Pam3CSK4 (Fig. 1D). Blocking of TLR6 before addition of 20:1 mono-acyl PS did result in inhibition of TLR2 activation, and this observed inhibition was similar to that of FSL-1, a well-known agonist of the TLR2/6 heterodimer (Fig. 1D). These results demonstrated that activation of TLR2 by 20:1 mono-acyl PS occurs via the TLR2/6 heterodimer. From these data, it can be concluded that 20:1 mono-acyl PS is a true agonist of the TLR2/6 heterodimer, and therefore, this heterodimer that is classically involved in the binding of di-acyl-lipopeptides, also accepts a mono-acyl phospholipid as a ligand.

To gain more insight into the interaction between mono-acyl PS and the TLR2/6 heterodimer, we used the available crystal structure of TLR2/6 to generate an *in silico* model of the potential interaction of 20:1 mono-acyl PS and the TLR2/6 heterodimer. A search space was defined around the active site of the TLR2/6 heterodimer, after which automatic fits of 20:1 mono-acyl PS were calculated. The generated models are available as PyMol workspace (see supplemental data and an online movie). In the optimal configuration in the *in silico* model, the hydrophobic acyl chain of 20:1 mono-acyl PS was positioned in the hydrophobic pocket of the TLR2 monomer such that it completely filled the pocket (Fig. 2A and 2B), with a theoretical binding affinity of −34.3 kJ/mol. Modelling of the other abundant mono-acyl PS variants present in *S. mansoni* (18:0, 22:4) demonstrated that the acyl chain of these mono-acyl PS species did not fit in the same hydrophobic pocket of the TLR2/6 receptor. These results are in agreement with the experimental data which showed that 20:1 mono-acyl PS was the most potent agonist for the TLR2/6 heterodimer. Fig. 2B shows an overlay of 20:1 mono-acyl PS with FSL-1, the well-known agonist of TLR2/6 heterodimers that was also used to produce crystals for X-ray crystallography. This image shows that the acyl chain of 20:1 mono-acyl PS mainly follows a similar configuration in the hydrophobic pocket of TLR2 as the lipid side chains of FSL-1. Finally, in the optimal configuration in the *in silico* model multiple hydrogen bonds are formed between the hydrophilic headgroup of 20:1 mono-acyl PS and amino acids in the ligand binding pocket of the TLR2/6 heterodimer (Fig. 2C); amino acid residue D327 of TLR2 and the residues K321 and F319 of TLR6. Of these amino acid residues D327 of TLR2 and F319 of TLR6 are known to form hydrogen bonds with FSL-1 during ligand (Jin et al., 2007), and therefore, binding of 20:1 mono-acyl PS might stabilize the TLR2/6 heterodimer similar to stabilizing effect of FSL-1 binding to TLR2/6.

The lower affinity of the TLR2/6 receptor for 20:1 lyso-PS compared to FSL-1 can be explained. Although the single acyl chain of 20:1 mono-acyl PS fills the hydrophobic pocket of the TLR2/6 receptor similar to the two acyl chains of FSL-1, the latter comprises two acyl chains that are both docking in the hydrophobic pocket, which results in a more stable interaction. In addition, the acyl chains are only part of the interaction between the 20:1 lyso-PS ligand and the TLR2/6 receptor, as the headgroup also interacts with the two subunits of the receptor. The headgroup of FSL-1 (CGDPKHPKSF) is completely different and substantially larger than the one of 20:1 lyso-PS. For these reasons, the interactions of lyso-PS and FSL-1 with the TLR2/6 receptor will be different.

4. Discussion

Although the general concept is that di- and tri-acylated compounds are ligands for the TLR2/6 and TLR2/1 heterodimer, respectively, three studies reported that lipid fractions derived from parasites containing mono-acyl phospholipids can activate TLR2 as well (Beutler et al., 2006; Carneiro et al., 2013; Magalhaes et al., 2010). In order to prove the TLR2 activating capacity of mono-acyl phospholipids, we developed a method for the enzymatic biosynthesis of several mono-acyl

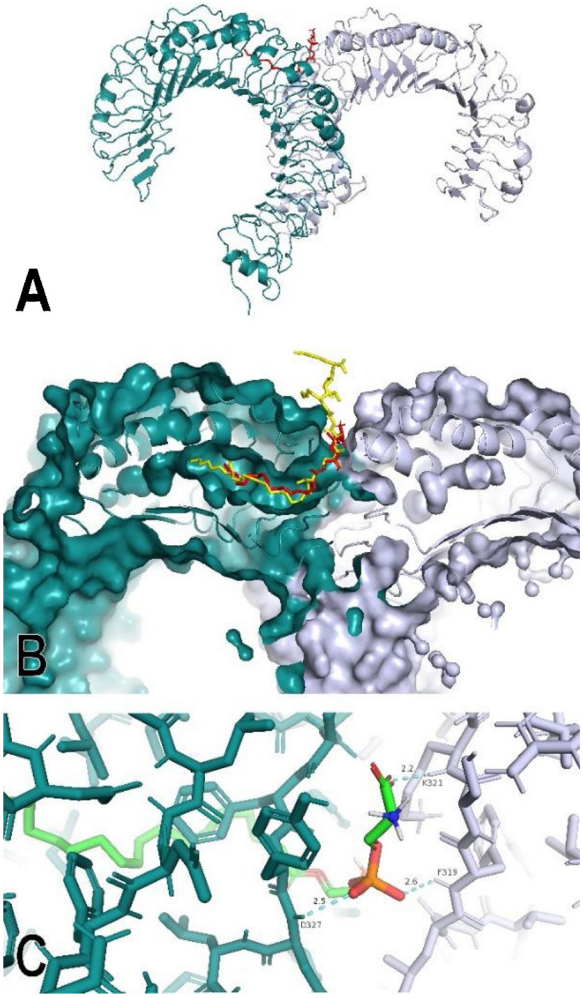

Fig. 2. *In silico* modeling of the ligand-receptor interaction of 20:1 mono-acyl PS with the TLR2/6 heterodimer. Panel A shows the interaction of 20:1 mono-acyl PS (red) with the TLR2/6 heterodimer (TLR2 green, TLR 6 gray) in an in silico prepared model using VINA and PyMol. The best fit positioned the acyl-chain of 20:1 mono-acyl PS in the hydrophobic pocket of TLR2 and the serine headgroup of mono-acyl PS interacting with amino acid residues of both TLR2 and TLR6. Panel B shows an identical projection compared to Panel A, but the TLR2/6 complex is now presented as a surface model and the ligands FSL-1 (yellow) and 20:1 mono-acyl PS (red) are shown in an overlay to visualize the similar position in the hydrophobic pocket of the TLR2 monomer of the acyl-chain of 20:1 mono-acyl PS compared to the acyl chains of FSL-1. Panel C shows the predicted hydrogen-bonds between 20:1 mono-acyl PS and residues D327 of TLR2, residues K321, F319 of TLR6, shown as dashed, cyan lines. Numbers indicate the distance in ångström. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

phospholipids. This biosynthetic approach has three advantages. First, it rules out the possibility that instead of mono-acyl PS, another minor compound present in the purified lipid fraction of *S. mansoni* was in fact the TLR2 activating compound (Van der Kleij et al., 2002). Second, the biosynthetic approach allows the preparation of several specific mono-acyl phospholipid variants, because mono-acyl phospholipids with distinct headgroups and/or acyl-chain can be synthesized. These synthesized specific mono-acyl phospholipid variants can then be used for structure-function analysis of TLR2 activation by mono-acyl phospholipid ligands. This is not possible using lipid fractions of schistosomes or other organisms, as those contain mixtures of different mono-acyl phospholipids. Third, the mono-acyl phospholipids that activate TLR2 can be synthesized in larger quantities than can be obtained purely by isolation from the parasite.

Since our method resulted in the preparation of several mono-acyl phospholipids, it allowed assessment of their TLR2 activating capacity in a cellular TLR2 reporter system. These experiments demonstrated that the biosynthesized 20:1 mono-acyl PS activated TLR2. This firmly confirms the earlier report in which a purified lipid fraction containing 20:1 mono-acyl PS from *S. mansoni* activated TLR2 (Van der Kleij et al., 2002).

TLR2-induced intracellular signaling requires the formation of heterodimers with TLR1 or TLR6 (Beutler, 2009; van Bergenhenegouwen et al., 2013). The TLR2/1 and TLR2/6 heterodimers each have their preferred ligands and the classical ligands for the TLR2/6 heterodimer are di-acylated lipopeptides, while tri-acylated lipopeptides are the classical ligands for TLR2/1 heterodimer (van Bergenhenegouwen et al., 2013; Oliveira-Nascimento et al., 2012). Our experiments showed that 20:1 mono-acyl PS activates the TLR2/6 heterodimer and not the TLR2/1 heterodimer. This is consistent with previous observations that activation of the TLR2/1 heterodimer requires a ligand with multiple acyl chains of which one or two are inserted in the hydrophobic pocket in TLR2 and another acyl chain in TLR1 (Kang et al., 2009).

Although mono-acylated ligands for TLR2 derived from pathogens seem to be scanty, many mono-acylated synthetic compounds have been prepared that can activate the TLR2/6 heterodimer (Agnihotri et al., 2011). These studies demonstrated that at least one acyl group and an appropriately oriented ester carbonyl group are essential for TLR2-agonistic activity (Agnihotri et al., 2011), which is consistent with our *in silico* modelling results, as well as with the observed *in vitro* activation of the TLR2/6 heterodimer by 20:1 mono-acyl PS. It seems that 20:1 lyso-PS fulfils the minimal demands for TLR2/6 interaction. However, the affinity of the TLR2/6 heterodimer for 20:1 mono-acyl PS seems low, as rather high concentrations (ED50 of 24 μM) of 20:1 mono-acyl PS were required for TLR2/6 activation compared to the classical TLR2 ligands derived from bacteria (e.g. lipopeptides, such as FSL-1) and reported synthetic ligands for TLR2/6 (nM to pM range) (Jin et al., 2007). This could also be in part be due to the headgroup-configuration of FSL-1, which consists of a small peptide (CGDPKHPKSF) rather than a single phosphoserine, in the case of 20:1 lyso-PS. Furthermore, the diacyl chains of the FSL-1 allow for a more extensive filling of the hydrophobic pocket present in TLR2 (See supplemental Fig. 1). Although the TLR2/6 receptor has a low affinity for lyso-PS, it could be a physiologically relevant ligand, as locally high concentrations of 20:1 mono-acyl PS might occur *in vivo* in *S. mansoni* infected hosts. Firstly, the outer-surface membranes of adult *S. mansoni* worms were shown to be highly enriched in this mono-acyl PS species (Retra et al., 2015). Secondly, adult schistosomes actively synthesize eicosaenoic acid (20:1) in high amounts (Brouwers et al., 1997). Thirdly, phospholipids in the outer-surface membranes of adult schistosomes have a high turnover rate (Brouwers et al., 1999). Therefore, 20:1 mono-acyl PS could be a clinical relevant agonist for TLR2 that affects dendritic cells such that mature dendritic cells gain the ability to induce the development of IL-10-producing regulatory T-cells (Van der Kleij et al., 2002).

In short, our results conclusively demonstrated that 20:1 mono-acyl PS is a true agonist for the TLR2/6 heterodimer, and is the TLR2-activating compound in lipid fractions derived from *S. mansoni.* As 20:1 mono-acyl PS can now be prepared in higher quantities by the developed biosynthetic method, future studies can be performed to further assess the functions of 20:1 mono-acyl PS as an immunological mediator.

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Declaration of Competing Interest

All authors declare that they have no conflicts of interest with the contents of this article.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2020.104951.

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