Schistosomal lysophosphatidylserine: an immunomodulatory factor

Kim Retra

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Schistosomal lysophosphatidylserine:
an immunomodulatory factor

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een immunomodulator

(met een samenvatting in het Nederlands)

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Contents

Chapter 1

General introduction

Partly based on:
Functions of the tegument of schistosomes: Clues from the proteome and lipidome

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Parasites

Parasites are organisms that live in or on the living tissue of a host organism at the expense of the host. "Parasites are among the world's most successful and sophisticated organisms. They can transform the inside of other creatures into hospitable homes. They can evade the onslaught of the immune system and even make it serve them. They can even control the minds of their hosts and force them to their bidding. And thanks to these skills, parasites make up the majority of all species" [1]

Helminths

The very great majority of parasites belong to one of the four phyla: Protozoa, Athropoda, Nematoda or Plathyhelminthes. Parasites from the phylum protozoa consist of a single cell and comprise for instance Plasmodia, Trypanosoma and Leishmania species. Parasites of the phylum arthropoda are insects such as ticks. Both groups will not be considered in this thesis. Helminths are divided into the two phyla, Nematoda and Platyhelminthes. Nearly 20,000 nematode (roundworm) species have been described. Many of them are parasites of insects, plants or animals, but free-living species also exist. Species of the phylum Platyhelminthes (flatworms) are present in all aquatic environments, and parasitic as well as non-parasitic species of flatworms exist. Most platyhelminthes are parasites of vertebrates, and all vertebrate species are prone to infection by one or more species of flatworms. The great majority of parasitic flatworms belong to the classes Aspidogastrea, Monogenea, Digenea (flukes) and Cestoda (tapeworms) and the first three classes are often together referred to as "trematodes". All trematodes residing in humans are Digeneans. They have indirect and complex life cycles with stages including parasitic stages, in snails and in others hosts such as humans. An example of such a parasite is the schistosome.

The family of schistosomatidae

Members of the family of schistosomatidae are parasitic worms that inhabit the vascular system of vertebrates. A general feature of the family is that the mature female is more slender than the male and is normally carried in a ventral groove of the male body. The family of schistosomatidae can be divided in three subfamilies, which together can be subdivided into 12 genera. Seven of these genera are confined to

birds and the other five to mammals. Only the genus *Schistosoma* is associated with man. Of the mammalian blood flukes, the genus *Schistosoma* has achieved the greatest geographical distribution and diversification in terms of numbers of identified species and different parasitized hosts. Five species are considered important parasites of man: *S. mansoni*, *S. haematobium* and *S. japonicum* are the most important and widespread while the other two, *S. intercalatum* and *S. mekongi* have a more localized distribution. Schistosomiasis is not only a human health problem, as also animals suffer from schistosomiasis. In southern and central Africa, *S. mattheei* is the predominant species infecting ruminants; in northern and eastern areas, *S. bovis* is more common. The latter parasite is also found in certain areas of southern Europe and the Middle East. Some species can cause human as well as veterinary schistosomiasis. Species such as *S. mattheei*, that normally infects primates and artiodactyla, are known to occasionally to infect man. Other species like *S. incognitum*, that normally infect Rodentia, Carnivora and Artiodactyla, must be considered as potential zoonoses [2].

S. haematobium

In 1852 *S. haematobium* was first described by Bilharz. This schistosome is a major pathogen of man and causes a disease called schistosomiasis or bilharziasis. *S. haematobium* is almost exclusively a parasite of man and adult worms of this species inhabit the venous drainage system of the bladder. Eggs, which have a terminal spine, work their way through the bladder wall and are voided with the urine. Haematuria, "red urine", is a common sign of infection. Occasionally, *S. haematobium* eggs are also observed in faecal samples, presumably due to some worm pairs migrating to the mesenteric veins of the large intestine. *S. haematobium* is found in Africa and in some parts of the Middle East [2].

S. japonicum

S. japonicum is responsible for a grave, debilitating and chronic form of intestinal schistosomiasis which affects both man and domestic animals. The disease is found in various areas of Asia. It is a true zoonotic disease and *S. japonicum* occurs as a natural parasite of a large number of mammalian species which play an important role in the epidemiology of the disease. Although many wild definitive hosts of *S. japonicum* are

known, domestic animals and especially cattle, pigs and dogs, are an important reservoir for this species. Throughout its range, *S. japonicum* is transmitted by populations of the snail *Oncomelania hupensis*, of which six subspecies are found in different geographical areas [2].

S. mansoni

S. mansoni is a very important pathogen, causing intestinal schistosomiasis in man. It is endemic in a large part of Africa, in some parts of the Middle East and in parts of South America (Brazil, Surinam and Venezuela) and the Caribbean. *S. mansoni* is thought to be introduced in the Americas relatively recently and probably via slave trade. The adult worms of this species are found in the mesenteric veins and the lateral-spined eggs are excreted with the faeces. Various, but not all, species of the *Biomphalaria* snail are susceptible to *S. mansoni*. Snails from different geographical areas tend to show variation in levels of susceptibility to different strains of *S. mansoni* and for example *B. glabrata* tends to be not as susceptible to parasites isolated from *B. tenagophila* and *vice versa*. Experimental studies have shown that *S. mansoni* strains from different geographical areas may display differences in their biological characteristics and in their infectivity or pathogenicity. There are numerous reports of infection with *S. mansoni* in a wide array of mammalian hosts. Among them are many Primates and Rodentia, but also some Insectivore, Artiodactyla, Marsupialia and Carnivore. Often the reports are restricted to the finding of lateral-spined eggs in faecal samples, and the importance of reservoir hosts in maintaining transmission is often uncertain [2].

Life cycle of schistosomes

The *S. mansoni* parasite has two hosts, the snail as an intermediate host and (for example) man as definitive host (Figure 1). Since only a few schistosomes in the free living stages succeed to infect a new host, the parasitic stages have to produce large numbers of offspring.

The detailed morphology of **eggs** of the genus *Schistosoma* varies greatly between species, and therefore, it can be used to identify schistosomal species. In general, eggs have an oval shape and a characteristic pointed expansion on their shell. By a still unclear mechanism, probably involving numerous interacting factors, the schistosome eggs pass through the wall of vessels and parachyma of intestine or bladder. The number of eggs excreted daily differs greatly

between species and varies from 20 to 2200 [3]. Schistosome eggs hatch only under certain conditions, in which temperature, light and especially osmotic pressure are important factors. It seems that hatching of schistosome eggs can also be induced by purely physical processes, as it even occurs when the miracidium is dead [4]. Upon contact with fresh water, water quickly penetrates into the vacuoles of the egg, which eventually causes a rupture in the shell.

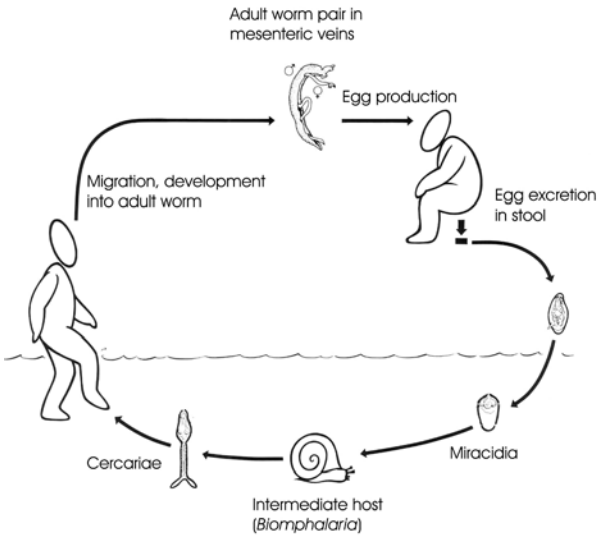

Figure 1: The life cycle of schistosomes (with courtesy of Dr. A.M. Polderman).

After hatching from the egg, the **miracidium** actively penetrates the snail. In temperatures close to those of the transmission biotopes (24-28°C), miracidia of *S. mansoni* remain infective for about 8 to 12 h [5,6]. After penetration of the snail, the miracidium develops into mother **sporocysts** by going through several morphological transformations. Within the mother sporocyst, daughter sporocysts differentiate, that contain germinal cells in their cavity. These cells then start to differentiate into cercariae, by a process called cercariogenesis. The production of cercariae of schistosomes occurs once a day and can occur daily. Generally, cercarial production can continue for periods

exceeding eight months and stops when the host dies, but some snails may spontaneously eliminate the parasite [7].

Cercariae leave the snail following a daily rhythm unique to each species of schistosome, based upon the light intensity and temperature of the environment [8-10]. After shedding under optimal conditions, cercariae remain infectious for 5 to 8 hours [11]. Cercariae can penetrate the skin of a mammal, during which the cercarial tail is shed. Twenty-four hours after cercarial penetration into the skin, the dermis contains a diffuse polymorphonuclear inflammatory infiltrate. Also vasodilation and migration of leucocytes to the penetrated skin occurs, but no significant leukocyte-mediated damage to schistosomula has been observed [12-14]. When the skin has previously been exposed to schistosomes, the next infiltration provokes a significantly greater inflammation. The observed inflammatory effect is not necessarily due to damage during the penetration, but may also be due to the release of molecules by the schistosomula, like for example eicosanoids.

The complete process of transformation from cercaria to **schistosomulum** takes less than one hour [15]. The plasma-membrane of the cercarial tegument, plus adherent glycocalyx material, is shed in the form of numerous microvilli, and is simultaneously replaced by a multilaminate configuration. Morphological changes in the tegument surface during transformation are reflected biochemically by a dramatic change in surface composition. Evidence points to the exposure of macromolecules pre-formed during cercarial development, rather than *de novo* synthesized during transformation. The majority of surface molecules appear to be glycosylated [16]. Subsequently, the parasite will locate a post-capillary venue, and penetrate the venule wall. Once within the lumen of the vessel, the parasite is rapidly carried away by the blood flow. The arrival of schistosomula in the lungs via the pulmonary artery is the prelude to a sequence of developmental changes which are presumably necessary for further migration. In the ensuing days, lung stage schistosomula do not significantly increase in volume, but their surface area increases by 50%, implying the synthesis of tegument plasma-membranes [17]. The lung schistosomulum thus becomes capable of great extension of its length, but is still able to contract to minimum size. The changes in extensibility are accompanied by remarkably few modifications of internal structure. The pits and troughs present in the tegument of contracted specimens function as devices to accommodate the body length changes. Thus, in fully

extended regions of the body, the pits disappear completely and the tegument surface is then almost smooth [17,18]. Schistosomula migrate from the lung to the hepatic portal system and become trapped in de liver. Soon after arrival in the liver, the elongated migrating parasites shorten to the dimension of the schistosomula stage in the skin. The tegument surface of a 10-day liver worm is highly ridged to accommodate the "surplus" membrane, and small numbers of spines begin to appear in the tegumental cytoplasm [17]. In the hepatic portal system, pairing of males and females takes place, thereafter females are carried in the gynaecophoric canal of the male worms. A new phase of migration then begins, up the hepatic portal vessels to oviposition sites in the mesenteric veins or rectal veins. Upon arrival in these veins, the **adult worm pairs** start producing eggs. The adult worm pairs are able to survive in the bloodstream and produce eggs for many years. Unfortunately, their survival is not without consequences for the host.

Pathology and Morbidity

According to World Health Organization (WHO), schistosomiasis is the most prevalent and serious parasitic disease after malaria [19]. Schistosomiasis affects 200 million individuals in tropical regions of Africa, Asia and South America, being endemic in 74 countries. The number of death per year due to schistosomiasis could be as high as 200,000 and it is estimated that 600 million people live in areas at risk [19]. The degree of morbidity caused by schistosomiasis depends on the schistosomal species, the intensity of infection and on environmental and host-related factors. In the case of intestinal schistosomiasis, the mortality is mainly due to liver fibrosis and portal hypertension, whereas in the case of urinary schistosomiasis, it is bladder cancer or renal failure. The disease weakens infected individuals and therefore schistosomiasis also has serious socio-economic consequences [20].

Three distinct syndromes caused by schistosomiasis have been described: cercarial dermatitis, acute schistosomiasis and chronic schistosomiasis.

Cercarial dermatitis (swimmer's itch) is caused by penetration of cercariae of schistosomes into human skin, which may provoke an acute inflammatory response. The diagnosis is difficult and treatment is usually not needed. In non-endemic areas, such as The Netherlands, cercariae from avian schistosomes that are not able to complete their life cycle in man, can often penetrate human skin and transform into

schistosomula. They persist for up to 10 days post infection and give rise to an allergic-type immediate type hypersensitivity reaction [21]. Swimmer's itch is prevalent in many parts of the world.

Acute schistosomiasis (katayama fever) can occur three to nine weeks after infection. The allergic reaction is characterized by fever, headache, cough, loss of appetite, abdominal pain, diarrhea and eosinophilia. The clinical symptoms in the majority of cases start when eggs are deposited in the host tissue. At this stage the clinical diagnosis is difficult because in general it is still too early to reliable demonstrate eggs in the excreta [22,23].

Chronic schistosomiasis may occur even without any recognizable symptoms and can last for decades. Morbidity arises slowly and is accompanied by pathological changes in affected organs. Schistosome eggs that become lodged within the host tissues are the major cause of pathology. Antigens secreted by eggs, through microscopic pores within the eggshell, induce a chronic granulomatous inflammatory response, which may eventually lead to fibrosis of the liver, or urinary bladder, depending on the species. The active acute granulomatous response gradually becomes down regulated into a chronic phase. Initial symptoms of chronic schistosomiasis are diarrhea, dysentery, abdominal pain, lack of appetite, weight loss, proteinuria and bloody stool or haematuria. Severe infections can results in portal hypertension, hepatomegaly, splenomegaly, ascites, bleeding varices (intestinal schistosomiasis), or obstruction of the urinary tract (urinary schistosomiasis).

Hence, schistosomiasis is a serious disease with a complex nature. The complexity is partly a result of manipulation of the host and its immune system by the parasite.

Immune response to a schistosome infection

Schistosomes live in intimate contact with human blood cells from the third or fourth day after infection of the mammalian host. Therefore, it seems almost impossible to avoid immune recognition and destruction. Next to the presence of adult worms, the passage of schistosome eggs to the exterior, involves disruption of the intestinal epithelial layer. Last but not least, highly immunogenic eggs get trapped in the liver of the host. It seems illogical that an adult parasitic worm, which on one hand seems to be able to make it self undetectable by its host, on the other hand produces highly immunogenic eggs. The highly immunogenic eggs

seem to play a role in the down regulation of the immune system of the host.

Early after infection with schistosomes, mice develop an antiparasitic Th1 cytokine response. This response changes dramatically into a dominant Th2 response after the onset of egg laying by mature female worms at about 5 weeks postinfection [24]. Together with the Th2 production, there is a down-regulation of Th1 cytokine synthesis [25]. CD4+ cells from schistosome-infected mice produce large quantities of IL-10 and this response is both temporally and mechanistically linked to the down-regulation in Th1 cytokine synthesis observed in these animals. The IL-10 production is only detected after egg deposition, and can be artificially reproduced by the injection of isolated eggs into naïve mice. Down regulation of the Th1 response is not observed in vaccinated animals [26]. IL-10 deficient mice challenged i.v. with schistosome eggs or infected with *Schistosoma mansoni* develop a completely non-polarized, codominant Th1/Th2-type immune response [27,28].

The acute T cell response peaks at week 8 and, thereafter, it is down-modulated as the disease becomes chronic. Hallmarks of the Th2-associated immune response in chronic infected patients include up-regulation of cytokines IL-4 and IL-13, down regulation of the cytokine IFN-γ, elevated serum IgE, sequestration of parasite eggs by eosinophil-enriched granulomas and development of tissue fibrosis. In addition, chronic schistosomiasis is characterized by a stage of elevated immune suppression. Infection experiments with cytokine-deficient mice revealed severe consequences for hosts with unbalanced cytokine responses to schistosome eggs [29]. Long-term survival of persistently infected hosts relies upon a carefully balanced and tightly regulated immune response. The anti-inflammatory cytokine IL-10 is pivotal for the generation of host-protective homeostatic conditions in schistosomiasis [29]. *In vitro* suppression of the immune system is, in part, reversible by blocking the IL-10 receptor [30]. Granulomas around eggs in the livers of WT mice are significantly smaller at week 15 than week 8, whereas those in IL-$10^{-/-}$ animals are larger at week 8 and show no reduction in size at week 15 [31]. Recently, CD4+CD25+ T cells were identified as a major source for IL-10 in schistosome infected mice. It is suggested that the presence of these regulatory T cells is of functional importance in schistosomiasis, since schistosome infection stimulates the functional maturation of CD4+CD25+ regulatory T cells in the presence of soluble egg antigens,

while these regulatory T cells protect the liver from egg-induced damage [30,32].

So the host–parasite interaction is complex and crucial for the development of a schistosome infection. The interaction involves various host receptors, like for example toll-like receptor 2 (see below), and several parasitic factors. This thesis will focus on phospholipid derived parasitic factors.

Lipid metabolism of schistosomes

Adult schistosomes have a characteristic double membrane on their outer surface, which so far has only been found in blood-dwelling trematodes [33]. The continuous renewal of this outer membrane complex is essential for the survival of these parasites in the host, because the double membrane forms a system that counteracts the threat of the immune system of the host in various ways [34]. This outer membrane contains among other compounds, cholesterol and more than 1000 different phospholipid molecules with much of the diversity originating from the large variety of fatty acyl chains that are esterified to the *sn-1* and *sn-2* positions of the glycerol backbone of the phospholipids (see Figure 2). Commonly, the *sn-1* position of a phospholipid is enriched in saturated acyl chain substituents whereas the fatty acyl chains at the *sn-2* position are primarily unsaturated species. Different tissues of multicellular organisms, and different cell types, have distinct phospholipid compositions.

Although schistosomes have high requirements for lipids because of the high turnover of phospholipids [35] and release of the outer membrane [36,37], schistosomes can not synthesize fatty acids *de novo*, and have to obtain fatty acids and also cholesterol from the host, a characteristic shared with many other parasitic helminths. As a result, nearly all lipids found in schistosomes are present in the blood of the host on which they feed, although several characteristic differences exist. Some fatty acids that are only present in very low amounts in the host are abundant in schistosomes. This is possible because schistosomes are able to make small modifications in fatty acids obtained from the host. They can elongate fatty acids (Figure 3) and the elongation of oleic acid (18:1) to eicosenoic acid (20:1) is quantitatively the most important modification [38-40]. Next to elongation, schistosomes can also introduce desaturations into fatty acids.

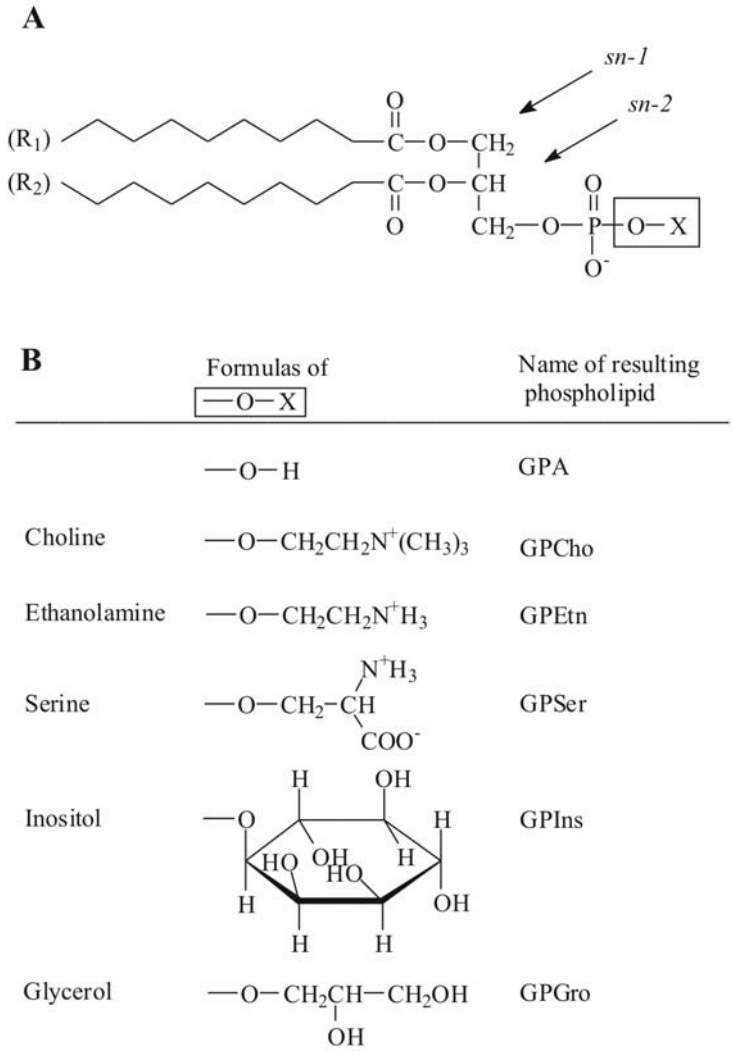

Figure 2: molecular structure of phospholipids
Structure of the molecular phospholipid subclasses. (A) The molecular structure of a phospholipid, R1 and R2 represent acyl chains that can vary in length, degree of unsaturation and type of linkage to the glycerol backbone, the head group is boxed. (B) Various head groups of the phospholipid subclasses are shown. Abbreviations: GPA, glycerophosphatidic acid; GPCho, glycerophosphocholine; GPEtn, glycerophospho-ethanolamine; GPSer, glycerophosphoserine; GPIns, glycerophosphoinositol and GPGro, glycerophosphoglycerol.

When fatty acids are saturated, the first bond is generally introduced in the Δ9 position, whereafter other desaturases can introduce bonds at other positions. Vertebrates possess Δ9, Δ6, Δ5 and possibly a Δ4 desaturase, while plants contain Δ6 (lower plants only), Δ9, Δ12 and Δ15 desaturase. Many insects are able to introduced double bonds at the 5, 6, 9,12 and 15 position [41]. In fatty acids derived from schistosomes, a single double bond at the Δ5 position has been identified. The octadec-5-enoic acid (18:1Δ5, Figure 4A) is a highly unusual fatty acid, which is absent in the blood of the host and a host fatty acid is therefore modified by the schistome to form this fatty acid. The glycerol which is used by the parasite to form the backbone of phospholipids and triacylglycerol is mainly synthesized from glucose obtained from the host [42,43].

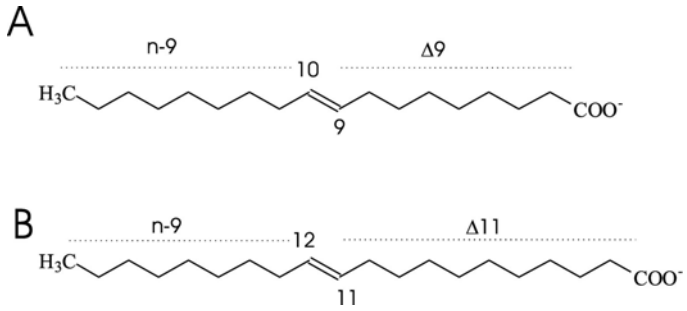

Figure 3: Elongation of fatty acids. Shown is a **(A)** 9–oleic acid (also named 9-18:1 or 18:1(n-9)) which can be chain elongated by two carbon atoms resulting in a longer carbon-chain fatty acid of the (n-9) family **(B)** 11-eicosenoic acid (also named 11-20:1 or 20:1 (n-9).

Lipids and the tegument

In 1977 it was demonstrated that all investigated blood-dwelling flatworms contain an outer surface covering, the tegument, that is unique in nature [33]. This tegument consists of a syncytium of fused cells surrounding the entire worm and has a single continuous double-bilayer outer-membrane. Hence, the host-interactive surface consists of two closely-apposed lipid bilayers that overlay the syncytium. The two outer-surface membranes of the tegument, which are detected as a heptalamellar layer, form many surface pits that substantially enlarge the surface area of the schistosome [33,44,45].

Since the host-interactive tegument is present in all blood-dwelling digenean parasitic worms, yet absent in those that inhabit the gut or other body cavities, it has been concluded that this structure is an important adaptation to survival in the bloodstream [33,46]. Regarding immune evasion, several mechanisms mediated by the tegument have been suggested, including antigenic mimicry, proteolytic degradation of 'attacking' host proteins, rigid biophysical membrane properties and a rapid tegumental membrane turnover [34].

Despite their inability to synthesize sterols *de novo*, adult schistosomes contain a relatively high amount of cholesterol. The molar ratio of sterols to phospholipids in adult *S. mansoni* is 0.8, whereas that in typical eukaryotic cells is only 0.3 to 0.5 [47]. The tegumental membranes of adult schistosomes are even more enriched and contain more cholesterol than phospholipids on a molar basis [48]. Similar to other eukaryotic cells, the phospholipids phosphatidylcholine (glycerophospho-choline, GPCho) and phosphatidylethanolamine (glycerophospho-ethanolamine, GPEtn) are the major constituents of membranes of both the worm and the tegument [49], but the composition of the fatty acids present in these phospholipids is very distinct from those found in mammalian blood cells. GPCho in schistosomes, and in particular GPCho in the tegumental membranes, contains more saturated fatty acids than most mammalian cells. For instance, nearly 50% of the acyl chains present in GPCho of the tegument is palmitate, a fatty-acyl chain of 16 carbon atoms without any desaturation (C16:0) [48]. Furthermore, tegumental membranes are highly enriched in GPCho- and especially GPEtn-species in which the fatty acid chain on the *sn*-1 position is linked by an ether bond instead of an ester bond (Figure 4B) [50]. The high amount of cholesterol, sphingomyelin and saturated (ether-linked) phospholipid species in the tegumental membranes results in a tight packing of the membrane, which makes the membrane more rigid in physical terms. These properties render the tegumental membrane stable and relatively inert, as it is less accessible to lipases and thereby less sensitive to degradation [51,52].

Next to the rigid biophysical properties and the high fraction of ether lipids, the lipids in the tegument are also of interest because the phospholipids contain several peculiar fatty acid species. One of the most abundant GPCho species in the tegumental membranes has as one of its two fatty-acyl chains the highly unusual fatty acid octadec-5-enoic acid (18:1(5Z)), a fatty acid that is not present in the host [53] (Figure

4A). Furthermore, schistosomes are relatively rich in phospholipids containing eicosaenoic acid (20:1) [48]. In GPCho derived from adult schistosomes, eicosaenoic acid is predominantly esterified to the sn-1 position in combination with a saturated fatty acid (16:0) at the sn-2 position (Figure 4C). This combination is very unusual as most phospholipids contain unsaturated fatty acids at the sn-2 position. In tegumental membranes eicosaenoic acid is predominantly present in the plasmalogen GPEtn species (16:0/20:1), an ether-lipid species that constitutes 27% of the GPEtn species in the outer-surface membranes and that is absent in blood of the host [50]. A yet unanswered question related to the ether lipid metabolism is whether or not the specific Δ1 desaturation of plasmalogens is performed by schistosomes, or that the plasmalogen backbones are host derived. If the modification is performed by schistosomes, the Δ1 desaturase would be the second lipid desaturase (next to the Δ5 desaturase, see above) found in schistosomes.

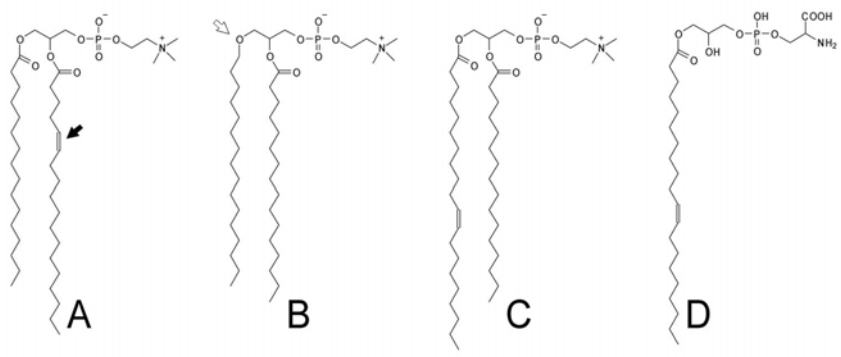

Figure 4: Unusual phospholipid species in tegumental membranes of *S. mansoni*. The chemical structures of schistosome-specific phospholipids are shown (see text for details and references). (A) GPCho with unusual position of the double bond in the unsaturated fatty acid, 1-palmitoyl-2-(5Z-octadecenoyl)-*sn*-glycero-3- phosphocholine (GPCho (16:0/18:1 (5Z)). (B) Specific ether phospholipid, 1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phophocholine (GPCho(O-16:0/16:0)), (C) phospholipid with unusual position of the unsaturated fatty acid, 1-eicosaenoyl-2-palmitoyl-sn-glycero-3-phosphocholine (GPCho(20:1/16:0)) and (D) lysophospholipid, 1-eicosaenoyl-2-lyso-*sn*-glycero-3-phosphoserine (lysoGPSer(20:1)). The filled arrow indicates the unusual position (5Z instead of 9Z) of the unsaturation (panel A), whereas the open arrow indicates the ether-linkage present in ether phospholipid species (panel B).

The tegumental membranes are continuously renewed from membranous bodies (see above), however, the exact turnover-rate of these membranes is debated, as several studies reported half-life values for tegumental proteins or glycoconjugates ranging from a few hours to several days [54-57]. Studies on the turnover of the phospholipids demonstrated that tegumental lipids have a shorter half-life than those in the worm body, and that lysophospholipids are excreted as degradation products into the environment [35,58].

Phosphatidylserine (GPSer)

Phosphatidylserine (glycerophosphoserine, GPSer) is a quantitatively minor membrane phospholipid, comprising 2-10% of the total phospholipids, but has important functions in signal transduction. The brain is enriched in GPSer compared to other mammalian tissues. Mammalian cells contain two GPSer synthases. GPSer synthase-1 is a serine-exchange enzyme that uses GPCho as a substrate. GPSer synthase-2 uses PE as a substrate and catalyzes the ethanolamine-exchange reaction. Both enzymes are localized in mitochondria-associated membranes. The major mechanism that has been identified for regulation of GPSer synthesis in mammalian cells is a feed-back mechanism whereby the activity of GPSer synthase is regulated by the end-product, GPSer. GPSer is widely distributed throughout various organelle membranes. However, as GPSer synthetases are localized to mitochondria-associated membranes, GPSer needs to be transported. Since GPSer is highly enriched on the cytosolic leaflet of the plasma membrane, an aminophospholipid translocase has been implicated in establishing and maintaining this asymmetric distribution. Several candidate proteins have been proposed for both processes, but the mechanisms are still poorly understood and under investigation (reviewed by Vance and Steenbergen [59]). GPSer is involved in various biological processes. The exposure of GPSer on the outside surface of cells, for which the asymmetrical distribution needs to be disturbed, is widely believed to play a key role in the removal of apoptotic cells and in initiation of the blood clotting cascade. GPSer is also the precursor of GPEtn that is made by GPSer decarboxylase in bacteria, yeast and mammalian cells. Furthermore, GPSer is required as a cofactor for several important enzymes such as protein kinase C and Raf-1 kinase, that are involved in signaling pathways (reviewed by Vance and Steenbergen [59]).

Next to diacylated phospholipids also monoacylated phospholipids (lyso-phosphorlipids) exist.

Lysophospholipids

Lysophospholipids are minor compounds in membranes but they play an important role in signal transduction. Lysophospholipids are generated in cells after hydrolysis of phospholipids by the phospholipase A enzymes. Lysophosphatidic acid (lysoGPA) can also be generated from lysophosphatidylcholine (monoacylglycerophosphocholine, lysoGPCho) by lysophospholipase D [60]. Lysophospholipids are intermediates of the phospholipid deacylation pathway [61], but they also show various biological activities themselves. Many of these activities follow upon activation of a G protein-coupled receptor (GPCR) by the lyso-phospholipids. There are several GPCRs known with lysophospholipids as ligands. The main ligands of these receptors are sphingosine 1-phosphate, lysoGPA, sphingosylphosphorylcholine and lysoGPCho [62-64]. Lysophospholipids are for example biologically active within the immune system. LysoGPCho has been shown to display immunoregulatory activities, via its GPCR, on differentiating monocytes to generate mature dendritic cells with the ability to stimulate IL-2 and IFNγ production by allogeneic T lymphocytes [65,66]. LysoGPIns accumulates in stimulated macrophages and oncogene-transformed fibroblasts [67,68]. Mitogenic activities by lysophospholipids through their GPCR have also been shown. LysoGPA and GPA can stimulate DNA synthesis and cell division when added extracellular to fibroblasts or epithelial cells in culture. Sphingosine 1-phosphate protects cells from apoptosis [69,70] and also lysoGPIns has mitogenic actions which are completely inhibited by suramin, a membrane-impermeant antagonist of GPCRs [67,71-73]. Lysophospholipids also play a role in host-parasite interactions. The major lysophospholipid excreted by *S. mansoni* schistosomula, lysoGPCho(16:0), has been suggested to have a function in lysis of red blood cells that subsequently attach to the parasite as ghost cells [74]. Therefore, lysophospholipids from schistosomes could promote the acquisition of host membrane components, resulting in surface exposure of host components that mask antigenic epitopes of the parasite. Beside these activities many other activities of lysophospholipids have been shown. LysoGPCho accumulates in oxidized low density lipoprotein and atherosclerotic aorta [75,76] and in lesions of ischemia [77]. LysoGPA and sphingosine 1-phosphate are produced

and released from activated platelets [78,79] and lysoGPGro inhibits calcium release induced by lysoGPA [80].
Besides the headgroup, also chain length and linkage type are important for GPCR activation as it has been shown that when the acyl-chain of lysoGPA is varied, the rank order of mitogenic potency is: 18:1 ≈ 16:0 > 14:0 > 12:0 > 10:0. An ether-linked lysoGPA had a much lower mitogenic activity when compared to the ester-linked analogue [73].

LysoGPSer
Lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) appears to have specific effects on mast cells, dendritic cells, T cells, cancer cells and neural cells. LysoGPSer, and also lysoGPA, has been found in ascites of ovarian cancer patients. LysoGPSer stimulates intracellular calcium increase in leukemic cells but not in normal human peripheral blood mononuclear cells. LysoGPSer also stimulates intracellular calcium increase in human leukemic THP-1 cells. These effects seem to be mediated by a Gi/G0-protein mediated signaling pathway and therefore suggests the presence of a lysoGPSer specific GPCR on cancer cells [81].
LysoGPSer is required for nerve growth factor (NGF)-induced secretion of histamine from rat mast cells. LysoGPSer is thought to be generated by deacylation of GPSer upon tissue damage and may be a soluble messenger of tissue injury. NGF in combination with lysoGPSer also strongly promotes the number of PC12 cells that develop neurites and the neurites were then also longer. LysoGPSer is therefore also a modifier of neuronal structure and/or function [82].
During a ligand fishing study for orphan G protein coupled receptor GPR34, lysoGPSer was found as a ligand. The activities on GPR34 of lysoGPSer with different acyl chain lengths were examined and it was found that the activity increased with the length of the acyl chain when lauroyl-, myristoyl-, and palmitoyl-lysoPS were tested. Lyso-phosphatidyl-D-serine, lysoGPCho, lysoGPEtn and lysoGPA are ineffective on GPR34 [83]. mRNAs of GPR34 are particular abundant in mast cells, but also present in for example human placenta, spleen, thymus, ovary, CD4+ cells and basophils [83].
LysoGPSer derived from schistosomes was shown to interact with the immune system of the host. Schistosomal lysoGPSer (Figure 6F) was found to activate Toll-like receptor 2 (TLR2) and to suppress IL-12 production. The co-culture of lysoGPSer exposed dendritic-cells with

naive T-cells resulted in IL-10 producing T-cells. The IL-10 producing T-cells were shown to have a suppressor function [84].

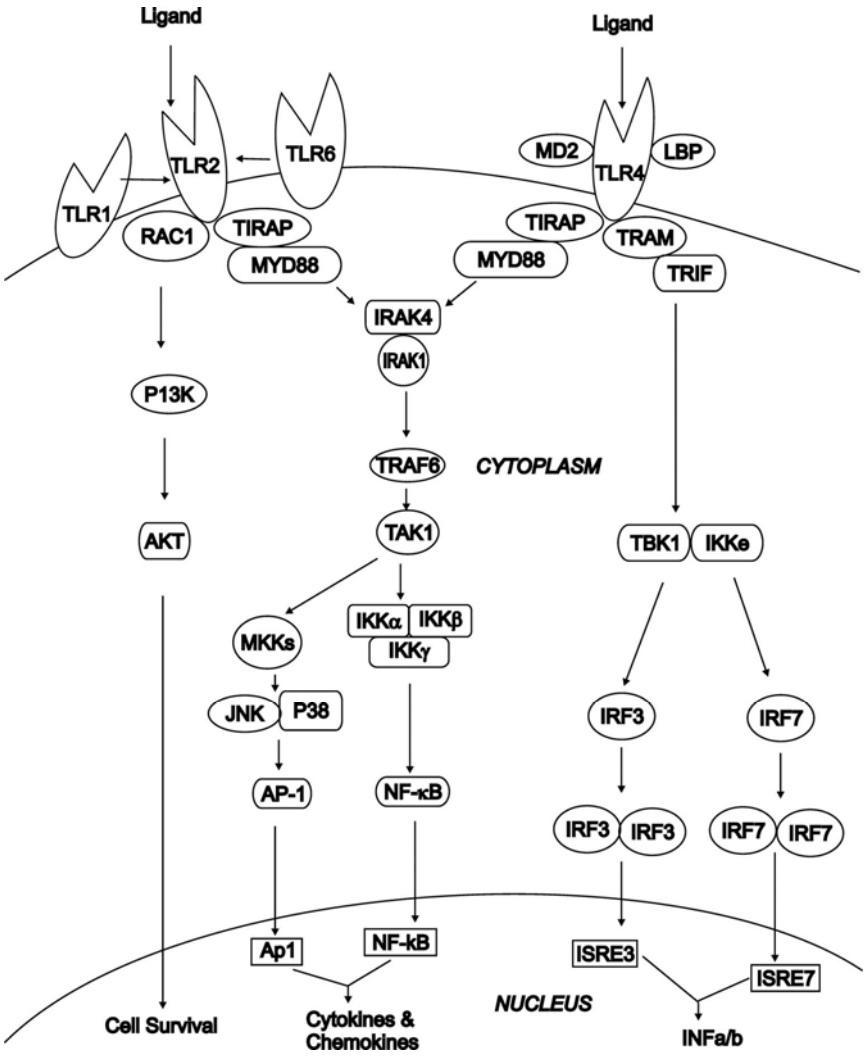

Figure 5: Signaling pathways downstream of Toll-like receptors A schematic overview of the signal transduction pathways of TLR2 and TLR4 is shown.

Toll-like receptors

Toll-like receptors (TLRs) play an essential role in initiating the innate immune response against infectious pathogens (see below). Up to now ten members of the TLR family protein (TLR1 to TLR10) have been identified in humans. TLRs recognize a broad variety of pathogen-associated molecular patterns from bacteria, viruses, fungi and parasites and elicit a broad array of antimicrobial responses. Upon engagement of TLRs with their ligands, several intracellular signal transduction pathways are inititated resulting in activation of various processes (Figure 5). TLRs have been shown to recognize a particular molecular structure associated with a pathogen: TLR4 recognizes lipopoly-saccharide from Gram-negative bacteria, TLR3 senses double stranded (viral) RNA, TLR5 recognizes bacterial flagellin, TLR7 and TLR8 sense single stranded viral RNA, whereas TLR9 recognizes bacterial CpG DNA. Among TLRs, TLR2 is a unique receptor that recognizes lipid- and/or carbohydrate-containing components in cooperation with other TLRs, namely TLR1 and TLR6. Via TLR1 or 6, TLR2 seems to be able to recognize the most diverse set of pathogen-associated motifs within the TLR family.

Toll-like receptor 2 ligands and heterodimers

There are many TLR2 ligands identified, including several lipopeptides. Many of these ligands are found to activate TLR2 via a heterodimer of the receptor (Figure 5). In general, triacyl lipopeptides from bacteria have been found to activate TLR2 via heterodimers of TLR2 and TLR1 [85]. An example of such a triacyl lipopeptide is Pam_3CSK_4 (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl]-(*R*)-cysteinyl-seryl-(lysyl)3-lysine), a synthetic lipoprotein analog (Figure 6A [86]). On the other hand, diacyl lipopeptides from bacteria have been found to activate TLR2 via a heterodimer of TLR2 and TLR6 [87]. An example of a diacyl lipopeptide is MALP-2 (2-kDa macrophage-activating lipopeptide) isolated from *Mycoplasma fermentans* (Figure 6B, [88]). In addition, FSL (follistatin-like)-1, which also has two acyl chains, also activates TLR2/6 heterodimers. FSL-1 is synthesized on the basis of the N terminal part of lipoprotein LP44 of *Mycoplasma salivarium* and has the same framework structure as that of MALP-2 but with differences in the amino acid sequence and length of the peptide portion (Figure 6C, [89,90]). Examination of a wide variety of lipopeptide derivatives indicates that recognition by human TLR1/2 does not strictly correlate with the number

or position of the acyl chains in a triacyllipopeptide [91]. On the other hand, human TLR2/6 heterodimers have been reported to exclusively respond to lipopeptides possessing a diacylgroup and not to other lipopeptides. Chirality of the central carbon of the diacylglycerol group is a structural determinant for human TLR2/6 recognition [91]. Furthermore, it has been shown that also the length of the fatty acids is important for the activation of the TLR heterodimers [89,92].

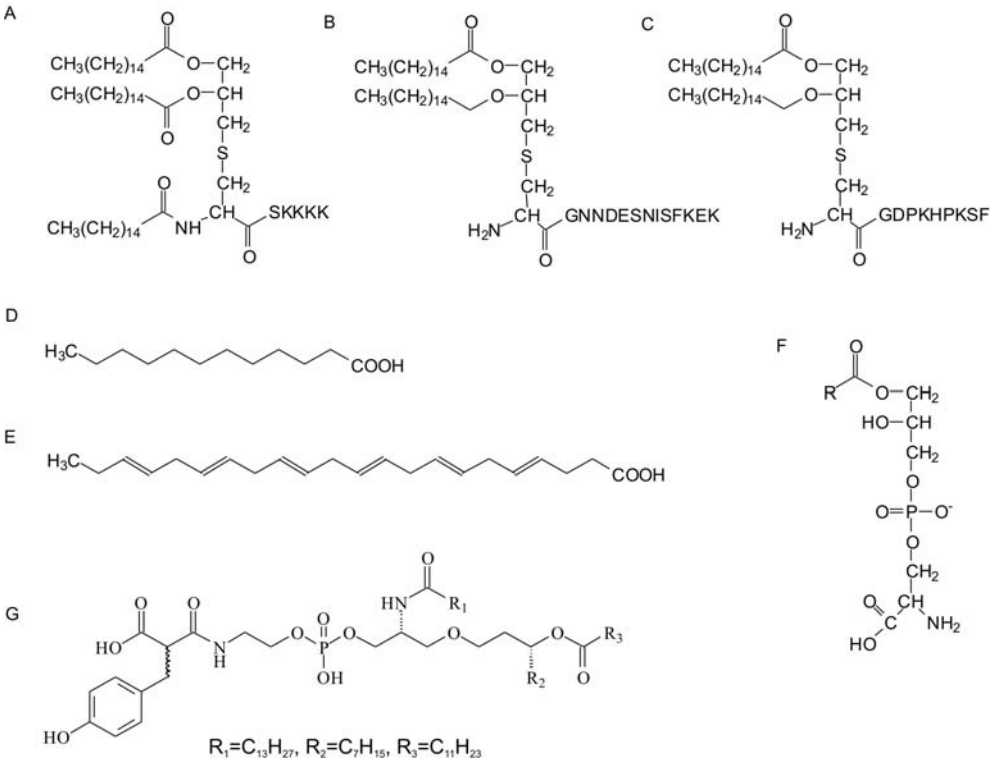

Figure 6: TLR2 ligands. The chemical structures of several TLR2 ligands are shown. (A) Pam_3CSK_4, (B) MALP-2, (C) FSL-1, (D) lauric acid (12:0), (E) docosahexaenoic acid (22:6) and (F) lysoGPSer (R represents a hydrocarbon chain that can vary in length and degree of unsaturation) (G) a synthetic TLR2 antagonist.

For various other TLR2 activating compounds it is still unknown whether they need a co-receptor and if so, which co-receptor they need. Next to activating ligands for TLR2, also a couple of antagonistic ligands have

been found. The fatty acid docosahexaenoic acid, a fatty acid with 22 carbon atoms and 6 double bonds (Figure 6E) has been found to inhibit the activation by lauric acid, a fatty acid with 12 carbon atoms (figure 6D) and a synthetic phospholipid (Figure 6G), yet nameless, has been found to inhibit the stimulation with Pam_3CSK_4 [93,94].

Binding to TLR-2 and receptor complexes

The heterodimers formed by TLR2 and TLR1 or TLR2 and TLR6 are not induced by a ligand but pre-exist in the membrane [95]. Upon stimulation by the specific ligand, they are thought to be recruited to lipid rafts [95]. Examination of chimeric receptors, generated by domain exchange between TLR1 and TLR6, has revealed that leucine-rich repeats of the extracellular domain enables these receptors to discriminate between structural similar lipopeptides. However, this region alone is not sufficient [91]. TLR2 is found to bind directly to bacterial lipopeptides. The leucine rich domain carries the specificity for binding and activation [96]. TLR2 mediated responses to lipoproteins are greatly increased when cells express a membrane-bound CD14 that is co-transfected with TLR2 or when they are incubated in the presence of soluble CD14. CD14 has been found to bind directly to TLR2 ligands, such as triacylated lipopeptides, and to facilitate the recognition of ligands by TLR2, but does not bind to the receptor complex [97]. Lipopolysaccharide binding protein (LBP) is a pattern recognition receptor transferring a variety of bacterial ligands including the two major types of lipopetides (triacylated and diacylated) to CD14 [98]. CD36 is a member of the scavenger receptor type B family. It has been found to facilitate the response of the TLR2/6 heterodimer but does not facilitate the response of the TLR2/1 heterodimers. CD36 is not absolutely required for a response [99]. The heterotypic associations of TLR2/6 with CD36 are not pre-formed but are ligand-induced [95].Next to TLR1 and TLR6, TLR10 might also play a role in TLR2 signaling. The human TLR10 receptor is an orphan member of the TLR family and its gene is present in a locus that also contains TLR1 and TLR6 [100]. Furthermore, it was demonstrated that TLR10 not only forms homodimers but can also heterodimerize with TLR1 and TLR2 [101]. Thus, TLR10 could potentially function together with TLR1 or TLR2, although the specific ligands for these combinations have yet to be identified.

Toll-like receptors and their role in immunology

Instructions for development of specific immune responses are largely mediated by dendritic cells, which are present in peripheral tissues as sentinel dendritic cells and upon activation migrate to the draining lymph nodes to activate naïve T cells, not only by presenting antigens but also by providing signals that determine polarization of T cell development toward Th1 (in general induced by intracellular pathogens), Th2 (in general induced by extracellular pathogens) or regulatory T cells (role in down modulation of the immune system). In this way, dendritic cells play a central role in providing information on the nature of the invading pathogen. Dendritic cells express several TLRs and these receptors play an important role in dendritic cell function.

LysoGPSer containing fractions from schistosomes have been found to activate TLR2 and affect dendritic cells such that mature dendritic cells gained the ability to induce the development of IL-10 producing regulatory T cells. This activity appears to be a unique property of schistosomal lysoGPSer, as neither a synthetic lysoGPSer(16:0) nor GPSer isolated from the mammalian host activates TLR2. This host-parasite interaction via lysoGPSer and TLR2 may be central to long term survival of the parasite and limited host pathology [84].

Scope of the thesis

Chronic schistosomiasis is a disease which is characterized by immune suppression. The suppression of the immune system of the host is, at least partly, induced by the parasite and allows the adult worm pairs to live within the host for decades. Pinpointing the factors the parasite uses to influence the host immune system, will provide important information about the parasite. Furthermore, these factors might be used against auto-immune diseases, in which the immune system is over stimulated. One of such factors is schistosomal lysophosphatidylserine (mono-acylglycerophosphoserine, lysoGPSer). This phospholipid activates TLR2, and via this activation it influences the immune system of the host. This study focuses on the various GPSer species present in *S. mansoni* eggs and worms, their capacity to activate TLR2 and their influence on cytokine production in children who live in an area endemic for schistosomes.

In order to be able to isolate and characterize schistosomal lysoGPSer, a new chromatographic method had to be developed. Chapter 2 of this

thesis describes this new liquid chromatography - mass spectrometry (LC-MS) method, which allows us to separate in a single HPLC run the molecular species of both monoacyl and diacyl forms of all phospholipid classes. In chapter 3 we used this new method to investigate the phospholipids of the tegumental membrane of schistosomes. We identified the presence of lysoGPSer species and investigated their distribution within the worm by comparing phospholipids of the tegument with the complete lipidome of the parasitic worm. Furthermore, we investigated the distribution of possible precursors for lysoGPSer. In chapter 4 we isolated various schistosomal lysoGPSer species and tested their TLR2 activating capacity. Furthermore, four lysoGPSer species were synthesized and tested for TLR2 activating capacity. We focused on the influence of the acyl chain on the activating capacity, and also tested the need for a serine head group and the influence of a second acyl chain. Chapter 5 reports on the influence of schistosomal lysoGPSer on cytokine production in a more complex cell system. Cytokine levels in children living in an area endemic for schistosomes were measured upon stimulation with schistosomal lysoGPSer. The influence was compared with two commercially available TLR2 ligands. Furthermore, lysoGPSer was also tested in the presence of diacylGPSer. Finally, chapter 6 summarizes the main conclusions of this thesis. The possible routes of biosynthetic formation of lysoGPSer in the parasite are discussed. Also the transfer of lysoGPSer from the adult worm to the TLR2 of the host is discussed.

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Chapter 2

A simple and universal method for the separation and identification of phospholipid molecular species

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submitted

Abstract

One of the major challenges in lipidomics is to obtain as much information about the lipidome as possible. Here, we present a novel HPLC-MS/MS method that separates molecular species of all phospholipid classes in one single run. The method is sensitive, robust and allows lipid fingerprinting using full scan mass spectrometry, as well as lipid class specific scanning. Excellent separation of isobaric- and even isomeric species is achieved, and original levels of lysolipids can be determined without interference from lysolipids formed from diacyl species by source fragmentation. Using this method, more than 400 phospholipid species were identified and quantified in crude lipid extracts from rat liver and the parasitic helminth *Schistosoma mansoni*.

Introduction

Phospholipids play a multitude of roles in biological systems. Phospholipids are involved in cell signaling, either directly (phosphoinositides, platelet activating factor, lysophosphatidic acid (monoacylglycerophosphatidic acid, lysoGPA), lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer), etc.) or indirectly, by supplying precursors for active compounds like prostaglandins and diacylglycerols. Phospholipids are main constituents of biological membranes and are thus involved in intracellular and extracellular compartmentalization. Furthermore, phospholipids play important roles in the maintenance of lipid homeostasis, in host-pathogen interactions and in (membrane-) protein function [1-5].

Phospholipids can be classified according to their polar head group, their backbone, the fatty acids that are linked to this backbone and the type of linkage at the *sn*-1 and *sn*-2 position of the glycerol backbone. Many hundreds of molecular species have been identified in cellular lipidomes [5-8] and this number only increases as analytical methods are gaining power and sensitivity.

Functions of phospholipids do, however, not only depend on the presence or absence of certain molecular species, but more on their concentration and the total lipid context. For instance, clustering of specific membrane proteins is supposedly driven by organization of (phospho-)lipids into micro-domains, with the phospholipids in these domains sharing a low level of unsaturation [9]. Lipidomics research is, therefore, more and more focusing at the systems-level, rather than investigating the function of just one lipid at a time.

To enable systems-level lipidomics, the lipidome should be defined in as much detail as possible. Hence, not only the lipid class, but also the exact molecular species composition within each class should be defined to allow an unbiased search for biomarkers. In the past years, it has become clear that both HPLC and (tandem) mass spectrometry are indispensable tools in lipidomics [10,11].

Here, we describe and apply a universal method for the separation of molecular species of phospholipids, their lysoforms and free fatty acids (FFA), and their subsequent identification by on-line, data-driven tandem mass spectrometry. The method exploits the possibilities of state-of-the-art mass spectrometry, and relies on HPLC to separate lipid species that can not be identified by mass spectrometry alone.

Materials and Methods

Chemicals and materials

All solvents used were from Lab-Scan, Dublin, Ireland and were of HPLC grade.
GPGro(18:1/18:1) and di-oleoyl lysobisphosphatidic acid (LBPA, also named bis-monoacylphosphatidic acid (BMP)) were purchased from Avanti polar lipids (Alabaster, AL, USA). Ammonium acetate, serine and silica-G for column chromatography were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Baker (St. Louis, MO, USA).

Preparation of samples

Schistosoma mansoni parasites were isolated from ether anesthetized hamsters, 45-48 days after infection, by perfusion of the heart at 37°C with 0.9% NaCl. Schistosomes were washed and homogenized in 0.9% NaCl (approx. 20% v/v). Livers isolated from male Wistar rats were homogenized in 0.9% NaCl (approx. 20% v/v). Lipids were extracted from both samples according to the method of Bligh and Dyer [12], with the minor modification that 0.5% 6M HCl was added to the second chloroform wash to increase recovery of acidic phospholipids. The phospholipids and free fatty acids were separated from neutral lipids (cholesterol, cholesterol esters and triacylglycerols) by fractionation on a 1 ml silica column prepared from 0.063-0.200mm silica 60 (Merck, Darmstadt, Germany). Lipid extracts were dissolved in chloroform and loaded on top of the silica column and eluted successively with acetone (4 volumes) and methanol (4 volumes). The last fraction, which contained the purified phospholipids, was dried under nitrogen and stored at -20°C until HPLC-MS analysis. A GPSer containing fraction was made from *S. mansoni* adult worms as described before [13].

High Performance Liquid Chromatography

The purified phospholipids were dissolved in methanol : acetonitrile : chloroform : water (46 : 20 : 17 : 17, v/v/v/v). Separation of molecular species was performed on a Synergi 4 µm MAX-RP 18 A column (250 x 3 mm) (Phenomenex, CA, USA). Elution was performed with a linear gradient of water in methanol/acetonitrile (60 : 40, v/v) decreasing from 12.5% to 0% in 25 min, followed by further isocratic elution for another 25 minutes. The flow rate was kept constant at 0.425 ml·min^{-1}

and 1 µM serine and 2.5 mM ammonium acetate were used in all solvents as additives.

Mass spectrometry

Mass spectrometry of lipids was performed using electrospray ionization, on a 4000QTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Source temperature was set to 450°C and nitrogen was used as curtain gas. The declustering potential was optimized using lipid standards. The optimal collision energy was dependent on the type of experiment performed and was set to +45V (precursor scanning *m/z* 184), -45V (precursor scanning *m/z* -196), +35V (neutral loss 141), -30V (precursor scanning *m/z* -241), and -40V (neutral loss scanning 87 Da) respectively. For quantification of molecular species, samples were measured in multiple reaction monitoring mode (MRM), monitoring for 95 head-group specific mass transitions with a total dwell time of 1 s, using the same settings as above.

Data analysis

Data analysis was performed with Analyst^{tm} v 1.4.1 software (Sciex, Toronto, Canada) including the LipidProfiler v1.0 script and the metabolomics export script (Sciex, Toronto, Canada).

Results and Discussion

Chromatography

Current chromatographic methods for the separation of lipids can be classified in two categories based on the stationary phase that is used (recently reviewed in [14]). The first category uses polar (normal phase) column material such as silica or diol modified-silica [6,15,16]. With these methods, phospholipids are separated primarily on the interaction of the polar head group with the column material, and the (crude) lipid extracts are thus separated into lipid classes. With the most modern column materials, even some sub-fractionation of a lipid class into molecular species may be observed, but resolution of isobaric species (such as 16:0/22:4; 18:0/20:4 and 18:1/20:3) cannot be achieved by these methods [6].

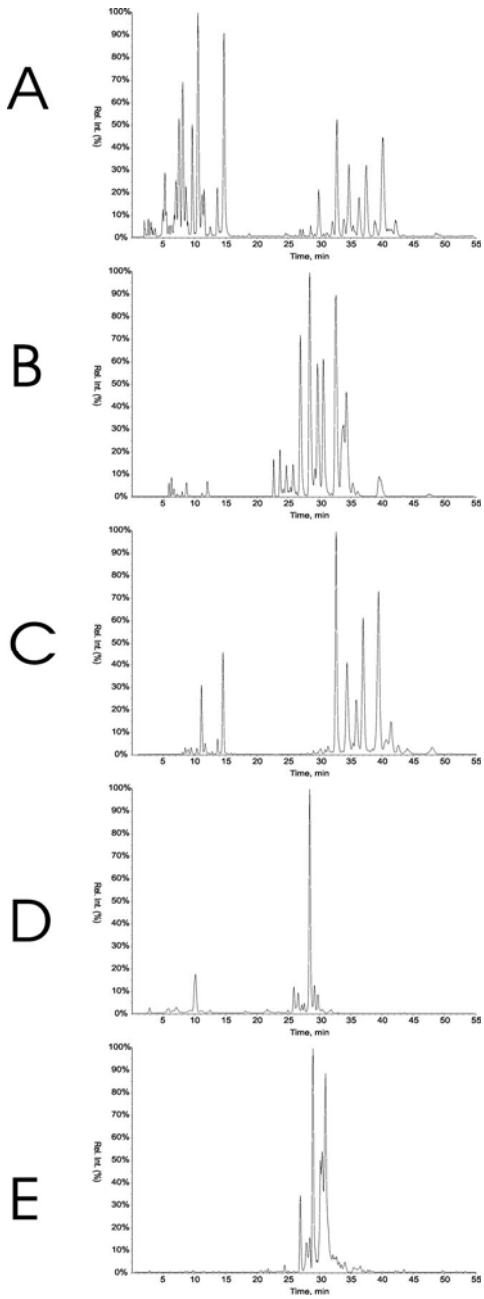

Figure 1: Analysis of a crude phospholipid extract of rat liver cells by HPLC-MS/MS. A: Base peak chromatogram as recorded by negative ionisation mass spectrometry. During the first 20 minutes, free fatty acids, lyso-lipids and other polar lipids elute. After 20 minutes, diradyl species elute. B: Precursor scanning for *m/z* +184, identifying GPCho and SM species. C: Neutral loss of +141 amu, identifying GPEtn species. D: Precursor scanning of *m/z* -241, identifying GPIns species. E: Neutral loss scanning of -87 amu, identifying GPSer species.

The second category, reversed-phase chromatography, typically uses a C18-modified stationary phase and separates lipids on the hydrophobic interaction between radyl chains and stationary phase. Advantage of reversed-phase chromatography is the high resolving power, and two lipid species that only differ in the intra-molecular position of a single double bond can be separated [17]. However, most reversed-phase methods are optimized for a single or just a few lipid classes [18-20] due to the need for class-specific additives to block aspecific interaction between headgroup and stationary phase, which makes these methods less suitable for the analysis of crude lipid extracts.
Our goal was to design a method that is compatible with crude lipid extracts, but also provides a high resolution species separation. In Figure 1A, the base peak chromatogram is shown as recorded during the separation of a crude lipid extract from rat liver with this newly developed reversed-phase method. This method combines the main advantages of the two methods described above: it is applicable to crude lipid extracts and benefits from the high resolving power of a reversed-phase stationary phase. The combination of latest generation reversed-phase material and the use of ammonium acetate and serine as additives in the mobile phase, made this method lipid class independent, and therefore, universally applicable for the separation of molecular species of phospholipids.

Using the freely available Metabolomics Export Script (Sciex, Toronto, Canada), more than 400 compounds having a signal to noise ratio exceeding 9, were identified in the sample of Figure 1A. Depending on the amount and origin of a lipid extract, the number of detected compounds may come close to a thousand (data not shown).

Lipid fingerprinting by full scan HPLC-MS

The negative charge on the phosphate(di-)ester of phospholipids has made negative ionization common practice in mass spectrometry of phospholipids. Phosphatidylglycerol (GPGro), phosphatidylinoositol (GPIns), GPSer, GPA and phosphoinositides are nearly exclusively measured in negative mode [21]. Phosphatidyletanolamine (GPEtn) may be measured both in positive- or in negative mode. In the positive mode, a higher sensitivity can be achieved (with the exception of ether-linked species) but the negative ionisation mode gives more informative ions upon fragmentation [22]. Phosphatidylcholine (GPCho) and

sphingomyeline are most often measured in positive mode, which is related to the positively charged quaternary nitrogen in the head group. Nevertheless, also these lipid classes can, and were, detected in negative mode ionization (see below) [17].

Lipid class specific scanning

Full lipid fingerprinting by HPLC-MS/MS generates large amounts of data, and depending on the experiments at hand, there will often be a significant amount of redundancy in the data. For instance, if one is interested only in the GPCho lipid class, the presence of ions from other lipid classes is often confusing. Obviously, this may be prevented by isolation of GPCho preceding HPLC analysis, but tandem mass spectrometry offers a more efficient and attractive alternative in the exploitation of head group specific fragmentation.

Head group specific fragmentation has been described in detail elsewhere [10,23]. In brief, each phospholipid class shows fragmentation at the phospho(di-)ester linkage, resulting in a diagnostic fragment ion. For GPCho, this diagnostic ion is phosphorylcholine ($(HO)_2POOCH_2CH_2N(CH_3)_3^+$, *m/z* +184) and scanning for precursor ions that produce a fragment of *m/z* +184 will visualize GPCho and SM molecules only. Similar scans can be programmed to specifically visualize GPEtn, GPIns, GPSer, GPGro and even phosphoinositides. This head group specific scanning can be done on-line, and Figure 1B-E shows phospholipid-class specific scanning during the chromatography of a crude lipid extract of rat liver. Thus, head group-specific scanning reduced the complexity of the data.

Lysolipids and preparative HPLC

Assessment of lysolipids and FFA in crude lipid extracts by MS has been troublesome due to the fact that lysolipids and FFA may be artificially produced from diacyllipids during the ionization process (so called source fragmentation). In fact, the difference in efficiencies in which the *sn*-1 and *sn*-2 fatty acyls are lost from the glycerol backbone has successfully been used to assign positional isomers of phospholipids [24-26]. However, when FFA, lysolipids and diacyllipids are simultaneously ionized, it is not possible to discriminate between endogenous lysolipid (or FFA) and lysolipid (or FFA) resulting from source fragmentation. Notably, during the first 20 min of chromatography, the FFA and lysoforms of phospholipids elute, followed

by elution of the diacyl species (Figure 1A-E). In our method, FFA, lysolipids and diacyl species are now separated by HPLC prior to MS analysis, which allows accurate assessment of endogenous lysolipids and FFA in crude lipid extracts.

The high resolving power of reversed-phase HPLC enables isolation of highly purified lipid species. With the exception of the low amount of serine, the mobile phase is fully volatile, so concentration of purified compounds is easily achieved. As an example, Figure 2 shows the separation of (lyso)GPSer species, isolated from the parasitic helminth *Schistosoma mansoni*, and partially purified by anion exchange chromatography. In contrast to rat liver GPSer (Figure 1E), *S. mansoni* contained relative high amounts of lysoGPSer and it has been suggested that these lyso species are responsible for the T-cell hypo-responsiveness observed during chronic schistosome infections [4]. It is also known that this biological activity is not shared among all lysoGPSer species [4]. Using a post-column flow splitter, this method now enabled us to individually isolate all molecular species of lysoGPSer, and test these for their biological activity.

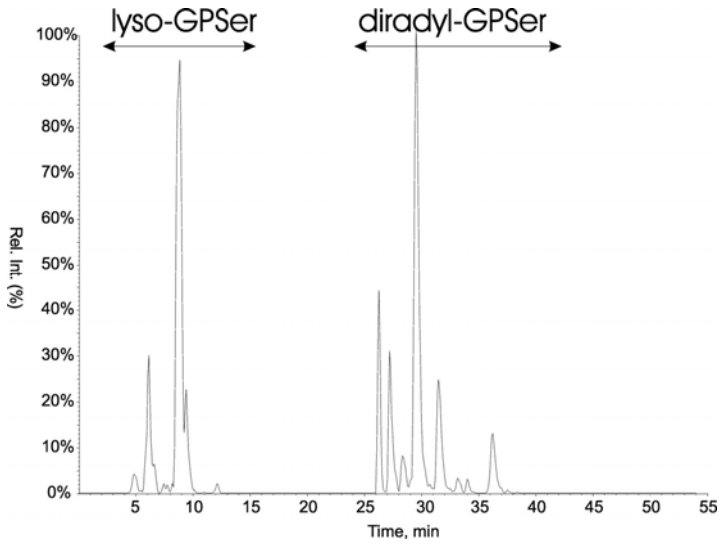

Figure 2: Neutral loss scan of a purified GPSer fraction from *S. mansoni*, showing clear separation between lysophospholipids and diradylphospholipids. With a simple post-column flow splitter the biological active lysolipids can be isolated without contamination of diradyl species.

Isobaric and isomeric species resolved by HPLC

One of the biggest challenges in lipidomics is the correct assignment of isobaric species. Isobaric lipid species have the same (nominal) mass, but contain different fatty acids and/or head groups, and therefore, these phospholipids will have distinct biological and biophysical properties.

In Figure 3A, a small fraction of the contour plot (plotting measured *m/z* values against elution times) shows several lipids, of which four (marked b-e) have the identical *m/z* ratio (766.7 amu). These isobaric lipids elute well separated within a six-minute time window. In Figure 3B-E the on-line recorded product spectra of the respective lipids are shown. Based on the observed headgroup fragments and the carboxylate ions, the lipid species were identified as *N,N*-dimethyl GPEtn(16:0/20:4) (B); GPEtn(20:2/18:2) (C); GPEtn(16:0/22:4) (D) and GPEtn(18:0/20:4) (E) respectively.

It should be noted that the detected *N,N*-dimethyl GPEtn(16:0/20:4) is derived from GPCho(16:0/20:4) after loss of CH_3^+ from the choline headgroup by source fragmentation. This is a well-known fragmentation occurring during negative mode ionization of phosphatidylcholine and illustrates an additional advantage of this method. In positive mode, the abundant, and easily ionized GPCho, often dominates the mass spectrum, leading to ion suppression and reduced sensitivity for other lipid classes such as GPEtn. Due to the reduced ion formation from GPCho in the negative mode and the reduced lipid concentration during ionization in HPLC-MS, this problem does not occur in the method described here. Of course, careful calibration is needed to correct for differences in ionization efficiencies between phospholipid classes in negative mode when quantitative analysis is required.

As an alternative to HPLC-MS, others have resolved isobaric species using only tandem mass spectrometry [8,27]. These "MS-only" methods however, require extensive computational processing to correct for the contributions of the ^{13}C isotopes of more unsaturated species. For example, to correctly quantify the component (*m/z* 768.7) eluting at 41 minutes in Figure 3A without HPLC separation, corrections have to be made for the abundance of the isobaric component eluting approximately 1 minute later, but one also has to correct for the ^{13}C contributions of components B-E.

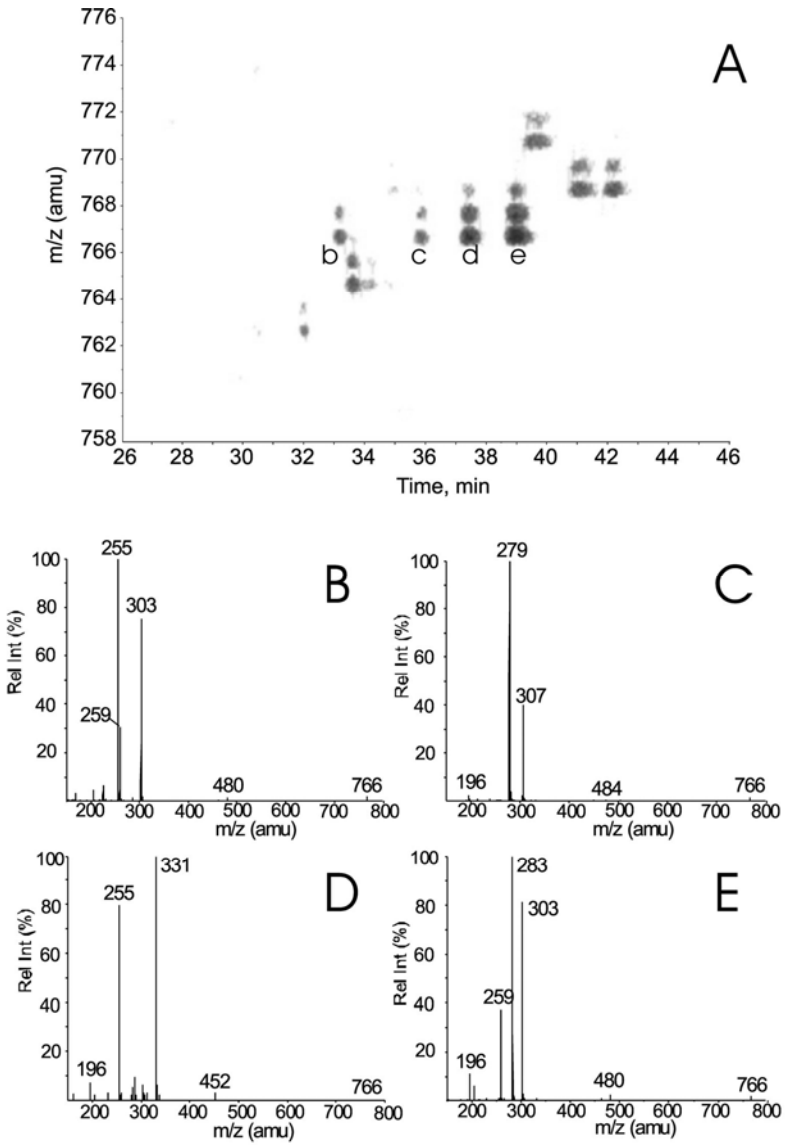

Figure 3: Detail of a contour plot (A) representing a part of the data from Figure 1. The separation of four isobaric species (labelled b-e) is shown in panel A. Product spectra identify these compounds as B: *N,N* dimethyl-GPEtn(16:0/20:4); C: GPEtn(18:2/20:2); D: GPEtn(16:0/22:4); E: GPEtn(18:0/20:4).

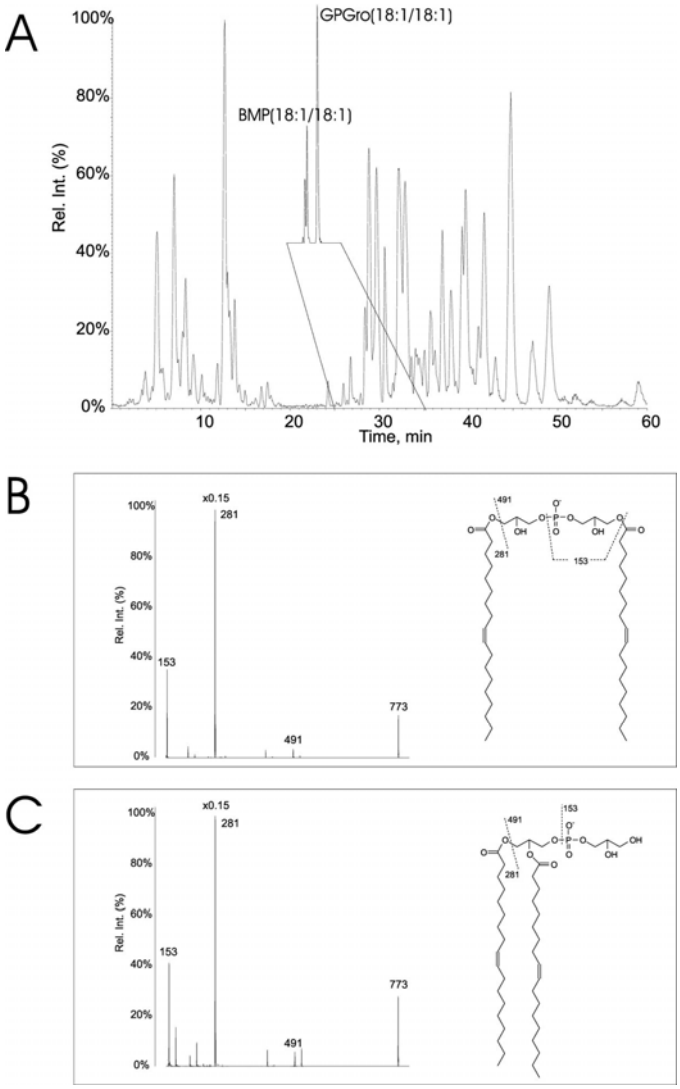

Figure 4: Analysis of a crude phospholipid extract of *S. mansoni,* spiked with GPGro(18:1/18:1) and 18:1/18:1 *bis*-monoacyl phosphatidic acid (BMP, also known as lysobisphosphatidic acid, LBPA). In panel A the base peak chromatogram is shown, and the insert shows the baseline separation of the two isobaric compounds. B: Fragmentation spectrum of BMP(18:1/18:1). C: Fragmentation spectrum of GPGro(18:1/18:1).

In Figure 4A the base peak chromatogram is shown recorded during chromatography of a total lipid extract of the parasitic helminth *Schistosoma mansoni*, that had been spiked with the isobaric compounds GPGro(18:1/18:1) and bis(mono-oleoylglycero)phosphate (BMP(18:1/18:1) also called lysobisphosphatidic acid (LBPA)). Despite the fact that these two compounds are structurally very similar (being build from two oleic acids, two glycerol and a phosphate group), their biological activity is very different. BMP is a marker for the late endocytic pathway, and it has been shown that this particular lipid plays a role in intra-membrane cholesterol transport [28,29] and endosome structure and function [30-32]. These functions are not shared between BMP(18:1/18:1) and GPGro(18:1/18:1). The insert in Figure 4A shows the extracted ion chromatogram of *m/z* 773.7 (the molecular ion of the compounds), demonstrating the clear base-line separation of the two compounds. The BMP even subfractionates into two peaks, likely to represent positional isomers of BMP [33,34]. The fact that HPLC is a prerequisite to discriminate between BMP and GPGro is further illustrated in Figure 4B-C, which show the fragmentation spectra of BMP and GPGro, respectively. These two fragmentation spectra are nearly identical, differing slightly in relative abundances of fragment ions only. This precludes accurate measurement of these two compounds in a mixture based on fragmentation spectra only.

Lipid species in rat liver and schistosomes

When using mass spectrometry for quantitative purposes, one has to consider the fact that the response factor of the machine (i.e. the amount of head group specific fragments produced at a given lipid species concentration) depends on the type of machine, the unsaturation of the species, the lipid concentration and the molecular weight (acyl length) of that species [35]. As a result, accurate molar quantification is only possible using a broad set of internal standards, closely resembling the species one wishes to quantify. In case of samples with an unknown composition, or when the sample contains unusual lipids such as in *S. mansoni*, this may prove problematic [6,17]. However, under the conditions described in this method, light (approximately 750 Da) and heavy (approximately 850 Da) molecular species had response factors that differed by less than 5% from the response factor of an average weight (approximately 800 Da) species, which is in good agreement with published results by others [23,35]. To

demonstrate the general applicability, we used the method above for the quantification of molecular species in rat liver and *Schistoma mansoni*, organisms with large differences in the relative abundance of various phospholipid species. By using on-line HPLC-MS/MS we identified over 400 compounds and quantified those contributing more than 0.5% to the total area under the curve (Table 1-4)*. Clear differences are seen between the two samples, reflecting the characteristic elongation of fatty acids obtained from the host by *S. mansoni* [36]. This chain elongation results in the relatively unusual fatty acid eicosenoic acid (20:1) being present in up to 20% in GPCho species of schistosomes (Table 1) and 22% in schistosomal GPEtn species (Table 2). The amount of 20:1 in the GPSer class was 14.5%, but the most notable characteristic of the molecular species of schistosome GPSer is the abundant presence of very long chain fatty acids: over 50% of the molecular species contained a fatty radyl of 22 carbon atoms or more, where, in rat liver, this was less than 10% (Table 3). GPIns contained only a trace amount of molecular species containing 20:1 (1.5%, Table 4), which is remarkable considering the high amounts of this schistosome-specific fatty acid in the other lipid classes. These data suggest that schistosomes either have highly specific (trans-)acylases responsible for the distinct acyl composition of the various phospholipid classes, or use very distinctive pools of diacylglycerol for the synthesis of the various glycerophospholipid classes. Considering the fact schistosomes are reported to have a high rate of acyl turnover [37], it is unlikely that lipid class species specificity obtained during phospholipid synthesis will be maintained. We are currently investigating the substrate specificity of schistosomal (trans-)acylases and exploring the likeliness that this parasitic characteristic may offer a candidate for drug development.

In conclusion, we have described a simple, rapid and broadly applicable technique for the separation and identification of phospholipid molecular species. Applying this method, allowed us to identify over 400 molecular species, and resulted in the identification of lipid class and parasite-specific characteristics.

* Tables can be found at the pages 48 to 53.

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Table 1: Molecular species of phosphatidylcholine detected in rat liver and *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPCho are shown. Percentages shown are the percentages of the total GPCho species mentioned in the table. Species are quantified by MRM with $[M+H]^+$ for MS1 and *m/z* 184 for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
720	16:0 / 16:0	1-alkyl, 2-acylGPCho	4.0 ± 0.30	2.8 ± 0.12
732	16:0 / 16:1	diacylGPCho	1.1 ± 0.08	1.6 ± 0.10
732	14:0 / 18:1	diacylGPCho	0.5 ± 0.07	*
734	16:0 / 16:0	diacylGPCho	9.0 ± 0.72	2.6 ± 0.27
746	16:0 / 18:0	1-*Z*-alkenyl, 2-acylGPCho	4.8 ± 0.12	*
758	16:0 / 18:2	diacylGPCho	5.8 ± 0.07	7.2 ± 0.32
760	18:0 / 16:1	diacylGPCho	*	4.6 ± 0.22
760	16:0 / 18:1	diacylGPCho	13.5 ± 0.29	2.8 ± 0.28
760	18:1 / 16:0	diacylGPCho	3.0 ± 0.20	*
762	16:0 / 18:0	diacylGPCho	9.8 ± 0.40	3.2 ± 0.10
774	16:0 / 19:1	diacylGPCho	*	2.2 ± 0.16
782	16:0 / 20:4	diacylGPCho	*	5.1 ± 0.09
784	18:1 / 18:2	diacylGPCho	*	3.5 ± 0.07
786	16:0 / 20:2 & 18:1 / 18:1	diacylGPCho diacylGPCho	*	6.6 ± 0.05
786	18:0 / 18:2	diacylGPCho	8.0 ± 0.31	*
786	18:1 / 18:1	diacylGPCho	1.0 ± 0.17	2.1 ± 0.12
788	16:0 / 20:1	diacylGPCho	*	10.0 ± 0.17
788	18:0 / 18:1	diacylGPCho	22.2 ± 0.46	4.3 ± 0.07
790	18:0 / 18:0	diacylGPCho	*	3.1 ± 0.17
790	16:0 / 20:0	diacylGPCho	1.5 ± 0.18	*
802	18:0 / 20:1	1-alkyl, 2-acylGPCho	*	0.9 ± 0.06
802	17:0 / 20:1	diacylGPCho	*	1.0 ± 0.03
806	16:0 / 22:6	diacylGPCho	1.6 ± 0.18	3.3 ± 0.16
808	18:1 / 20:4	diacylGPCho	*	2.5 ± 0.08
810	16:0 / 22:4	diacylGPCho	*	3.3 ± 0.20
810	18:0 / 20:4	diacylGPCho	4.4 ± 0.33	4.3 ± 0.13

812	20:1 / 18:2 & 18:1 / 20:2	diacylGPCho diacylGPCho	*	1.4 ± 0.04
812	18:0 / 20:3	diacylGPCho	0.8 ± 0.05	1.0 ± 0.02
814	18:1 / 20:1	diacylGPCho	*	3.1 ± 0.13
814	18:0 / 20:2	diacylGPCho	0.9 ± 0.11	*
816	16:0 / 22:1	diacylGPCho	1.6 ± 0.15	1.1 ± 0.08
830	18:2 / 22:6	diacylGPCho	0.6 ± 0.08	*
832	18:1 / 22:6	diacylGPCho	*	1.0 ± 0.02
834	20:2 / 20:4	diacylGPCho	*	1.0 ± 0.10
834	18:0 / 22:6	diacylGPCho	1.3 ± 0.09	2.6 ± 0.07
838	18:0 / 22:4	diacylGPCho	*	4.2 ± 0.33
840	18:0 / 22:3	diacylGPCho	*	0.9 ± 0.14
842	20:1 / 20:1	diacylGPCho	2.0 ± 0.23	2.3 ± 0.12
846	24:0 / 16:0	diacylGPCho	2.6 ± 0.22	1.4 ± 0.09
846	20:0 / 20:0	diacylGPCho	*	1.1 ± 0.09
848	#	diacylGPCho	*	1.1 ± 0.06
864	20:1 / 22:4	diacylGPCho	*	0.8 ± 0.14

Table 2: Molecular species of phosphatidylethanolamine detected in rat liver and in *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPEtn are shown. Percentages shown are the percentages of the total GPEtn species mentioned in the table. Species are quantified by MRM with $[M+H]^+$ for MS1 and $[M+H-141]^+$ for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
692	16:0 / 16:0	diacylGPEtn	0.7 ± 0.06	*
716	16:0 / 18:2	diacylGPEtn	7.1 ± 0.12	3.0 ± 0.05
718	16:0 / 18:1	diacylGPEtn	5.5 ± 0.11	2.3 ± 0.17
720	16:0 / 18:0	diacylGPEtn	4.0 ± 0.19	*
730	17:0 / 18:2	diacylGPEtn	0.7 ± 0.03	*
732	18:0 / 18:1	1-alkyl, 2-acylGPEtn	*	0.5 ± 0.08
732	17:0 / 18:1	diacylGPEtn	0.7 ± 0.05	*
734	17:0 / 18:0	diacylGPEtn	0.5 ± 0.01	*
740	16:0 / 20:4	diacylGPEtn	4.0 ± 0.10	0.7 ± 0.04
742	18:1 / 18:2 & 16:0 / 20:3	diacylGPEtn diacylGPEtn	*	3.1 ± 0,32
742	16:0 / 20:3	diacylGPEtn	2.2 ± 0.14	*
744	18:0 / 18:2	diacylGPEtn	*	9.2 ± 0.49
744	18:0 / 18:2 & 16:0 / 20:2	diacylGPEtn diacylGPEtn	12.1 ± 0.39	*
746	18:0 / 18:1 & 16:0 / 20:1	diacylGPEtn diacylGPEtn	8.4 ± 0.40	14.5 ± 0.55
748	18:0 / 18:0	diacylGPEtn	3.3 ± 0.35	*
760	19:0 / 18:1	diacylGPEtn	*	0.6 ± 0.13
760	17:0 / 20:1	diacylGPEtn	0.5 ± 0.09	1.1 ± 0.08
764	16:0 / 22:6	diacylGPEtn	12.3 ± 0.51	1.5 ± 0.12
766	18:1 / 20:4	diacylGPEtn	3.3 ± 0.10	1.5 ± 0.04
766	18:0 / 20.5	diacylGPEtn	0.9 ± 0.02	*
768	18:1 / 20:3	diacylGPEtn	*	1.0 ± 0.03
768	16:0 / 22:4	diacylGPEtn	1.0 ± 0.10	1.5 ± 0.04
768	18:0 / 20:4	diacylGPEtn	9.5 ± 0.69	6.1 ± 0.35
770	18:0 / 20:3	diacylGPEtn	3.5 ± 0.68	2.0 ± 0.12

770	16:0 / 22:3	diacylGPEtn	1.5 ± 1.39	2.5 ± 0.11
772	18:1 / 20:1	diacylGPEtn	*	6.0 ± 0.18
772	18:0 / 20:2	diacylGPEtn	0.9 ± 0.13	*
774	18:0 / 20:1	diacylGPEtn	0.8 ± 0.02	11.4 ± 0.35
776	#		*	1.1 ± 0.04
786	#		0.6 ± 0.16	*
788	20:4 / 20:4	diacylGPEtn	0.6 ± 0.06	*
790	18:1 / 22:6	diacylGPEtn	1.1 ± 0.05	*
790	18:2 / 22:5	diacylGPEtn	0.7 ± 0.02	*
792	18:0 / 22:6	diacylGPEtn	9.1 ± 0.82	4.6 ± 0.26
794	18:0 / 22:5	diacylGPEtn	1.6 ± 0.11	2.7 ± 0.19
794	20:1 / 20:4	diacylGPEtn	0.5 ± 0.04	*
796	18:0 / 22:4	diacylGPEtn	1.9 ± 0.08	13.8 ± 1.03
798	20:1 / 20:2	diacylGPEtn	*	2.2 ± 0.10
798	18:0 / 22:3	diacylGPEtn	*	0.9 ± 0.11
800	#		*	2.1 ± 0.18
814	20:3 / 22:6	diacylGPEtn	0.5 ± 0.12	*
818	20:1 / 22:6	diacylGPEtn	*	0.6 ± 0.08
822	18:0 / 24:5 & 20:1 / 22:4	diacylGPEtn diacylGPEtn	1.6 ± 0.11	0.9 ± 0.07
824	18:0 / 24:4	diacylGPEtn	*	1.6 ± 0.08
824	20:0 / 22:4	diacylGPEtn	*	1.1 ± 0.14

Table 3: Molecular species of phosphatidylserine found in rat liver and in *S. mansoni* adult worms. Species are quantified by MRM with $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole %)**	**Abundance in *S. mansoni* (mole %)**
734	16:0 / 16:0	diacylGPSer	0.8 ± 0.10	0.8 ± 0.10
760	16:0 / 18:1	diacylGPSer	1.9 ± 0.05	1.4 ± 0.13
762	#		7.7 ± 0.28	*
786	18:0 /18:2 & 18:1 / 18:1	diacylGPSer diacylGPSer	*	6.6 ± 0.21
786	18:0 / 18:2	diacylGPSer	7.1 ± 0.18	*
788	18:0 / 18:1	diacylGPSer	33.8 ± 1.14	8.0 ± 0.07
790	18:0 / 18:0	diacylGPSer	6.4 ± 0.57	*
810	18:0 / 20:4	diacylGPSer	17.1 ± 1.24	8.4 ± 0.20
812	18:0 / 20:3	diacylGPSer	5.4 ± 1.25	3.1 ± 0.37
814	#		*	2.8 ± 0.22
814	18:0 / 20:2	diacylGPSer	1.1 ± 0.58	*
814	18:1 / 20:1	diacylGPSer	0.9 ± 0.62	*
816	18:0 / 20:1	diacylGPSer	*	5.9 ± 0.83
816	20:0 / 18:1	diacylGPSer	4.2 ± 0.50	*
834	18:0 / 22:6	diacylGPSer	3.9 ± 0.82	10.3 ± 0.54
836	20:1 / 20:4	diacylGPSer	*	8.6 ± 0.38
836	18:0 / 22:5	diacylGPSer	*	2.3 ± 0.21
836	20:2 / 20:3	diacylGPSer	3.5 ± 0.66	*
838	18:0 / 22:4	diacylGPSer	5.8 ± 1.11	27.7 ± 0.85
840	18:0 / 22:3	diacylGPSer	*	2.4 ± 0.18
852	19:0 / 22:4	diacylGPSer	*	0.6 ± 0.14
852	20:0 / 22:4	1-alkyl, 2-acylGPSer	*	0.5 ± 0.07
864	20:0 / 22:5	diacylGPSer	*	1.7 ± 0.11
864	18:0 / 24:5	diacylGPSer	*	0.9 ± 0.12
866	20:0 / 22:4	diacylGPSer	*	2.2 ± 0.17
866	18:0 / 24:4	diacylGPSer	*	2.9 ± 0.32

Table 4: Molecular species of phosphatidylinositol detected in rat liver and *S. mansoni* adult worms. Species that comprise more than 0.5% of the total GPIns are shown. Percentages shown are the percentages of the total GPIns species mentioned in the table. Species are quantified by MRM with $[M-H]^-$ for MS1 and *m/z* 241 for MS2. Shown is the average ± standard deviation of a single lipid extract injected and analyzed in triplicate. Only species contributing more than 0.5% are indicated. * Less than 0.5%. # Unidentified.

m/z	**component**		**Abundance in rat liver (mole%)**	**Abundance in *S. mansoni* (mole%)**
795	16:0 / 15:0	diacylGPIns	*	1.3 ± 0.20
809	16:0 / 16:0	diacylGPIns	0.8 ± 0.03	0.7 ± 0.32
821	15:0 / 18:1	diacylGPIns	*	1.4 ± 0.11
833	16:0 / 18:2	diacylGPIns	3.3 ± 0.05	4.0 ± 0.24
835	16:0 / 18:1	diacylGPIns	1.3 ± 0.02	6.6 ± 0.63
837	16:0 / 18:0	diacylGPIns	4.7 ± 0.09	1.9 ± 0.05
847	#	diacylGPIns	*	0.5 ± 0.17
847	18:0 / 18:2 & 18:0 / 18:1	1-alkyl, 2-acylGPIns 1-*Z*-alkenyl, 2-acylGPIns	*	1.3 ± 0.04
849	17:0 / 18:1	diacylGPIns	*	1.2 ± 0.20
857	16:0 / 20:4	diacylGPIns	7.8 ± 0.09	2.0 ± 0.19
859	16:0 / 20:3	diacylGPIns	2.1 ± 0.01	2.0 ± 0.36
861	18:0 / 18:2	diacylGPIns	6.2 ± 0.05	19.1 ± 0.44
863	18:0 / 18:1	diacylGPIns	2.5 ± 0.06	17.6 ± 1.44
865	18:0 / 18:0	diacylGPIns	2.7 ± 0.04	1.0 ± 0.34
871	17:0 / 20:4	diacylGPIns	1.0 ± 0.02	*
881	16:0 / 22:6	diacylGPIns	1.5 ± 0.04	*
883	18:1 / 20:4	diacylGPIns	2.5 ± 0.03	*
885	18:0 / 20:4	diacylGPIns	38.7 ± 0.12	25.8 ± 0.91
887	18:0 / 20:3	diacylGPIns	13.9 ± 0.06	5.8 ± 0.06
889	18:0 / 20:2	diacylGPIns	1.8 ± 0.08	3.2 ± 0.39
891	18:0 / 20:1	diacylGPIns	*	1.5 ± 0.29
909	18:0 / 22:6	diacylGPIns	4.9 ± 0.02	1.4 ± 0.39
911	18:0 / 22:5	diacylGPIns	1.1 ± 0.06	*
911	18:1 / 22:4	diacylGPIns	0.8 ± 0.14	*
913	18:0 / 22:4	diacylGPIns	2.4 ± 0.17	1.7 ± 0.30

Chapter 3

The phosphatidylserine and lysophospholipid species composition of the tegumental outer-surface membranes of *Schistosoma mansoni*

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Abstract

The tegumental outer-surface structure of schistosomes is unique in nature and consists of a syncytium of fused cells covered by two closely-apposed lipid bilayers that form the interactive surface with the host. In order to identify parasite-specific lipids enriched in the tegument, the species composition was analysed of phosphatidylserine and lyso-phospholipids in adult schistosomes, in isolated tegumental membranes of adult schistosomes and in blood cells of the host. It was shown that the tegument comprises many schistosome-specific and tegument-specific lipids and that the species composition of most phospolipid classes in the tegument differs dramatically of that from total worms and from that of host blood cells. Furthermore, it was shown that the tegument is enriched in lysophosphatidylserine en lysophosphatidyl-ethanolamine.

Introduction

Schistosoma mansoni is a parasitic worm that causes schistosomiasis, a disease that afflicts over 200 million people and numerous animals [1]. Schistosomiasis is endemic in many rural areas in tropical countries, where it has a significant effect on the economy and welfare. Infection of the mammalian host occurs via penetration of the skin by the cercarial stage, which thereafter transforms into the schistosomulum stage. These juvenile schistosomes then reside in the skin for 2 to 4 days before they enter a blood vessel. Within the vascular system, juvenile *S. mansoni* worms migrate via a complex route to the mesenteric veins where they mature and form male-female couples that produce about 300 eggs a day [1]. Part of these produced eggs, fail to leave the blood vessel and then cause obstruction of capillaries in the liver, which subsequently results in severe inflammations.
Adult *S. mansoni* worms are able to maintain themselves for many years in the blood vessels of their mammalian hosts [1]. Despite their relatively large size compared to veins (0.5 cm long with a diameter of 0.5 mm) and their continuous exposure to immune cells present in the blood of the host, this parasite apparently prevents an adequate immune response [2,3]. Although the underlying molecular mechanisms involved in long term parasite survival are not yet completely understood, the tegumental outer-surface structure of schistosomes is of crucial importance for parasite survival and modulation of the host response [4,5].
The tegumental outer-surface structure of schistosomes is unique in nature and consists of a syncytium of fused cells covered by two closely-apposed lipid bilayers that form the interactive surface with host [6]. Recent studies characterized the protein composition of these outer-surface membranes, which demonstrated that these membranes contain nutrient transporters, structural membrane proteins, several proteins derived from the host and many schistosomal proteins with a yet unknown function [7,8]. The tegument is also enriched in proteins that share no sequence similarity to any sequence present in databases of species other than schistosomes, demonstrating that the unique tegumental structures comprise multiple unique proteins that are likely to fulfil schistosome-specific functions involved in parasite survival [5,7,8].

Next to proteins, the tegumental membranes contain many distinct lipids and multiple studies on the lipid composition of the tegumental membranes showed that the species composition of the phospholipids phosphatidylcholine (glycerophosphocholine, GPCho) and phosphatidyl-ethanolamine (glycerophosphoethanolamine, GPEtn) in the tegumental membranes differs not only drastically from that of blood cells of the host but also from that of the complete worm [8,9]. For instance, the tegumental membranes contain a large amount of ether-linked GPEtn species [9], and one of the most abundant GPCho-species in the tegument contains a highly unusual fatty acid, octadec-5-enoic acid (18:1(5Z)), which is absent in the host [10]. So far the diacylphospholipid species composition of the tegument has been characterized for the phospholipid classes GPCho and GPEtn, which are the most abundant phospholipid classes in schistosomal membranes [11]. However, the species composition of the less abundant phospholipid classes, such as phosphatidylserine (glycerophosphoserine, GPSer) and phosphatidylinositol (glycerophosphoinositol, GPIns) have not been characterized in the tegument. These phospholipids are known to function as precursors for the production of potent signalling molecules. Furthermore, schistosomal lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) species have been shown to activate Toll-Like Receptor 2 (TLR2) and affect dendritic cells of the host in such a manner that mature dendritic cells induced the development of IL-10 producing regulatory T cells, a process known to result in a down regulation of the immune response [12]. The present study analysed the species composition of GPSer, GPIns and lysophosphopolipids in adult *S. mansoni* worms, in isolated tegumental membranes and in hamster blood, in order to identify parasite-specific lipids enriched in the tegument, which forms the host-parasite interface.

Materials and Methods

Chemicals and materials

All solvents used were from Lab-Scan (Dublin, Ireland) and were of HPLC grade. Ammonium acetate, serine and Silica 60 for column chromatography were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Baker (St. Louis, MO, USA) and CM-52 was obtained from Serva (Heidelberg, Germany).

Biological materials
Adult *S. mansoni* parasites were obtained from ether-anesthetized hamsters at 45-48 days after infection by perfusion of the heart with 0.9% NaCl. Subsequently, they were washed and homogenized in 0.9% NaCl (approx. 20% v/v). Isolated adult *S. mansoni* worms were washed in Hanks Balanced Salt Solution [13] (HBSS) before tegumental membranes were isolated by a freeze-thaw method according to Roberts *et al* [14] as described by Brouwers *et al* [15]. Briefly, adult worms in HBSS were drop by drop plunged into liquid nitrogen. After thawing on ice, worms were extensively washed with ice-cold Tris-buffered saline (20 mM Tris-HCl, 0.9% [w/v] NaCl, containing protease inhibitors). The tegumental membrane complex was removed from the worms by applying 10 vortex pulses of 1 sec. The supernatant, containing the tegumental membranes, was passed over a fine stainless steal mesh and the filtrate was centrifuged at 5000 × g for 30 minutes at 4°C. The isolated tegumental membrane pellets were frozen at -20°C until further use. Blood was drawn from non-infected ether-anesthetized hamsters by a heart puncture. Blood was diluted (1:5, v/v) in water in order to lyse the red blood cells by hypotonic shock, shortly before lipid extraction.

Lipid extraction
Lipids were extracted from the biological samples according to the method of Bligh and Dyer [16], with the minor modification that 0.1% 6 M HCl was added at the second chloroform wash to increase recovery of acidic phospholipids. Neutral lipids were removed from the extracted lipids by fractionation on a 3 ml silica column prepared from 0.063-0.200 mm Silica 60. Lipid extracts were dissolved in chloroform and loaded on top of the silica column. Subsequently, neutral lipids were eluted with acetone (4 volumes) after which phospholipids and free fatty acids were eluted with methanol (4 volumes). This latter fraction was dried under nitrogen and stored at -20°C until HPLC-MS analysis of the phospholipids.

Quantification and identification of phospholipid species composition
Samples were analyzed by a HPLC-MS method as previously described (see chapter 2 for details). In short, phospholipids were separated by HPLC, using a Synergi 4 µm MAX-RP 18A column (250 x 3 mm) (Phenomenex, CA, USA). Elution was performed within 55 minutes with a decreasing linear gradient of water in methanol/acetonitrile, with

serine and ammonium acetate used as additives. Mass spectrometry was performed using electrospray ionization on a Sciex 4000QTRAP (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Samples were analyzed in a multiple reaction mode (MRM) or by neutral loss and precursor scanning. For ionization details of the mass spectrometric analysis see chapter 2. Data analysis was performed according to the manufacturers' protocols with Analyst^{tm} software version 1.4.1 (Sciex, Toronto, Canada) including the metabolomics export and Lipid-Profiler v1.0 script (Sciex, Toronto, Canada).

Results and discussion

This study aimed to provide a comprehensive characterization of the GPSer, GPIns and lysophospholipid species composition of the tegumental membranes of adult schistosomes, in order to detect parasite and tegument-specific lipids that might be involved in host-parasite interactions. We used a novel and universal HPLC-mass spectrometry method (see chapter 2) for the identification of the molecular species of the four most significant phospholipids classes: GPCho, GPEtn, GPSer and GPIns in adult schistosomes, in isolated tegumental membranes of adult schistosomes, and in blood cells of the host. This method separates isobaric species of all phospholipid classes prior to identification and quantification by mass spectrometry. This method also allows detection and quantification of lysophospholipid species.

Tegumental membranes were isolated by a well established freeze-thaw method originally developed by Roberts *et al* [14]. An improved version of this method has been used in our laboratory to characterize the proteome and the species composition of the phospholipids GPCho and GPEtn in the tegumental membranes of adult schistosomes [7,9]. Results of the current analysis of the species composition of GPCho and GPEtn in membranes of total schistosomes, tegumental membranes and host blood cells (see supplementary data, Table 1 and 2), confirmed the previously reported results [9]. Schistosomal membranes were again found to be enriched in the diacylGPEtn species 18:0/18:1 and 18:0/22:4 when compared to host blood cells. The ether-linked GPEtn species, which are especially enriched in the tegumental membranes, are not optimal detected in the positive ionisation mode that was used in

the present study, and therefore, the enrichment of ether-linked GPEtn species could not be confirmed by this method. The tegumental membranes were also found to be specifically enriched in diacylGPCho(16:0/16:0), alkyl,acylGPCho(16:0/16:0) and the diacyl-GPCho(16:0/18:1, delta 5) (see supplementary data, Table 2). This confirms earlier observations [9,10,17], and validates the purity of the isolated tegumental membranes used in the present study.
GPSer species were identified and quantified by multiple reaction monitoring (MRM) using the specific fragmentation of GPSer species (the neutral loss of 87 dalton). The elution pattern of GPSer species derived from tegumental membranes differed drastically from that of membranes of entire worms and from that of membranes from blood cells (Figure 1). Identification and quantification of the distinct diacyl-GPSer species demonstrated that the schistosomal membranes comprised many distinct GPSer species hardly present in the membranes of blood cells, such as for instance GPSer(18:0/20:1) and GPSer(18:0/22:4) (Table 1). Compared to membranes of blood cells, schistosomal membranes contained many GPSer species with longer acyl chains, as relatively many GPSer species were detected with one acyl chain containing 22 or more carbon atoms (Table 1).

In total, there were over 250 different molecular species of GPSer observed in the membrane preparations (data not shown). In order to identify which of these molecular species were specific for any type of membrane, and hence could be considered to be (bio-) markers for that type of membrane, we performed a disciminant analysis on the GPSer data. This statistical method defines two new variables, discriminant 1 (D1) and discriminant 2 (D2), which optimally separate the different membrane types. Each of these two variables is a linear combination of all molecular species with calculated loading coefficients: $D1 = (loading_1 \cdot GPSer_1 + loading_2 \cdot GPSer_2 + loading_3 \cdot GPSer_3 + \ldots\ldots)$. The result of the discriminant analysis can be graphically represented by: 1) The scores plot (Figure 2A), in which the calculated D1 and D2 values of each membrane preparation are plotted and 2) The loadings plot (Figure 2B), in which the contribution (loading) of each and every molecular species to D1 and D2 is plotted.

Table 1: Molecular species of diacylglycerophosphoserine (GPSer).
Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood. GPSer species were quantified by MRM with $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. Percentages shown are the average percentages of the total GPSer species ± standard deviation, of species comprising more than 1% of the total GPSer. The three most abundant species in each membrane preparation are printed bold and species marked by an * comprise less than 0.5% of the total GPSer in that membrane preparation. Attached acyl-chains are sometimes given in total number of carbon atoms and unsaturations. Rt, retention time in minutes; #, not identified; $, tentative identification based on *m/z*, retention time and head group fragmentation, full product spectra could not be obtained.

m/z	Rt	Acyl chains	Component	Total worm (n=5) % ± S.D.	Tegument (n=3) % ± S.D.	Hamster blood (n=5) % ± S.D.
760	24.7	16:0 / 18:1	diacylGPSer	0.7 ± 0.10	1.5 ± 0.99	1.3 ± 0.14
762	27.7	16:0 / 18:0	diacylGPSer	*	1.2 ± 0.28	*
772	29.7	16:0 / 20:1	plasmalogen GPSer	*	2.7 ± 3.56	*
782	21.7	16:0 / 20:4	diacylGPser	*	*	1.8 ± 0.21
784	22.7	18:1 / 18:2	diacylGPSer	*	*	1.3 ± 0.09
786	25.1	18:0 / 18:2	diacylGPSer	2.8 ± 0.37	2.2 ± 1.15	2.8 ± 0.14
788	28.5	18:0 / 18:1	diacylGPSer	**6.8 ± 0.79**	7.2 ± 1.94	3.0 ± 0.44
796	22.8	17:0 / 20:4	diacylGPSer	*	*	2.4 ± 0.08
798	31.4	#		2.4 ± 0.14	1.8 ± 2.24	*
800	35.8	38:2 $	etherlipid-GPSer	5.4 ± 1.07	4.7 ± 5.75	*
804	29.6	37:0 $	diacylGPSer	*	7.5 ± 1.20	*
806	19.9	18:2 / 20:4	diacylGPSer	*	*	2.2 ± 0.09
808	22.0	18:1 / 20:4	diacylGPSer	*	*	**15.1 ± 0.70**
810	24.1	18:0 / 20:4	diacylGPSer	5.2 ± 0.32	3.3 ± 1.61	**43.7 ± 1.75**
812	25.6	18:0 / 20:3	diacylGPSer	1.5 ± 0.24	0.8 ± 0.24	0.7 ± 0.29
814	28.1	18:0 / 20:2	diacylGPSer	2.2 ± 0.23	1.5 ± 1.49	*
816	32.1	18:0 / 20:1	diacylGPSer	4.8 ± 0.66	**8.0 ± 1.32**	*
818	32.1	18:0 / 20:0	diacylGPSer	*	3.1 ± 1.47	*
822	29.0	#		1.5 ± 0.32	*	*
824	25.3	19:0 /20:4	diacylGPSer	*	*	1.0 ± 0.28
830	19.2	20:4 / 20:4	diacylGPSer	*	*	**4.7 ± 0.25**
830	29.8	#		*	4.6 ± 1.10	*

832	22.0	#		*	*	2.3 ± 0.22
832	35.0	40:0	alkyl,acyl-GPSer	*	**10.6 ± 2.20**	*
834	22.0	18:1 / 20:5	diacylGPSer	*	*	2.3 ± 0.22
834	22.8	20:2 / 20:4	diacylGPSer	*	*	1.2 ± 0.08
834	23.2	18:0 / 22:6	diacylGPSer	**9.1 ± 1.24**	5.3 ± 3.11	3.4 ± 0.23
836	24.1	18:1 / 22:4 & 20:4 / 20:1	diacylGPSer diacylGPSer	*	*	2.1 ± 0.30
836	25.2	18:0 / 22:5	diacylGPSer	6.2 ± 0.63	4.4 ± 1.70	2.6 ± 0.56
838	26.1	18:0 / 22:4	diacylGPSer	**42.6 ± 3.14**	**16.8 ± 3.12**	2.6 ± 0.15
846	37.8	#		*	2.6 ± 2.02	*
854	27.9	#		*	1.7 ± 0.69	*
864	27.0	#		1.8 ± 0.09	0.8 ± 0.04	*
866	29.4	20:0 / 22:4	diacylGPSer	1.5 ± 0.15	0.6 ± 0.24	*
866	30.0	18:0 / 24:4	diacylGPSer	1.7 ± 0.24	*	*

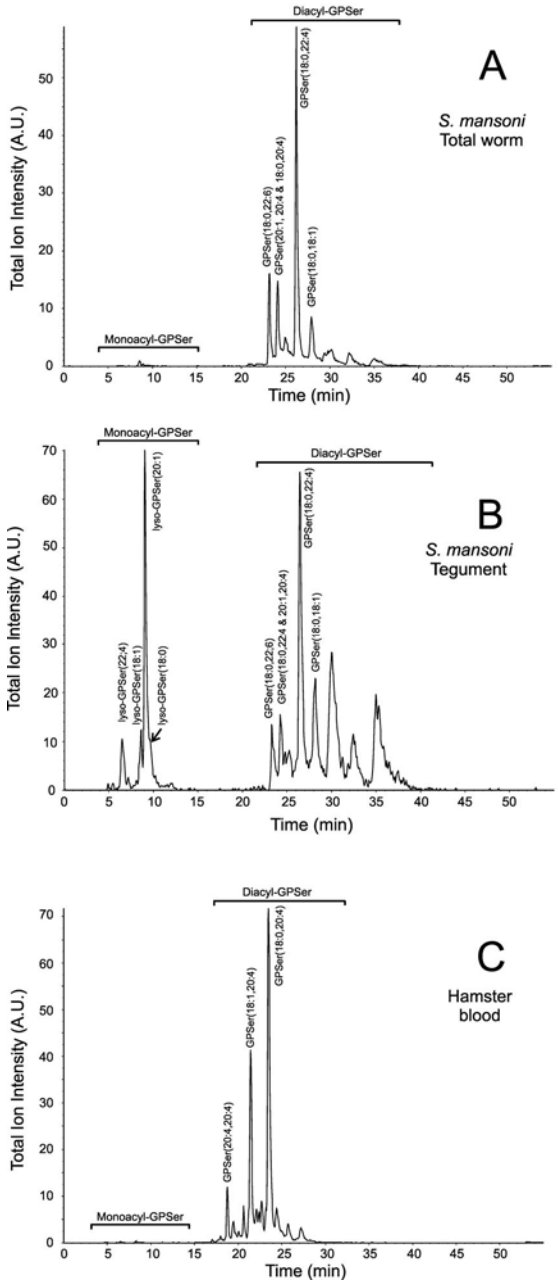

Figure 1. Analysis of the phosphatidylserine species composition by HPLC-mass spectrometry. Molecular species were determined in homogenates of total *S. mansoni* worms (panel A), in isolated tegumental membranes from adult *S. mansoni* worms (panel B) and in hamster blood (panel C). Phosphatidylserine (GPSer) species were detected and quantified by multiple reaction monitoring using $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. Shown is the total ion intensity in arbitrary units during elution.

This discriminant analysis revealed that the variance observed in five independent replicates of each membrane preparations was very small compared to the differences found between the three types of membranes, as independent preparations of each membrane type clustered closely together, whereas there was a clear separation between the different types of membranes (Figure 2A). It should be noted that D1 made a clear distinction between schistosome derived membranes and blood derived membranes, as the two schistosomal membranes had the same (negative) D1 score, whereas the hamster blood derived membranes had a large positive D1 score. D2 separated all three membranes: hamster blood had a score of approximately zero, tegumental membranes had a positive score, whereas whole worm homogenates had a negative D2 score (Figure 2A).

The loadings plot (Figure 2B) displays all GPSer molecular species as a dot, whose position shows the contribution of that species to D1 and D2. Molecular species that ended up close to the origin, did not contribute much to the separation of the three different types of membranes. In contrast, species further away from the origin were increasingly better (bio-)markers. Notably, the species showed to be organized along three vectors in the loadings plot (marked 1, 2 and 3 in Figure 2B). Each of these vectors pointed into a direction corresponding to the location of a specific membrane type in Figure 2A. Vector 1 thus identifies tegument-specific GPSer species, of which lysoGPSer(20:1) (*m/z* 550) was the most notable one, as it was furthest away from the origin along vector 1. Other tegument-specific species were the diradyl species with *m/z* 832, *m/z* 804 and *m/z* 830, and lysoGPSer(22:4) with *m/z* 572 (Figure 2B). The diradylGPSer species *m/z* 832 and *m/z* 830 are likely to contain an ether-linked acyl chain, because of their delayed elution time compared to diacylGPSer species with ester linked acyl chains. Therefore, the GPSer species composition in the tegumental membranes seemed to be enriched in ether-linked GPSer species, which is comparable to the already reported enrichment of ether-linked GPEtn species in the tegument [9].

Vector 2 identified whole worm specific GPSer species, of which GPSer(18:0/22:4) with *m/z* 838, located in the lower left hand corner of the loadings plot (Figure 2B), was the most notable one. Indeed, this was corroborated by the data in Table 1, which shows that GPSer(18:0/22:4) made up for 42.6% of the whole worm GPSer, whereas it made only up for 16.8% of the tegumental GPSer and as

little as 2.6% of hamster blood GPSer. In contrast, the molecular species GPSer(18:0/20:4) and GPSer(18:1/20:4) with *m/z* 810 and *m/z* 808 respectively, together accounted for 59% of hamster blood GPSer, but were found to be very minor compounds in schistosome derived GPSer (Table 1). This corresponded to the position of these two species in Figure 2B, where they were located at the end of vector 3, thus indicating to be hamster blood specific.

These results showed that, like previously reported on the species compositions of GPCho and GPEtn [9], the GPSer species composition in tegumental membranes differ substantially from that of entire adult worms as well as from that of blood cells. However, regarding the length and degree of unsaturation of the acyl chains, it were distinct species for GPCho, GPEtn and GPSer that were enriched in the tegument (Table 1 and supplementary data Table 1 and 2). Hence, the tegument should comprise enzyme machinery to achieve this tegument specific enrichment of distinct species in the different phospholipid classes.

Analysis of the species composition of GPIns showed that large differences exist between that of blood cells and that of the schistosomal preparations (see supplementory data, Table 3). Blood cells contained predominantly GPIns(18:0/20:4) and GPIns(18:1/20:4) with *m/z* ratios of 885 and 883, respectively. On the other hand, both schistosomal membrane preparations contained in addition to GPIns(18:0/20:4) a large amount of GPIns(18:0/18:1) with an *m/z* ratio of 863, which was virtually absent in hamster blood (Supplementary data, Table 3). When compared to the differences observed in species composition of GPCho, GPEtn and GPSer, the differences in GPIns species composition were remarkably small between tegumental membranes and those of total worms. GPIns species are well known to be precursors of potent signalling molecules, and the absence of specific enrichment of certain GPIns species in the tegument, suggests that any tegument-specific functions involving GPIns related signaling, does not require the presence or use of tegument-specific GPIns species.

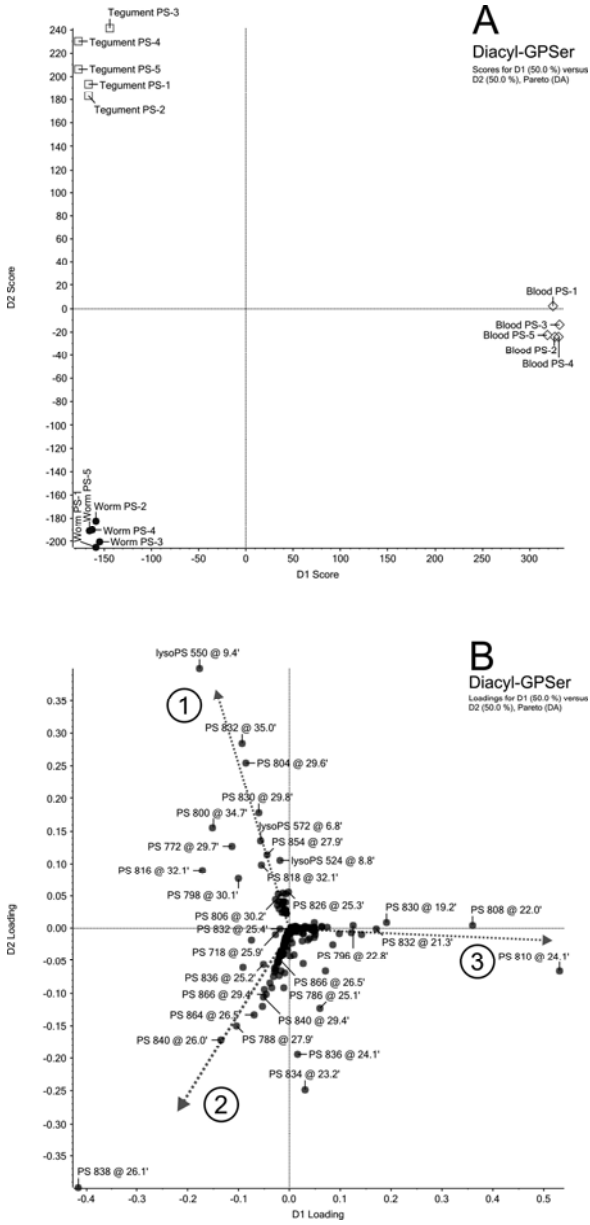

Figure 2. Discriminant analysis of the phosphatidylserine species composition. Molecular species were determined in homogenates of total *S. mansoni* worms, in isolated tegumental membranes from *S. mansoni* worms and in hamster blood. Panel A shows the scores plot of the discriminant analysis in which five independent preparations of the three different membrane types are plotted based on their values of the calculated discriminants D1 and D2. Panel B shows the loading plot, in which the contributions (loadings) of the more than 250 detected GPSer species to D1 and D2 are plotted. Arrows 1, 2 and 3 correspond to tegument specific, whole worms specific and hamster blood specific species respectively. GPSer species are labeled with their *m/z* ratio @ retention time, as listed in Table 1.

Next to analysis of the diacylphospholipid species, we also investigated the presence of lysophospholipid species of the above mentioned phospholipid classes, as it had been reported that both lysoGPCho and lysoGPSer species are important in the host parasite interaction [12,18]. Lysophospholipids (monoacylphospholipids) and diacylphospholipids do not ionize with the same efficiency, and therefore, these phospholipids are not detected with the same efficiency by mass spectrometry. For this reason, the molar ratio between lysophospholipids and diacylphospholipids cannot be estimated by mass spectrometry unless carefull calibration with a large array of standards is performed. However, the ratio of the detected signals of lysophospholipid species over that of diacylspecies can be compared between the distinct fractions in order to determine whether one of these fractions is relatively enriched in lysophospholipid species. Table 2 shows the ratio of detected signal derived from lysophospholipid species over that of diacylphospholipids for each phospholipid class in the three analysed membrane preparations. These results show that the tegumental membranes indeed comprise lysoGPCho species as earlier reported [18,19], but that the tegumental membranes are especially enriched in lysoGPSer species.

Table 2: Ratio of lysophospholipids over diacylphospholipids.

Molecular species were determined and quantified as described in the materials and methods in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood. Ratios shown are the average ratios of detected lysophospholipid species over that of diacylphospholipids species ± standard deviation of each phospholipid class in arbitrary units.

	Total worm ratio ± S.D. (n=5)	**Tegument ratio ± S.D. (n=4)**	**Hamster blood ratio ± S.D. (n=4)**
lysoGPSer / GPSer	100 ± 42	1833 ± 515	62 ± 84
lysoGPEtn / GPEtn	100 ± 106	775 ± 238	253 ± 54
lysoGPCho / GPCho	100 ± 87	96 ± 58	35 ± 28
lysoGPIns / GPIns	100 ± 11	398 ± 29	17 ± 22

Analysis of the lysophospholipid species of the distinct phospholipid classes demonstrated that in blood cells the species compositions of the

minor amounts of lysoGPCho, lysoGPEtn and lysoGPSer were very similar to each other, because over 75% of the lysophospholipid species in these classes comprised acyl chains with 16 or 18 carbon atoms and none or a single desaturation (not shown). On the other hand, the lysoGPIns species composition in blood also contained a large amount of lysoGPIns(20:4) in addition to the C16 and C18 species (not shown). Membranes of total worms comprised a lysophospholipid species composition that is also dominated by species containing saturated acyl chains of 16 or 18 carbon atoms (Table 3). However, schistosomal membrane preparations of total worms also contained significant amounts (over 20%) of lysoGPSer(20:1) and lysoGPEtn(20:1). Interestingly, these two lysophospholipid species were by far the most abundant lysoGPSer and lysoGPEtn species present in the tegumental membranes (Table 3). This eicosaenoic acid (20:1) is an abundantly present fatty acid in schistosomes, but virtually absent in the host [9,20]. Eicosaenoic acid (20:1) is synthesized by the parasite by chain elongation of oleic acid (18:1) [21]. On the other hand, the enrichment of eicosaenoic acid in lysoGPSer and lysoGPEtn in the tegument was not observed in lysoGPCho and lysoGPIns, as the species composition of these lysophospholipid classes was dominated by species containing saturated acyl chains of 16 or 18 carbon atoms (Table 3). Therefore, no major differences exist in the lysophopholipid species composition of GPCho and GPIns between tegumental membranes and membranes of total worms. On the other hand, the tegumental membranes were drastically enriched in eicosaenoic acid (20:1) containing lysoGPEtn and lysoGPSer species.

In conclusion, the tegumental outer-surface membranes of adult schistosomes, a unique biological structure which forms the site of interaction with the host, comprise many schistosome-specific and tegument-specific lipids. In addition to the already reported tegument-specific enrichment of several diacylGPCho and diacylGPEtn species [9], the present analysis showed that also multiple diacylGPSer species are specifically enriched in the tegument. On the other hand, no tegument-specific GPIns species could be detected. The species compositions of GPEtn and GPSer in tegumental membranes differ drastically from that of total worms. However, this phenomenon is not true for all phospholipid classes, as the species composition of GPIns in tegumental membranes did not differ from that of total worms. Furthermore, the

enrichment in the tegument of lysophospholipids containing eicosaenoic acid was only observed for the phospholipid classes GPSer and GPEtn and not for GPCho or GPIns. These results, therefore, suggest that the tegument contains specific enzymes that facilitate the enrichment of certain phospholipids in the tegumental membranes. The function of most of these tegument-specific lipids is not yet known, but lysoGPSer species of schistosomes activate toll-like receptor 2 on dendritic cells of the host, resulting in a down-regulation of the host immune response [8,12]. It is, therefore, likely that the schistosome-specific lipids enriched in the tegument, which forms the site of interaction with the host, are involved in yet unknown mechanisms employed by the parasite to manipulate its host in order to remain in the blood vessel for years.

Table 3: Molecular species of lysoglycerophospholipids.

Molecular species were determined in homogenates of *S. mansoni* worms and in isolated tegumental membranes from *S. mansoni* worms. LysoGPSer species were quantified by MRM with $[M-H]^-$ for MS1 and $[M-H-87]^-$ for MS2. LysoGPEtn species were quantified by MRM with $[M+H]^+$ for MS1 and $[M+H-141]^+$ for MS2. LysoGPCho species were quantified by MRM with $[M+H]^+$ for MS1 and *m/z* 184 for MS2. LysoGPIns species were quantified by MRM with $[M-H]^-$ for MS1 and *m/z* 241 for MS2. Percentages shown are the average percentages of the total detected lysophospholipid species ± standard deviation of species comprising more than 1% of the total lysophospholipids. Species marked by an * comprise less than 1.0% of the total lysophospholipids in that membrane preparation. Species containing an ether-linkage are indicated by the addition alk and the most abundant lysophospholipid species in each fraction are printed in bold.

	lysoGPSer % ± S.D.		**lysoGPEtn % ± S.D.**		**lysoGPCho % ± S.D.**		**lysoGPIns % ± S.D.**	
	Total worm (n=5)	Tegument (n=5)	Total worm (n=5)	Tegument (n=5)	Total worm (n=5)	Tegument (n=5)	Total worm (n=5)	Tegument (n=5)
16:0	1.7 ± 1.0	2.9 ± 1.9	7.2 ± 4.8	8.3 ± 3.4	**46.4 ± 12.4**	**53.5 ± 7.7**	1.1 ± 1.5	6.8 ± 2.0
18:1 alk	8.4 ± 2.2	4.4 ± 4.8	*	*	*	*	*	*
18:0 alk	*	*	*	*	*	*	5.4 ± 0.8	2.4 ± 2.8
18:2	*	*	*	*	1.4 ± 2.0	2.8 ± 1.9	*	5.3 ± 1.4
18:1	*	*	8.5 ± 4.3	12.4 ± 2.5	6.7 ± 3.8	7.9 ± 5.3	2.8 ± 2.8	13.6 ± 3.6
18:0	**51.9 ± 4.3**	11.3 ± 5.2	**62.8 ± 8.0**	20.7 ± 9.9	32.0 ± 2.5	26.6 ± 7.4	**85.1 ± 4.9**	**68.1 ± 7.7**
20:4	*	*	*	*	*	*	*	3.0 ± 1.9
20:2	*	*	*	*	2.1 ± 2.9	3.0 ± 1.9	*	*
20:1	26.3 ± 3.6	**65.6 ± 6.2**	21.5 ± 9.9	**57.8 ± 9.1**	8.2 ± 4.9	6.3 ± 4.9	*	1.4 ± 1.9
20:0	1.4 ± 1.0	1.5 ±1.4	*	*	*	*	*	*
22:4	7.8 ± 1.5	8.7 ± 0.7	*	*	*	*	*	*
22:1	*	1.4 ± 1.3	*	*	*	*	5.7 ± 1.5	*
24:1	*	*	*	*	1.4 ± 2.0	*	*	*

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Supplementary data chapter 3

Supplementary Table 1: Molecular species of phosphatidylethanolamine.
Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood. Species are quantified by MRM with $[M+H]^+$ for MS1 and $[M+H-141]^+$ for MS2. Percentages shown are the average percentages of the total GPEtn species ± standard deviation. The 10 most abundant species in each membrane preparation are shown, the three most abundant species in each fraction are printed in bold and species marked by an * comprise less than 0.5% of the total GPEtn of that membrane preparation. Attached acyl chains are given in total number of carbon atoms and unsaturations. Rt, retention time in minutes.

			Total worm (n=5)	**Tegument (n=3)**	**Hamster blood (n=5)**
m/z	**Acyls**	**Rt**	**% ± S.D.**	**% ± S.D.**	**% ± S.D.**
716	34:2	31.5	2.3 ± 0.31	2.7 ± 0.22	4.1 ± 0.15
718	34:1	37.2	3.7 ± 0.37	7.9 ± 1.12	**9.0 ± 0.57**
740	36:4	29.9	0.7 ± 0.08	0.7 ± 0.37	4.5 ± 0.08
742	36:3	31.9	2.3 ± 0.34	2.9 ± 0.71	**9.6 ± 0.24**
744	36:2	37.4	**9.4 ± 0.64**	**10.6 ± 1.76**	8.0 ± 0.22
746	36:1	45.3	**12.5 ± 0.67**	**8.4 ± 5.62**	4.1 ± 0.21
766	38:5	29.8	*	1.9 ± 0.49	**8.9 ± 0.51**
768	38:4	33.7	5.5 ± 0.32	5.3 ± 0.41	3.3 ± 0.18
768	38:4	35.4	2.1 ± 0.45	2.9 ± 0.33	5.2 ± 0.60
770	38:3	37.8	4.1 ± 0.10	5.4 ± 0.61	0.7 ± 0.05
772	38:2	45.5	6.0 ± 0.46	7.1 ± 0.78	*
788	40:8	30.4	*	*	4.3 ± 0.34
790	40:7	28.4	*	*	4.1 ± 0.40
792	40:6	32.7	4.1 ± 0.48	3.1 ± 0.34	1.4 ± 0.10
794	40:5	34.7	4.1 ± 0.49	5.2 ± 0.40	3.1 ± 0.25
796	40:4	40.4	**20.4 ± 1.17**	**16.1 ± 2.64**	0.9 ± 0.17
818	42:7	40.5	3.8 ± 0.40	2.7 ± 0.37	*

Supplementary Table 2: Molecular species of phosphatidylcholine.
Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood. Species are quantified by MRM with $[M+H]^+$ for MS1 and *m/z* 184 for MS2. Percentages shown are the average percentages of the total GPCho species ± standard deviation. The 10 most abundant species in each sample are shown, the three most abundant species in each fraction are printed in bold and species marked by an * comprise less than 0.5% of the total GPCho in that sample. Attached acyl-chains are given in total number of carbon atoms and unsaturations. Species containing an ether-linkage are indicated by the addition alk. Rt, retention time in minutes.

m/z	Acyls	Rt	Total worm (n=5) % ± S.D.	Tegument (n=3) % ± S.D.	Hamster blood (n=5) % ± S.D.
720	32:0alk	40.9	3.1 ± 0.21	3.8 ± 0.32	*
720	32:0alk	41.2	*	2.6 ± 2.11	*
734	32:0	35.69	2.2 ± 0.27	**17.9 ± 4.17**	7.4 ± 0.89
746	34:1alk	41.60	3.6 ± 0.26	2.9 ± 1.10	*
758	34:2	30.8	1.5 ± 0.13	4.9 ± 1.69	**25.2 ± 1.70**
760	34:1	35.8	3.0 ± 0.26	9.9 ± 1.43	**15.8 ± 0.89**
760	34:1	37.4	**4.2 ± 0.13**	**11.6 ± 2.71**	*
762	34:0	43.0	3.8 ± 0.16	1.9 ± 0.35	1.7 ± 0.12
782	36:4	29.2	3.1 ± 0.31	1.7 ± 0.26	5.6 ± 0.32
784	36:3	31.2	1.2 ± 0.90	1.2 ± 0.37	1.8 ± 1.14
786	36:2	36.5	**5.0 ± 2.52**	**23.2 ± 7.72**	**14.6 ± 0.67**
788	36:1	43.2	3.3 ± 0.29	6.2 ± 1.11	*
788	36:1	44.0	**5.3 ± 0.44**	*	2.7 ± 0.23
806	38:6	27.5	2.5 ± 0.40	2.2 ± 1.40	7.8 ± 1.32
810	38:4	34.3	3.3 ± 0.23	1.3 ± 0.05	
810	38:2	43.6	*	*	3.7 ± 0.39
814	38:2	44.2	3.1 ± 0.21	*	*
834	40:6	31.8	1.8 ± 0.85	1.0 ± 0.42	2.4 ± 0.23

Supplementary Table 3: Molecular species of diacylphosphatidylinositol (GPIns). Molecular species were determined in homogenates of adult *S. mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood. Species are quantified by MRM with $[M-H]^-$ for MS1 and *m/z* 241 for MS2. Percentages shown are the average percentages of the total GPIns species ± standard deviation. The three most abundant species in each sample are shown in bold and species marked by an * comprise less than 0.5% of the total GPIns. Attached acyl-chains are given in total number of carbon atoms and unsaturations. Species containing an ether-linkage are indicated by the addition alk. Rt, retention time in minutes, #, not identified.

			Total worm (n=5)	**Tegument (n=3)**	**Hamster blood (n=5)**
m/z	**Acyls**	**Rt**	**% ± S.D.**	**% ± S.D.**	**% ± S.D.**
795	#	25.4	*	1.3 ± 0.4	*
807	32:1	38.8	1.7 ± 1.18	*	*
823	#	29.0	*	0.7 ± 0.15	*
831	34:3	32.9	1.3 ± 0.93	*	*
833	34:2	21.8	2.3 ± 0.25	3.2 ± 0.71	8.3 ± 0.42
833	34:2	39.2	2.9 ± 2.03	*	*
835	34:1	23.8	*	3.8 ± 1.57	2.0 ± 0.30
837	34:0	27.6	2.0 ± 0.28	5.4 ± 0.94	*
847	36:2alk	27.0	0.9 ± 0.51	*	*
849	36:1alk	29.5	*	0.7 ± 0.13	*
857	36:4	20.1	*	*	5.5 ± 0.42
857	36:4	21.3	0.9 ± 0.11	0.9 ± 0.10	5.5 ± 0.42
859	36:3	22.2	1.3 ± 0.14	0.9 ± 1.12	2.3 ± 0.22
861	36:2	24.3	**19.1 ± 2.59**	**15.8 ± 0.56**	**13.3 ± 0.67**
863	36:1	27.3	**25.8 ± 1.99**	**28.2 ± 2.47**	0.9 ± 0.09
865	36:0	30.4		1.8 ± 0.23	*
865	36:0	32.2	0.9 ± 0.06	*	*
881	38:6	20.4	*	*	1.1 ± 0.10
883	38:5	21.4	*	0.5 ± 0.46	3.3 ± 0.34
883	38:5	21.9	*	*	**30.0 ± 0.10**
885	38:4	23.0	*	*	0.5 ± 0.14
885	38:4	23.6	**31.5 ± 1.49**	**22.4 ± 5.05**	**41.7 ± 2.27**
887	38:3	24.9	*	*	3.0 ± 0.11
887	38:3	25.5	2.4 ± 0.37	3.1 ± 0.85	0.6 ± 0.04
889	38:2	27.0	2.4 ± 0.14	3.1 ± 0.74	0.5 ± 0.13
891	38:1	31.0	1.6 ± 0.14	2.6 ± 0.35	*

909	40:6	22.6	*	06 ± 0.59	2.1 ± 0.27
911	40:5	23.4	*	0.9 ± 0.48	2.6 ± 0.18
911	40:5	24.5	*	*	0.6 ± 0.09
913	40:4	25.8	1.5 ± 0.15	1.2 ± 0.53	1.2 ± 0.15
943	42:3	24.2	*	1.0 ± 0.47	*
945	42:2	27.2	1.4 ± 0.23	2.3 ± 0.13	*

Chapter 4

Activation of Toll-like receptor 2 by synthetic and schistosomal lysophosphatidylserine

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in preparation

Abstract

Schistosome-specific lysophosphatidylserine (monoacylglycerophospho-serine, lysoGPSer) activates TLR2 and affect dendritic cells in such a way that mature dendritic cells gained the ability to induce the development of IL-10 producing regulatory T cells [1]. The present study shows that four synthesized lysoGPSer species (lysoGPSer(18:1), lysoGPSer(18:3), lysoGPSer(20:1) and lysoGPSer(24:1)) activate TLR2. However, different lysoGPSer species activate the TLR2 to various extends. Hydrogenized lysoGPSer species from *Schistosoma mansoni* eggs were fractionated and it was shown that lysoGPSer species with saturated acyl chains with 16 to 20 carbon atoms had very poor TLR2 activating capacity**.** Non-hydrogenized lysoGPSer species from *S. mansoni* eggs were also fractionated and we showed that long chain lysoGPSer species (22 carbon atoms and longer) have a potent TLR2 activating capacity. Furthermore, both the serine head group and the number of acyl chains are required as lysoGPCho and diacylGPSer have no TLR2 inducing capacity.

Introduction

Schistosomes are parasitic worms that cause schistosomiasis, a chronic disease that is associated with a Th2 response and during the chronic phase of infection the disease is also associated with enhanced IL-10 production and suppressed T cell proliferation against parasite and third party antigens [2-5]. The anti-inflammatory responses induced by parasitic worms seem to enable parasite survival within the host by limiting inflammatory responses that otherwise might be destructive to host tissues. This controlled immune response, central to chronic helminth infections, may arise from signals received from the pathogen.
Recognition of invading micro-organisms by cells of the immune system involves pathogen-associated molecular patterns (PAMPs) which bind specific receptors on the host cells. The Toll-like receptors (TLRs) form a family of pattern recognition receptors for PAMPs. Among TLRs, TLR2 is a unique receptor that recognizes lipid-and/or carbohydrate-containing components in cooperation with other TLRs. Via heterodimers with TLR1 or TLR6, TLR2 is able to recognize a highly diverse set of pathogen-associated motifs within the TLR family [6]. TLR2 recognizes a variety of bacterial components such as tri- and diacylated lipopeptides and peptidoglycans [7], but also components from fungi and parasites.
Several PAMPs have been shown to contain lipid moieties that are essential for the activation of TLR2. For example, the bacterial lipopeptides MALP-2 [8] and Pam_3CSK_4 [9] and the glycosyl-phosphatidylinositol (GPI) anchor from the protozoan parasite *Trypanosoma cruzi* and the lysophosphatidylinositol-anchored lipophosphoglycan (LPG) from the protozoan parasite *Leishmania major*, activate TLR2 [10,11]. Lipids are important factors in TLR2 activation, and thus can actively participate in triggering innate immune responses and the subsequent shaping of adaptive immune responses. The interaction of these lipid moieties with the immune system and the role they may play in immunopathologies or immune regulation are of great interest [12].
We previously showed that schistosome-specific lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) activated TLR2 and affected dendritic cells in such a way that mature dendritic cells gained the ability to induce the development of IL-10 producing regulatory T cells [1]. However, it is still unclear whether all lysoGPSer species have a TLR2

activating capacity or whether special features of the attached acyl chain are required.
Here, we show that four synthesized lysoGPSer species (lysoGPSer(18:1), lysoGPSer(18:3), lysoGPSer(20:1) and lysoGPSer-(24:1)) induced TLR2 activation in a dose dependent manner. We also show that the serine head group is essential for TLR2 activating capacity, and that diacylGPSer has no TLR2 activating capacity. Furthermore, we show that lysoGPSer species with saturated acyl chains with 16 to 20 carbon atoms have a very poor TLR2 activating capacity, whereas lysoGPSer species with acyl chains of at least 22 carbon atoms, have a potent TLR2 activating capacity, independent on saturation.

Material en methods

Reagents
Dioleoylglycerophosphocholine (GPCho(18:1/18:1)) was purchased from Sigma (St. Louis, MO, USA). DilinolenoylGPCho (GPCho(18:3/18:3)), dieicosenoylGPCho (GPCho(20:1/20:1)), dinervonoylGPCho (GPCho-(24:1/24:1)), 1-palmitoyl,2-oleoylGPSer (GPSer(16:0/18:1)), 1-palmitoyl,2-hydroxyGPCho (lysoGPCho(16:0)) and 1-oleoyl,2-hydroxy-GPSer (lysoGPSer(18:1)) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Phospholipase D (*Streptomyces* species) and phospholipase A_2 (bovine pancreas) were obtained from Sigma (St. Louis, MO, USA). Silica and prefab LiChrolut columns were from Merck (Darmstadt, Germany). Triethylaminoethyl cellulose was obtained from Serva (Heidelberg, Germany). All organic solvents were of HPLC grade and purchased from Lab-scan (Dublin, Ireland). All other chemicals were of analytical grade.

Synthesis of lipids
Lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) was synthesized from diacylglycerophosphocholine (GPCho). GPCho was converted to GPSer using phospholipase D according to Yamane *et al* [13]. In short, 5mg of GPCho was dissolved in 1 ml of chloroform, after which 25 mg of silica (kieselgel 60 for column chromatography) was added. Subsequently, the mixture was stirred for 30 min and carefully dried under a stream of nitrogen. Next, 1 ml of 100mM acetate buffer (pH 5.6) containing 100mM $CaCl_2$, 3.8M serine and 10 units of

phospholipase D enzyme (*Streptomyces* species) was added to the silica and the suspension was incubated for 24 to 36 hrs at 30°C while it was shaken continuously. The suspension was centrifuged at 120g for 10 min, after which the buffer was removed and the silica was washed once with 1 ml of 100mM Tris-HCl buffer (pH 7.4) containing 4mM $CaCl_2$. Subsequently, the silica was incubated with 1 ml of 100mM Tris-HCl buffer (pH 7.4) containing 4mM $CaCl_2$ and 10 units of phospholipase A_2 (bovine pancreas) for 3 hrs at 37°C while it was shaken continuously.A prefab LiChrolut RP-18 (40-63 µm) column was successively washed with 1 ml of methanol, 1 ml of water and 1 ml of 1M hydrochloric acid, after which 0.5 ml of the silica suspension was loaded on the column, immediately followed by 0.5 ml 1M hydrochloric acid. This procedure was repeated until all silica was loaded on to the column. Subsequently, the column was washed with 1 ml of 1M acetic acid, after which lysoGPSer was eluted in a plastic tube, with 1 ml of methanol. The methanol was evaporated under a stream of nitrogen and the samples were used within 24 hrs for LC-MS purification and analysis.
Monoacylglycerophosphocholine (lysoGPCho) and diacylglycerophosphoserine (GPSer) were synthesized as described above, but without the PLD incubation step or PLA_2 treatment, respectively.

Purification and quantification of synthesized lipids by LC-MS
The synthesized lysoGPSer species were purified, characterized and quantified by a LC-MS method as previously described (chapter 2). In short, lipids were separated by HPLC, using a Synergi 4 µm MAX-RP 18A column (250 x 3 mm) (Phenomenex, CA, USA). Elution was performed within 55 min with a decreasing linear gradient of water in methanol/acetonitrile, with serine and ammonium acetate as additives. Using a splitter, one third of the eluted solvent was infused in a mass spectrometer, whereas two thirds of the eluted volume was used to collect the synthesized lipid. Mass spectrometry was performed using electrospray ionization on a Sciex 4000QTRAP (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). Samples were analyzed in a multiple reaction mode (MRM) or by neutral loss or precursor scanning. For ionization details of the mass spectrometric analysis see chapter 2.
Collected lipid fractions were dried, dissolved in sodium sulfate dried methanol, and evaporated again under a stream of nitrogen. These lipid fractions were tested for TLR2 activation within 12 hrs after collection.

Purified lysoGPSer species, GPSer(18:1/18:1) and lysoGPCho(18:1) were quantified by comparison of the MRM peak areas with a calibration curve of commercially available lysoGPSer(18:1), GPSer(16:0/18:1) and lysoGPCho(16:0), respectively.

Toll-like receptor activation assay.
Human Embryonic Kidney (HEK) cell lines, stably transfected with either CD14 and TLR2 or with CD14 and TLR4 were maintained in DMEM medium supplemented with 10% FCS, 10 µg/ml ciprofloxacin and 5 µg/ml puromycine. The medium for HEK-CD14-TLR4 cells was also supplemented with supernatant of MD-2-transfected cells. For stimulation experiments, cells were seeded at 35 x 10^3 cells/well in 96-well flatbottom plates and were stimulated the next day with various concentrations of lipids dissolved in medium. After 22 hrs IL-8 production was measured in the supernatants using a commercial ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

LysoGPSer isolation from S. mansoni eggs
S. mansoni eggs were collected from livers of golden hamsters 45-48 days after infection. A lysoGPSer containing fraction was prepared from *S. mansoni* egg homogenates by the following procedure. Chloroform extraction in accordance with the method described by Bligh and Dyer [14] was used as pre purification step, whereby over 95% of the GPSer was removed. The water-methanol phase, containing over 90% of the lysoGPSers, was collected and solvents were evaporated in a rotor evaporator. The residue was dissolved in 1M hydrochloric acid, methanol and chloroform (2.4 : 7 : 2.5, v/v/v) and stirred for 30 min after which water and chloroform (2.5 : 2, v/v) were added. The mixture was stirred for 10 min and centrifuged at 1000g for 5 min at room temperature, after which the chloroform phase was collected. Because of the presence of hydrochloric acid, the chloroform phase now contains the lysoGPSers. The remaining acidic water-methanol phase was washed with 2 ml chloroform and centrifuged at 1000g for 5 min, at room temperature, after which the chloroform phase was again collected. The combined chloroform extracts were dried in a rotor evaporator, dissolved in chloroform and the lipids were separated in various classes by using triethylaminoethyl cellulose column chromatography, as described by

Rouser *et al*. [15]. LysoGPSer was eluted from the coloumn with glacial acetic acid.

Hydrogenation, separation and identification of schistosomal lysoGPSer
Where indicated, the schistosomal lysoGPSer species were dissolved in water and methanol (1 : 1, v/v) and hydrogenated by hydrogen gas in the presence of platinum (IV) oxide for 8 hrs. Subsequently, liquid chromatography coupled to mass spectrometry was used to separate and identify the schistosomal lysoGPSer species (as described above). At the same time, by using a splitter, 5 min fractions were collected to test for TLR2 activating capacity.

Results and discussion

Toll-like receptor 2 stimulating activity of synthetic lyso-phosphatidylserine (monoacylglycerophosphoserine, lysoGPSer)
Several lysoGPSer species were synthesized from diacylglycerol-phosphatidylcholine (GPCho) species by phospholipase D (PLD) treatment in the presence of serine, followed by treatment of the resulting diacylglycerophosphoserine (GPSer) with phospholipase A_2 (PLA_2). The synthesized lipids were purified and identified by LC-MS. Identity was confirmed by multiple reaction monitoring (MRM) scanning. To analyze the TLR2 stimulating capacity of the synthesized lysoGPSer species, these compounds were incubated with human embryonic kidney (HEK) cell-lines stably transfected with CD14 and TLR2, by monitoring IL-8 production in the supernatants (Figure 1). The synthesized lysoGPSer(18:1), lysoGPSer(18:3), lysoGPSer(20:1) and lysoGPSer-(24:1) all appeared to activate TLR2 (Figure 1, panel A-D, respectively), since all these lysoGPSer species induced IL-8 production in TLR2 transfected cells in a dose dependent manner. It can be seen that the shape of the dose- response curves of lysoGPSer(18:1) and lysoGPSer(18:3) is different compared to the shape of the dose-response curves of lysoGPSer(20:1) and lysoGPSer(24:1). This might be due to, for example, a different binding pocket in the TLR2, or to a yet unknown variability in the experiments.
To investigate whether the IL-8 production of the cells upon incubation with lysoGPSer was a specific reaction of these cells on the stimulation of the TLR2, we incubated a new preparation of lysoGPSer(24:1) not

only with HEK cells stably transfected with CD14 and TLR2, but also with HEK cells stably transfected with CD14 and TLR4. As expected, lysoGPSer(24:1) induced IL-8 production in cells transfected with TLR2. However, no significant IL-8 production was induced in cells transfected with TLR4 (Figure 2). The induced IL-8 production must therefore be the result of a specific activation of the transfected TLR2 receptors and not of an aspecific reaction of the cells to lysoGPSer, since the same cell-line was used to generate both stably transfected cell-lines.

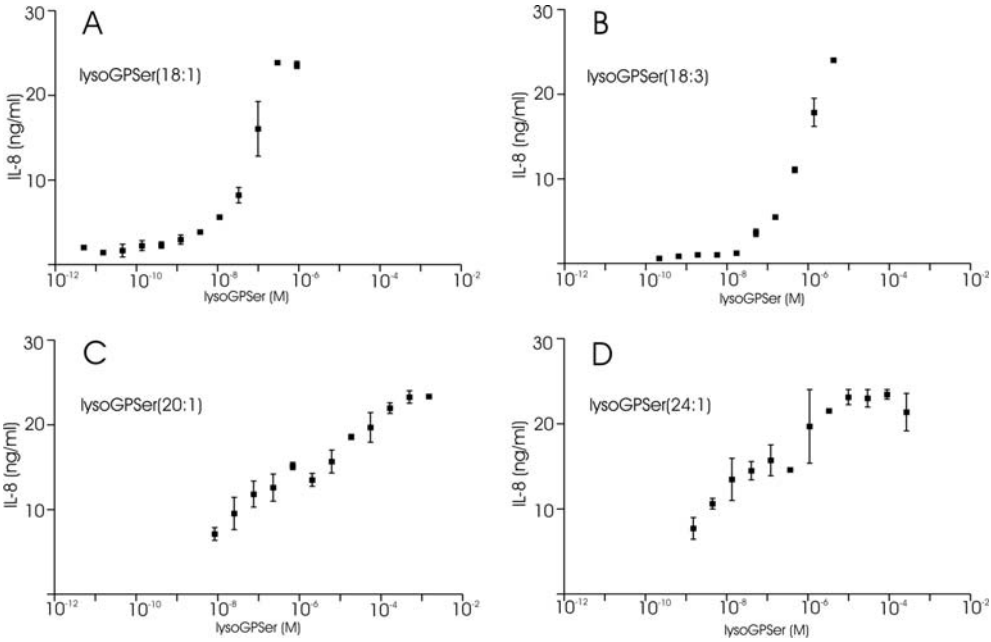

Figure 1: Activation of Toll-like receptor 2 (TLR2) by distinct synthetic lysoGPSer species. Shown is the IL-8 production by HEK 293 cells transfected with CD14 and human TLR2, that were stimulated for 22 hours with the indicated amounts of synthetic lysoGPSer(18:1) (panel A), synthetic lysoGPSer(18:3) (panel B), synthetic lysoGPSer-(20:1) (panel C) and synthetic lysoGPSer(24:1) (panel D).

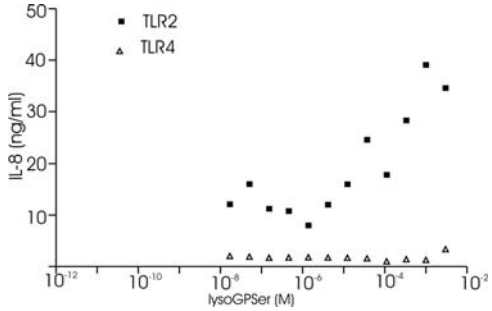

Figure 2: Stimulation of Toll-like receptor 2 (TLR2) and TLR4 by synthetic lysoGPSer(24:1). Shown is the IL-8 production by HEK 293 cells transfected with CD14 along with either human TLR2 or human TLR4, that were stimulated for 22 hours with the indicated amounts of synthetic lysoGPSer(24:1).

To investigate which parts of the lysoGPSer are required for TLR2 activation, a new batch of GPSer(18:1) was synthesized. This batch activated TLR2 (Figure 3A), which confirms the result of Figure 1. Parallel to the synthesis of lysoGPSer(18:1) a negative control was prepared. In this case the same method using phospholipase D and A_2 (see material and methods section) was used, however no GPCho(18:1/18:1) or any other lipid substrate was added. The negative control was further treated identical to the synthesized lysoGPSer(18:1) sample during purification. The absence of lysoGPSer(18:1) in the sample was confirmed by LC-MS. This negative control was tested for TLR2 stimulating capacity and no significant IL-8 production in TLR2 transfected was detected (Figure 3B). Therefore, TLR2 activation is specific for the synthesized lysoGPSer species, as other possible TLR2 activating components in our lipid samples resulting from the synthetic procedure can be excluded.
In addition to the synthesis of lysoGPSer(18:1), GPSer(18:1/18:1) was synthesized from GPCho(18:1/18:1) by phospholipase D treatment in the presence of serine. LysoGPCho(18:1) was synthesized from GPCho(18:1/18:1) by phospholipase A_2 treatment. Both lipids were purified by a prefab LiChrolut column and subsequently by LC-MS, see material and methods for details. Upon incubation of TLR2-transfected cells with either GPSer(18:1/18:1) or lysoGPCho(18:1) no significant IL-8 production was detected (Figure 3, panel C and D, respectively). Since

neither of the two show TLR2 inducing capacity in the same concentration range as lysoGPSer(18:1), it seems that the number of acyl chains and serine head group both are essential for the TLR2 activating capacity of lysoGPSer(18:1).
The concentration range in which the lysoGPSer(18:1) activates TLR2, varies between experiments (Figure 1, panel A and Figure 3, panel A). This indicates that the lysoGPSer might be unstable. Differences might therefore be due to differences in storage time and conditions prior to incubation of the cells. Also the differences in the shape of the two curves of lysoGPSer (24:1) (Figure 1, panel D and Figure 2) could be due to variability in the experiments.

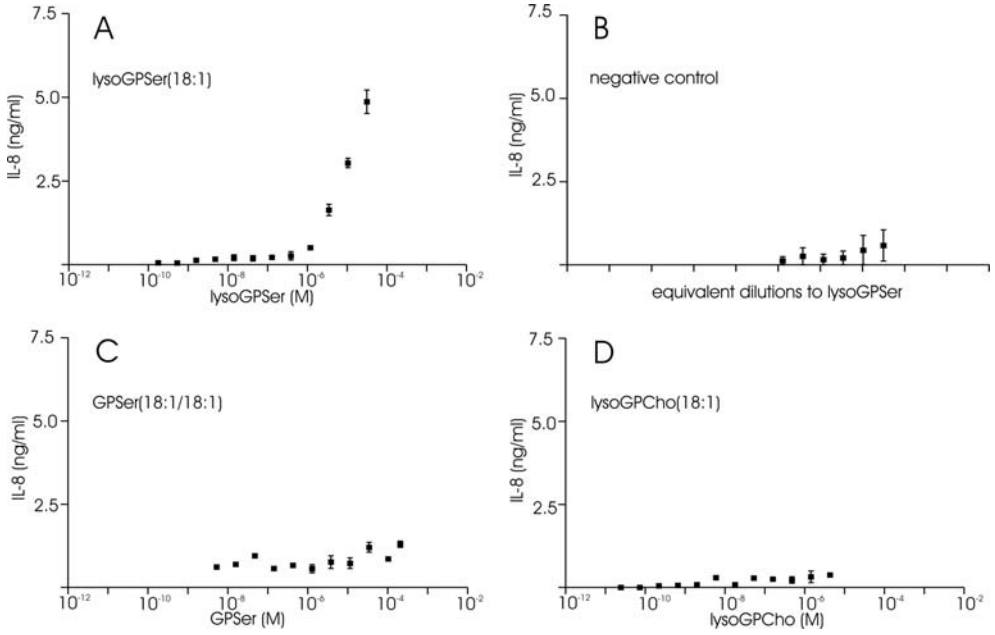

Figure 3: Activation of Toll-like receptor 2 by synthetic phospholipids. Shown is the IL-8 production by HEK 293 cells transfected with CD14 and human TLR2, that were stimulated for 22 hours with the indicated amounts of synthetic lysoGPSer(18:1) (panel A), a negative control sample (panel B) for possible activating compounds derived from the biosynthetic process was used in the same dilutions as the synthetic lysoGPSer (panel A), synthetic GPSer(18:1/18:1) (panel C), and synthetic lysoGPCho(18:1) (panel D).

Structural analysis of lysoGPSer species derived from *S. mansoni* eggs in relation to toll-like receptor 2 activation

We earlier reported that lysoGPSer-containing fractions of both eggs and adult worms of the parasite *Schistosoma mansoni* contain TLR2 stimulating capacity [1]. To investigate which of these natural occurring lysoGPSer species in *S. mansoni* are most active in TLR2 stimulation, we further fractionated the schistosomal lysoGPSer-containing preparation and tested these fractions for TLR2 activating capacity (Figure 4). Liquid chromatography coupled to mass spectrometry was used to separate and identify the lysoGPSer species (Figure 4, panel A-C). At the same time, by using a splitter, 5-min fractions were collected to test their TLR2 activating capacity (Figure 4, panel D). LysoGPSer species with long saturated acyl chains elute later from the LC column than lysoGPSer species with short saturated acyl chains. For instance, lysoGPSer(22:0) elutes after approximately 18 min, whereas lysoGPSer(20:0) elutes after approximately 13.5 min. However, every double bond (unsaturation) in an acyl chain results in a shorter elution time of the lysoGPSer species. For instance, lysoGPSer(22:2) elutes after approximately 11 min whereas lysoGPSer(22:1) elutes after approximately 14.5 min. Therefore, the different fractions contain a mixture of lysoGPSer species. For example, the 10 to 15 min fraction derived from *S. mansoni* eggs contains mainly lysoGPSer(18:0) but also, among others, lysoGPSer(20:1) and lysoGPSer(22:2) (panel C). It appeared that the first 5 collected fractions activated TLR2 (Figure 4, panel D). The TLR2 activating species present in the fraction collected from 25 to 30 min were below the detection limit of the mass spectrometry in this sample.

To further investigate the structural requirements of the attached acyl chain of the lysoGPSer species, the double bonds in the attached acyl chain were reduced by hydrogenation. The resulting saturated lysoGPSer species were separated and identified by LC-MS, after which the TLR2 activating capacity of the collected fractions was analyzed (Figure 4, panel E-H). The LC chromatogram before and after saturation are comparable, since both samples predominantly contain the saturated lysoGPSer(18:0), which elutes at approximately 10 min. However, the amount of lysoGPSer(20:0), which elutes around 14 min, clearly increased upon hydrogenation (Figure 4, panel A & E). Saturation of the double bonds in lysoGPSer species by hydrogenation was confirmed by mass spectrometry. For example, lysoGPSer(24:1) with an m/z of 606 is

present before (panel B), but not after hydrogenation, since it is reduced to lysoGPSer(24:0) with an m/z of 608 (panel F). Also lysoGPSer(20:1) with an m/z of 550 is present before (panel B), but not after hydrogenation, since it is reduced to lysoGPSer(20:0) with an m/z of 552 (panel F). The hydrogenation resulted in the expected shift of the eluted lysoGPSer species. For example, unsaturated species with a chain length of 26 carbon atoms, were originally detected in the 15-20 and 20-25 min fraction, but after hydrogenation these species were detected in the 25-30 min fraction (Figure 4, panel C&G). Hydrogenation resulted in a significant shift in the elution pattern of TLR2 activating components (Figure 4, panel D&H). The TLR2 activating capacity of the first 2 fractions (collected between 5 and 15 min) was strongly reduced after hydrogenation, whereas that of the last fraction (30-35 min) was strongly increased after hydrogenation. Although the first two fractions of the hydrogenated sample contained over 90% of the saturated lysoGPSer species (panel E), it induced almost no TLR2 activation (panel H). Since these two fractions only contained saturated lysoGPSer species with acyl chains ranging from 16 to 20 carbon atoms (panel G), it can be concluded that lysoGPSer species with saturated acyl chains with 16 to 20 carbon atoms have a very poor TLR2 activating capacity.

On the other hand, the fractions collected between 15 and 35 min, with saturated lysoGPSer species with acyl chains of 22 carbon atoms and longer did contain TLR2-activating capacity (Figure 4, panel D), which demonstrates that these long chain lysoGPSer species have a potent capacity to activate TLR2. The first two fractions of non-hydrogenated schistosomal lysoGPSer species, collected between 5 and 15 min, contained lysoGPSer species with saturated as well as unsaturated acyl chains. The saturated lysoGPSer species in this fraction have acyl chains containing 16 to 20 carbon atoms, were shown to be incapable of TLR2 activation (see above). Therefore, TLR2 activation must be the result of unsaturated lysoGPSer species that are present in these two fractions, such as lysoGPSer(18:1), lysoGPSer(20:1), lysoGPSer(22:2) and lysoGPSer (24:4). Hence, these lysoGPSer species with unsaturated acyl chains with 18 carbon atoms or longer, have a good TLR2 activating capacity. This is in agreement with the observed TLR2 activating capacity of the synthesized lysoGPSer species lysoGPSer(18:1), lysoGPSer(18:3), lysoGPSer(20:1) and lysoGPSer(24:1) (Figure 1).

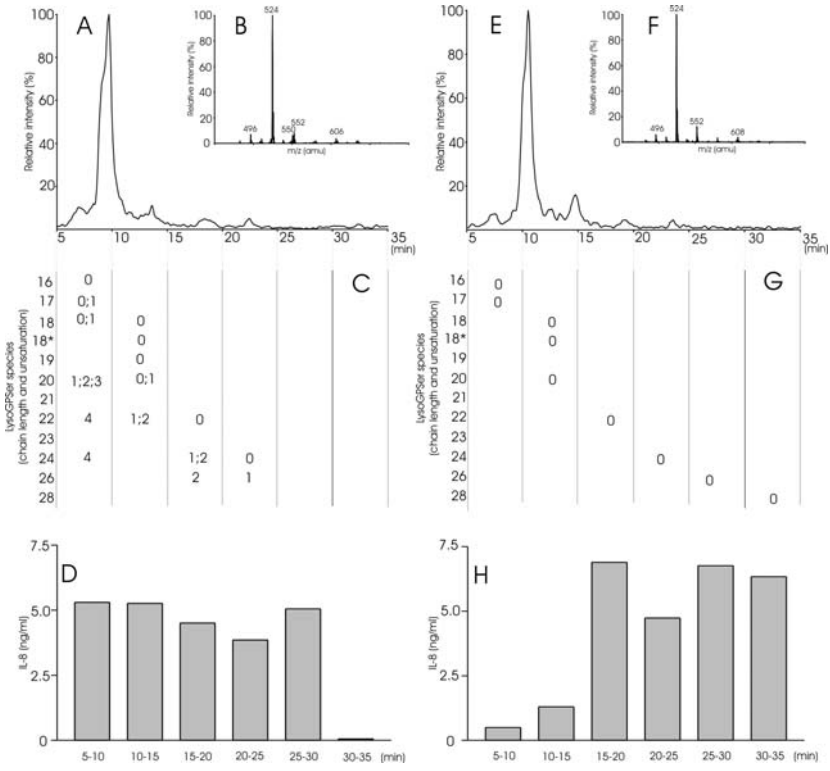

Figure 4: Structural analysis of lysoGPSer species of *S. mansoni* eggs in relation to Toll-like receptor 2 activation. LysoGPSer species of *S. mansoni* eggs, were separated and analysed by LC-MS and at the same time eluting lipids were collected in 5-min fractions (see material and methods). Neutral loss scans of 87 amu, corresponding to the loss of serine head group from the phospholipid, of an untreated lysoGPSer sample of *S. mansoni* eggs are shown in panel A and B, and scans of a hydrogenized lysoGPSer sample of *S. mansoni* eggs are shown in panel E and F. The total ion counts (TIC) during elution of schistosomal egg lysoGPSer species and that of hydrogenated lysoGPSer species are shown in panel A and E, respectively. The mass/charge ratios of the detected molecular species in panel A and E, are shown in panel B and F, respectively. Panel C and G show the composition of the lysoGPSer species detected in the corresponding 5-min fractions. The acyl chain of all *S. mansoni* lysoGPSer species in Panel C and G is linked to the glycerol back-bone via an ester bond, except for 18* which is linked via an ether bond. HEK 293 cells, transfected with CD14 and human TLR2, were stimulated with corresponding 5-min fractions from untreated or hydrogenated schistosomal egg fractions and the resulting IL-8 production is shown in panel D and H, respectively.

The abundance of lysoGPSer species seems unique for *S. mansoni*, since no lysoGPSer species could be detected in mammalian liver or mammalian blood [1]. Further more, we show here that *S. mansoni* eggs contain lysoGPSer species with uncommon long and polyunsaturated fatty acids. Also other lysoglycerophospholipids are abundant in *S. mansoni* like for example lysoglycerophosphor-ethanolamine, however, lysopglycerophosphoinositol and lysoGPCho are less abundantly present [chapter 3]. We previously showed that also the diacylglycerophospholipid species of *S. mansoni* contain long and unsaturated fatty acid acyl chains [16,17]. Long acyl chains of 22 or more carbon atoms are significantly more abundant in *S. mansoni* compared to mammalian liver or mammalian blood and this is especially the case in GPSer [chapter 2,chapter 3].
In conclusion, we show here that *S. mansoni* eggs possesses unique lysoGPSer species that contain TLR2 activating capacity. However, not all lysoGPSer species contain a significant TLR2 activating capacity. In short, lysoGPSer species with saturated acyl chains with 16 to 20 carbon atoms have a very poor TLR2 activating capacity, whereas lysoGPSer species with acyl chains with 22 or more carbon atoms have a potent TLR2 activating capacity. LysoGPSer species with unsaturated acyl chains with 18 carbon atoms or longer, also have a good TLR2 activating capacity. These conclusions were confirmed by the significant TLR2 activating capacity of four synthesized lysoGPSer species.

Acknowledgements

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Chapter 5

Immunologic activity of schistosomal, bacterial and synthetic TLR2 ligands in Gabonese children

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submitted

Summary

The effect of two Toll-like receptor 2 (TLR2) activating schistosomal lipid fractions on the immune system of Gabonese children living in an area endemic for schistosomiasis was studied. One of the lipid fractions contained lysophosphatidylserine (monoacylglycerophosphoserine, lyso-GPSer) plus diacylphosphatidylserine (diacylglycerophosphoserine, GPSer) while the other one contained lysoGPSer and only a trace of GPSer. The effect of these schistosomal lipid fractions was compared with the known TLR2 ligands PAM_3CSK_4 and MALP-2. PAM_3CSK_4 and MALP-2 had preferential IL-10 activating capacities, while the fraction containing lysoGPSer plus GPSer had a strong TNF-α inducing capacity. The fraction containing lysoGPSer was neutral with respect to pro- versus anti-inflammatory effects. When the Th1 and Th2 cytokines were analysed, the schistosomal lipid fraction containing lysoGPSer plus GPSer showed a stronger Th2 response compared to PAM_3CSK_4, MALP-2 and lysoGPSer alone.

Introduction

Toll-like receptors (TLR) form a family of pattern recognition receptors for pathogen-associated molecular patterns (PAMPs). TLR2 is essential for the signaling in response to a range of PAMPs, including a variety of bacterial components such as tri- and diacylated lipopeptides and peptidoglycans [1]. This receptor also recognizes components from fungi and parasites. TLR2 forms heterodimers with TLR1 and TLR6 for the recognition of different ligands. More specifically, diacylated MALP-2, a lipopeptide of *Mycoplasma fermentans*, acts via TLR2/TLR6 heterodimers [2], while the bacterial lipopeptide PAM_3CSK_4 and other triacylated lipopeptides act via TLR2/TLR1 heterodimers [3]. The lipid moieties of these bacterial molecules are essential for the activation of the TLRs [4]. Lipids can thus actively participate in triggering innate immune responses and very likely the subsequent shaping of adaptive immune responses. The interaction of lipids with the immune system and the role they may play in immunopathologies or immune regulation are of great interest. The Platelet Activating Factor (PAF) receptor, for example, induces the production of anti-inflammatory mediators when activated by PAF or oxidized diacylglycerophosphatidylcholine (GPCho) [5]. Monoacylglycerophosphatidylcholine (lysoGPCho) has been shown to affect immune response generation, via interaction with its G-protein coupled receptor (GPCR) on differentiating monocytes, to generate mature dendritic cells with the ability to stimulate IL-2 and IFN-γ production by allogeneic T lymphocytes [6,7]. Also the *Schistosoma* parasite contains several PAMPs, such as pseudo-Lewis^Y glycolipids, which bind to C-type lectins [8], or GPSer containing fractions, which activate TLR2. It has been shown that a schistosomal GPSer fraction can modulate dendritic cells to induce Th2 as well as regulatory T cell responses [9]

TLR signaling is mostly associated with a Th1 response, characterized by Th1 related cytokines such as IFN-γ. However, helminths are known to signal through TLRs and yet induce Th2-skewed immune responses, characterized by production of the cytokines IL-4, IL-5 and IL-13 [10]. Helminths can also induce regulatory T cell responses or lead to elevated anti-inflammatory cytokines such as IL-10 and TGF-β at the chronic phase of infection. It is thought that this immune down regulation may protect the host from excessive pro-inflammatory responses that, if unchecked, may lead to organ damage.

We previously reported that a fraction containing diacylphosphatidylserine (diacylglycerophosphatidylserine, GPSer) plus lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) of *S. mansoni* worms can stimulate peripheral blood mononuclear cells (PBMC) of children living in areas where schistosomiasis is endemic [11]. However, to distinguish the responses of the two components, the GPSer and the lysoGPSer, of which the latter is known to activate dendritic cells via TLR2, we developed a method to obtain a lipid fraction containing lysoGPSer and only a trace of GPSer. We tested this fraction containing lysoGPSer and the other fraction containing lysoGPSer plus GPSer immunological activity in school children from areas where schistosomiasis is endemic. We stimulated whole blood cultures and analysed the cytokine profiles in response to these schistosomal lipids and compared them with cytokine profiles of two known TLR2 ligands.

Materials and methods

Study population
Lambaréné, Gabon, is an area with little urbanisation, high vaccine coverage and high prevalence of helminth infections. This study presents data from schoolchildren attending two schools in the area of Lambaréné (Table 1). One school (PK15) is located in a rural area with no paved roads, located 15 km from the Albert Schweitzer Hospital. The other school (Lalala) is located in semi-urban Lambaréné close to the Albert Schweitzer Hospital.

Table 1: Characteristics of the participating schoolchildren in the study, from a semi urban area and a rural area in Gabon.

Characteristic	**Semi-urban**	**Rural**
N	34	37
Age, mean (range), years	10.8 (8-13)	10.3 (5-14)
Sex, M:F	17:17	19:18
S. haematobium infected (%)	29	73
Infected with intestinal helminths (%)	35	46
Infected with malaria (%)	6	27

The study has been approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné, Gabon. Written, informed consent was obtained from parents or guardians of the children participating in the present study.

Lipid isolation

S. mansoni adult worms were collected by perfusion of golden hamsters 45-48 days after infection. Lipid containing fractions were made from *S. mansoni* adult worms as described before [12]. In brief, a lipid extract was prepared using chloroform extraction in accordance with the method described by Bligh and Dyer [13]. Lipids were separated into different fractions by use of triethylaminoethyl cellulose column chromatography as described by Rouser *et al*. [14], which resulted in 8 fractions containing various lipid classes. One of these fractions was the lysoGPSer plus GPSer containing fraction. In addition, a fraction containing lysoGPSer and only a trace of GPSer was made from the same homogenate of adult *S. mansoni* worms. In brief, the remaining water-methanol phase after the chloroform extraction according to Bligh and Dyer (described above), was collected and solvents were evaporated. The residue was dissolved in 1M hydrochloric acid, methanol and chloroform (2.4 : 7 : 2.5, v/v/v). The mixture was stirred for 30 minutes, after which water and chloroform (2.5 : 2, v/v) were added. The mixture was stirred for 10 minutes and centrifuged at 1000g for 5 min, at room temperature. The chloroform phase was collected. The remaining acidic methanol phase was washed with 2 ml chloroform and centrifuged at 1000g for 5 min, at room temperature, after which the chloroform phase was again collected. The lipids from the combined chloroform phases were separated into different fractions by use of triethylaminoethyl cellulose column chromatography, as described above. One of the fractions was the lysoGPSer fraction. Mass spectrometry was used to confirm the presence of GPSer and lysoGPSer in the two GPSer fractions. In short, lipids were separated by HPLC using a Synergi 4 µm MAX-RP 18A column (250 x 3 mm) (Phenomenex, CA, USA). Elution was performed within 55 minutes with a decreasing linear gradient of water in methanol/acetonitrile, with serine and ammoniumacetate used as additives. Mass spectrometry was performed using electrospray ionization on a Sciex 400QTRAP (Applied Biosystems, Nieuwerkerk aan de IJsel, The Netherlands. For further details see chapter 2.

Cell lines
HEK-CD14-TLR2 and HEK-CD14-TLR4 cell lines (a gift from Dr. E. Latz, University of Massachusetts, USA) were maintained in DMEM medium supplemented with 10% FCS, 10 µg/ml ciprofloxacin and 5 µg/ml puromycine. For stimulation experiments, cells were seeded at 35 x 10^3 cells/well in 96-well flatbottom plates and were stimulated the next day with pure LPS (*E. coli*, Sigma, St Louis, USA), Pam_3CSK_4 (EMC microcollections GmbH, Tübingen, Germany, 12.5 ng/ml final concentration), MALP-2 (EMC microcollections GmbH, Tübingen, Germany, 12.5 ng/ml final concentration) or schistosomal lipids (derived from approximately 1, 2.5 or 6.25 worm pairs). A 8 fold diluted supernatant of MD-2-transfected cells was added for stimulation of HEK-CD14-TLR4 cells. IL-8 production was measured in supernatants after 22h using a commercial ELISA kit (Sanquin, Amsterdam, The Netherlands) following the manufacturer's recommendations.

Whole blood culture
Four milliliters of venous blood was obtained in heparin-coated tubes (BD Vacutainer Systems, Plymouth, UK). The blood was aseptically distributed, 100 µl per well, in duplicate 96-well tissue culture plates (NUNC, Roskilde, Denmark). Blood cells were incubated with stimulating agents dissolved in 100 µl RPMI-1640 medium (Invitrogen, Breda, The Netherlands). *S. mansoni* GPSer containing lipid fractions were dissolved in RPMI and added to the blood cell cultures at such a concentration that each incubation contained lipids derived from approximately one worm pair. In addition, Pam_3CSK_4 (100 ng/ml final concentration), MALP-2 (12.5 ng/ml final concentration) and medium only, were also used to stimulate blood cell cultures. After 24 and 72 hours, culture supernatants (150 µl per well) were collected and stored at -20 °C. For further analysis, supernatants were transported in frozen condition to Leiden, The Netherlands.

Cytokine measurements in whole blood cultures
Concentrations of the cytokines IFN-γ, IL-5, TNF-α and IL-10 were determined simultaneously in the supernatants by using the Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA), equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK). Buffer reagent kits and Luminex cytokine kits (BioSource, Camarillo, CA, USA) were used and cytokines were measured according to the

manufacturers protocol, with minor modifications. Briefly, assays were performed in 96-well roundbottom plates (Nunc, Roskilde, Germany) at room temperature. A mix of beads was incubated in the dark under continuous shaking for 2 hours with standards, samples, or blanks in a final volume of 50 µl. Subsequently, plates were washed twice and incubated with a cocktail of biotinylated antibodies (25 µl/well) for 1 hour. After removal of excess biotinylated antibodies by another washing step, Streptavidin-RPE was added and incubated for 30 minutes. Subsequently plates were washed a final time and analysed using the Luminex-100 cytometer. Concentrations of the cytokines IFN-γ and IL-5 were determined in plasma supernatants collected after 72 hours and concentrations of the cytokines TNF-α and IL-10 were determined in supernatants collected after 24 hours. The lower detection limit of the assays was 3 pg/ml, 5 pg/ml, 5 pg/ml and 10 pg/ml for IL-5, IL-10, IFN-γ and TNF-α, respectively. Samples with concentrations below the detection limit were given the value of this threshold.

Statistical analysis
One-way ANOVA with Bonferroni's Multiple Comparison Test post test on paired samples and the calculation of geomeans were performed by using GraphPad Prism (GraphPad Software, San Diego, California, USA.)

Results

Preparation of schistosomal phosphatidylserine fractions

The preparation of lipid fractions from schistosome adult worms resulted in two lysophosphatidylserine (monoacylglycerophosphoserine, lyso-GPSer) preparations, one containing lysoGPSer plus diacylphosphatidyl-serine (diacylglycerophosphoserine, GPSer) and one containing lysoGPSer with only a trace of GPSer, as confirmed by MS (Figure 1). Liquid chromatography coupled to mass spectrometry was used to separate and identify the GPSer species present in each of the two prepared samples (chapter 2). Panel A and panel B show the total ion count (TIC) of the LC separation of lysoGPSer plus GPSer and of the fraction containing lysoGPSer with only a trace GPSer, respectively. LysoGPSer species elute approximately between 5 and 20 minutes, while GPSer species elute approximately between 25 and 40 minutes (Figure 1). Panel C and panel D show the masses of the detected GPSer species

in the isolated fractions. LysoGPSer species have masses from 500 to 650 amu, depending on the attached acyl chain, and the most abundant species were lysoGPSer(20:1), lysoGPSer(22:4), lysoGPSer(22:1) and lysoGPSer(18:0). GPSer species have masses from 750 to 900 amu and the most abundant species were GPSer(18:0/22:4), GPSer(18:0/20:4), GPSer(20:1/20:4) and GPSer (18:0/18:1). The lysoGPSer fraction contains a very small amount of GPSer species and only the species which are abundant in the other fraction, such as GPSer(18:0/22:4) and GPSer(18:0/18:1) could be detected.

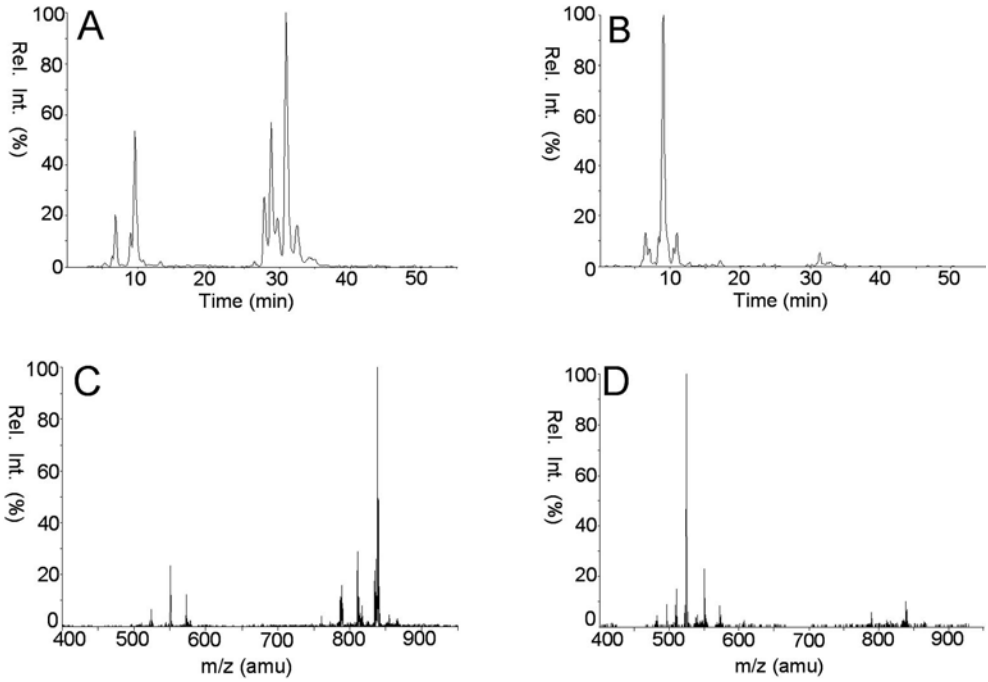

Figure 1: Structural analysis of schistosomal preparations. Samples were analyzed by LC/MSMS in the negative mode. Neutral loss scans of 87 amu, corresponding to the loss of serine from the phospholipid are shown. Panel A and B show the TIC (total ion count) of the separated GPSer components in the schistosomal GPSer plus lysoGPSer fraction and the TIC of the schistosomal lysoGPSer fraction with only a trace of GPSer, respectively. The mass/charge ration of the detected molecular species in panel A and B is shown in panel C and D, respectively.

Toll-like receptor-2 stimulating activity

To determine the TLR2 activity of the lysoGPSer fraction, we stimulated human embryonic kidney (HEK) cell-lines, which were stably transfected with either TLR2 or TLR4, and monitored IL-8 production in the supernatants (Figure 2). The commercially available Pam_3CSK_4 and MALP-2 stimulate the TLR2 transfected cells, whereas the commercially available LPS stimulates the TLR4 transfected cells (Figure 2A). Both the fraction containing lysoGPSer plus GPSer as well as the lysoGPSer fraction activated TLR2 (Figure 2B), which is in agreement with our previous findings [9]. Neither of the two fractions activated TLR4, excluding the possibility of endotoxin contamination.

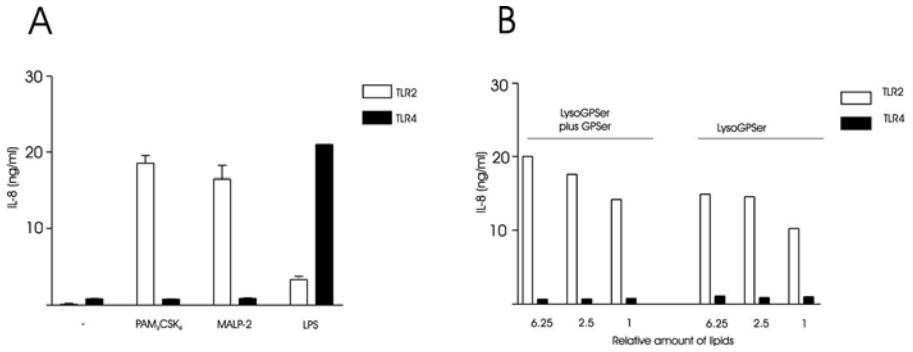

Figure 2: Activation of TLR2 by schistosomal GPSer. HEK 293 cells were transfected with CD14 and human TLR2 or TLR4. (A) Cells were stimulated with Pam_3CSK_4, MALP-2 and LPS. (B) Cells were stimulated with lysoGPSer plus GPSer and cells were stimulated with lysoGPSer (derived from an extraction of approximately 1 to 6.25 worm pairs).

Cytokine induction by TLR2 ligands

To study the differences in effect of exposure to lysoGPSer plus GPSer, compared to a lysoGPSer fraction, we examined the cytokine responses of Gabonese school children to these two fractions. These effects were compared with the effects of the known TLR2 ligands, Pam_3CSK_4 and MALP-2.

Whole blood was cultured and stimulated with the TLR2 ligands Pam_3CSK_4, MALP-2, and with the schistosomal GPSer fractions. As shown in Figure 3, MALP-2 and Pam_3CSK_4 had a preferential IL-10 inducing capacity. LysoGPSer plus GPSer stimulated strong TNF-α production, and therefore, seems more pro-inflammatory. The lyso-

GPSer fraction was more neutral concerning pro- or anti- inflammatory responses. These responses were similar in children from rural or semi-urban schools and were also similar in children with a schistosome infection or free of it.

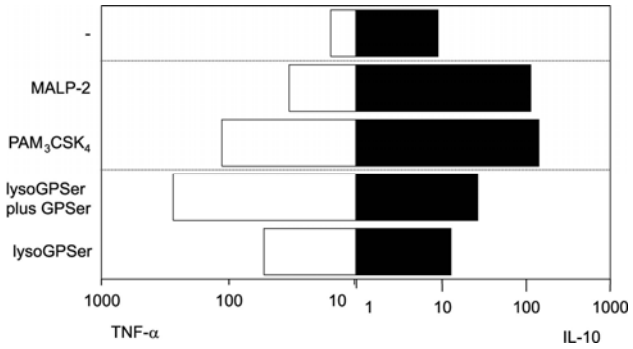

Figure 3: Responses of pro- and anti-inflammatory cytokines in Gabonese schoolchildren. TNF-α and IL-10 were measured in supernatants of whole blood cultures stimulated for 24 hrs with MALP-2, Pam_3CSK_4, lysoGPSer plus GPSer and the lysoGPSer fraction. Cytokine levels are given in pg/ml and the geometric means are shown.

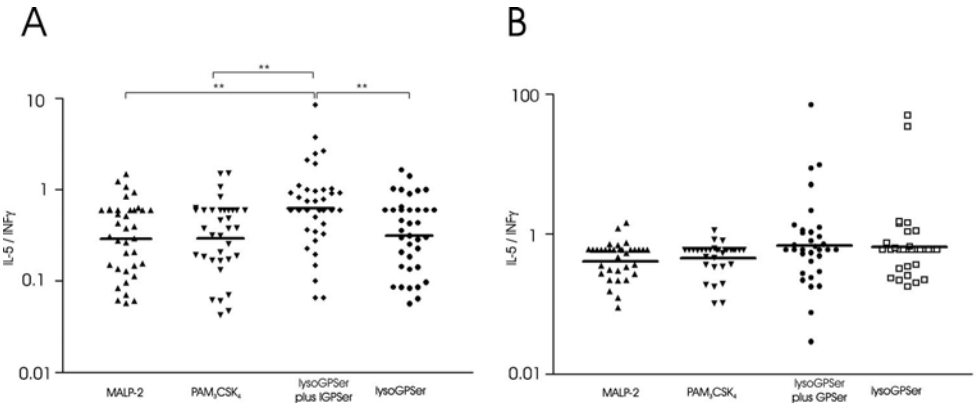

Figure 4: Responses of Th1 and Th2 cytokines in Gabonese schoolchildren. IFN-γ and IL-5 were measured in supernatants of whole blood cultures stimulated for 72 hrs with MALP-2, Pam_3CSK_4, lysoGPSer with GPSer and the lysoGPSer fraction. In each plot the horizontal line indicates the geometric mean, and the P-value of a Bonferroni's Multiple Comparison Test is indicated. Panel A shows cytokine responses from children living in rural areas. Panel B shows cytokine responses from children living in semi-urban areas. **P<0,01.

When the ratio of Th2 to Th1 cytokines was analysed, it was found that in children who resided in rural areas, where schistosome infections are more intense and more prevalent, the lysoGPSer plus GPSer induced stronger Th2 responses compared to the TLR2 ligands Pam_3CSK_4, MALP-2 and to the lysoGPSer fraction (Figure 4A). For children living in urban areas, there were no significant differences in Th2 inducing activity of the tested TLR2 ligands (Figure 4B). There were no differences found between children with schistosome infections or free of it, within the two areas.

Discussion

Stimulation of whole blood with TLR2 ligands derived from adult schistosomes may reflect closely the role of these ligands *in vivo*, since adult worms reside in the blood stream and their released products are expected to interact with this compartment directly. In addition, this method requires minimal blood volumes and minimum *ex vivo* manipulations [15].

Schistosomes contain several PAMPs that are recognized by pattern recognition receptors such as TLRs. We previously reported that a schistosomal diacylglycerophosphoserine (GPSer) and lysophosphatidyl-serine (monoacylglycerophosphoserine, lysoGPSer) containing fraction of *S. mansoni* worms can stimulate peripheral blood mononuclear cells of children living in areas where schistosomiasis is endemic [11]. Here the role of schistosomal lysoGPSer separately was determined additionally. We examined the cytokine response also to two other TLR2 ligands, MALP-2 and Pam_3CSK_4, to investigate possible differences in host responses. It is most likely that parasitic factors, such as lysoGPSer, will not be exposed to the immune system separately, and therefore, we also investigated the cytokine responses to lysoGPSer plus GPSer.

Our results demonstrated that the two TLR2 ligands Pam_3CSK_4 and MALP-2 both have a stronger IL-10 activating compared to their TNF-α activating capacity, and therefore, tend to trigger a more anti-inflammatory response in whole blood cultures (Figure 3). Although the lysoGPSer fraction also activates TLR2 (Figure 2B), it seems to have a neutral effect, since both pro- and anti-inflammatory responses are triggered to the same extent. To our knowledge, no data are available on the ability of various TLR2 ligands to trigger cytokine responses in

whole blood cultures. For the TLR2 ligand Pam_3CSK_4, it has been shown that it induces enhanced extracellular signal-regulated kinase (ERK) signalling in dendritic cells, resulting in an enhanced IL-10 production [16], but no other TLR2 ligand was tested for comparison. In PBMC (peripheral blood mononuclear cells) cultures, Taylor *et al*. have shown that TLR2 ligands lead to varying patterns of cytokine production, where pansorbin (heat killed preparation of *Staphylococus aureus*) induces more TNF-α than IL-10, whereas Staphylococcal LTA (lipoteichoic acid) triggers stronger IL-10 compared to TNF-α, and Pam_3CSK_4 appears to be more neutral [17].
The cytokine profile, concerning TNF-α and IL-10, induced by lysoGPSer plus GPSer is different from the cytokine profiles induced by the lysoGPSer fraction. The observed preferential TNF-α activating capacity compared to the IL-10 activating capacity of the lysoGPSer plus GPSer must be attributed to the activity of GPSer, or to a synergistic effect of the two compounds (Figure 3). In studies of apoptotic cells, it has been shown that GPSer, isolated from bovine brain, can indeed enhance TNF-α release in human whole blood cultures [18]. During apoptosis, the asymmetry of plasma membrane phospholipids is lost, leading to the exposure of GPSer in the outer leaflet of the plasma membrane. Interestingly, Serinkan *et al*. have shown that the production of TNF-α in Jurkat cells can be significantly increased when GPSer-enriched cells are added to the TLR2 ligand zymosan [19]. In addition, Lucas *et al*. showed that the combination of apoptotic cells and a TLR2 ligand can enhance (early) secretion of TNF-α [20]. It is therefore possible that the combination of schistosomal GPSer and schistosomal lysoGPSer works synergistically to lead to high levels of TNF-α in our whole blood cultures.
The levels of the cytokine IL-5 compared to INF-γ indicate the Th1 / Th2 balance. In Gabonese schoolchildren from rural areas lysoGPSer plus of GPSer resulted in a more Th2 skewed profile compared to the TLR2 ligands MALP-2, Pam_3CSK_4 or the lysoGPSer (Figure 4a). However, in children from semi-urban areas, no significant differences were found in the ability of the investigated ligands to skew the IL-5/INF-γ balance (Figure 4b). Schoolchildren who live in a rural area have a higher prevalence for parasitic infections, and therefore, a Th2 biased immune response. The ability of GPSer plus lysoGPSer to induce a response that favors Th2, is likely to be due to the triggering of cells that already have the ability to release Th2 cytokines and are increased in numbers due to

the exposure to helminth infections, highly prevalent in rural areas. Preferential triggering of Th2 cytokines is likely to result from GPSer, since the lysoGPSer preparation does not show this effect. This is in line with our previous finding in dendritic cells [9].
The current study indicates that, when whole blood is exposed to different TLR2 ligands derived from bacteria or from parasites, the generated cytokine profiles can be widely different. Moreover, the cytokine profile upon stimulation of whole blood with the schistosome derived TLR2 ligand lysoGPSer only, is different from that when GPSer is also present at the same time. Information on these molecules, when studied as single entities, can be exploited for specific immune modulation, whereas data on their combined effects is needed to fully understand immune or immunopathological processes during infection.

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Chapter 6

Summarizing discussion

Introduction

The disease caused by schistosomes develops when schistosomal eggs are unable to leave the body of the host via the stool or urine. The adult worms are able to survive for years in the blood vessel of the host for years and seem to be harmless to the host [1]. Early after infection with schistosomes, an anti-parasitic Th1 cytokine response develops, but this response changes into a dominant Th2 response upon egg production by the female worms. After the initial acute phase of infection, the T-cell response is down-modulated during the chronic phase of the disease via regulatory mechanisms involving, among others, IL-10 producing regulatory T cells [2]. We previously showed that schistosome-specific lysophosphatidylserine (monoacylglycerophosphoserine, lysoGPSer) activates Toll-like receptor-2 (TLR2) and affects dendritic cells such that mature dendritic cells gain the ability to induce the development of IL-10 producing regulatory T-cells [3]. In the studies described in this thesis we further investigated schistosomal lysoGPSer in relation to toll-like receptor 2 activation and its effects on the immune response.

Lysophospholipid research

Most definitions for lipids contain the words "water-insoluble". This definition excludes, for example, lysoGPSer, since it is very well soluble in water. The exclusion of lysophospholipids, such as lysoGPSer, especially in the older definitions, already suggests that the interest in lysophospholipids started relatively recently. Nowadays, various lysophospholipids have been studied extensively and many of them have been shown to function as signaling molecules [4,5]. These lysophospholipids have a broad range of biological effects on a variety of cellular systems through numerous signaling pathways.

To investigate the function of schistosomal lysophospholipids, as described in this thesis, it was necessary to evaluate whether classical, well-established methods for lipid isolation and characterization are also suitable for lysophospholipids. For example, the lipid extraction method according to Bligh and Dyer [6] is based on the biophysical property that lipids are rather water in-soluble, and therefore can be extracted from a water-phase when it is exposed to organic solvents, such as chloroform. LysoGPSer, however, is soluble in water and is therefore poorly extracted from a water-phase (less than 10%) using chloroform. However, acidification of the water phase significantly increases the

recovery of lysoGPSer in the chloroform phase, since over 90% of lysoGPSer can then be extracted (see chapter 4).
In addition to optimalisation of extraction methods, adaptations to high performance liquid chromatography (HPLC) methods for separation and identification of phospholipids were needed, because existing methods were not suitable for proper separation of monoacyl species. In chapter two we describe a new HPLC-MS method that separates the molecular species of both monoacyl and diacyl forms of all phospholipid classes in a single run. Detection of diagnostic fragment-ions of specific phospholipid classes can be used to monitor the elution of a particular class of phospholipids, even when a complex phospholipid mixture is injected. This method allows separation of isomeric species such as 16:0/22:4, 18:0/20:4 and 18:1/20:3, after which these species could be quantified separately. Furthermore, isobaric species such as dioleoylglycerophosphatidylglycerol (GPGro((18:1/18:1)) and bis(mono-oleoylglycero)phosphate (BMP(18:1/18:1)) also called lyso-bisphosphatidic acid (LBPA), can be separated from each other, despite the fact that these compounds are structurally very similar (being build from two oleic acids, two glycerol and a phosphate group). As their biological functions are very different, discrimination and separate quantification is required. In addition, levels of lysophospholipids that are naturally present can be determined without interference of lysophospholipids formed from diacyl species by source fragmentation in the mass spectrometer. Therefore, the development of this method was essential for studies on the presence and characterization of lysoGPSer species in schistosomes.

LysoGPSer in *S. mansoni*

In chapter two we showed that adult *S. mansoni* worms contain the lysoGPSer species lysoGPSer(18:0), lysoGPSer(20:1) and lyso-GPSer(22:4). LysoGPSer species are also present in the eggs of *S. mansoni* (see chapter four). However, the composition of lysoGPSer species in eggs is not identical to the composition of lysoGPSer species in adult worms. The major difference is in the presence of lysoGPSer species with longer acyl chain in eggs, such as lysoGPSer(24:0) and lysoGPSer(26:1).
In chapter three we further investigated the presence and location of lysoGPSer and other (lyso)phospholipids in adult worms. We compared (lyso)phospholipid class and species composition between adult *S.*

mansoni worms and mammalian blood, the natural environment of these schistosomes. Furthermore, we compared the (lyso)phospholipid class and species composition of adult worms with the class and species composition of the outer surface membranes of the worm, the tegument. We showed that lysoGPSer(20:1) is more abundant in the tegument compared to the whole worm and is nearly absent in mammalianr blood.

Formation of lysoGPSer in schistosomes

In theory, lysoGPSer may be synthesized in schistosomes via various biosynthetic pathways. LysoGPSer could be synthesized via head-group exchange of other lysophospholipids or via deacylation of diacylGPSer (Figure 1).

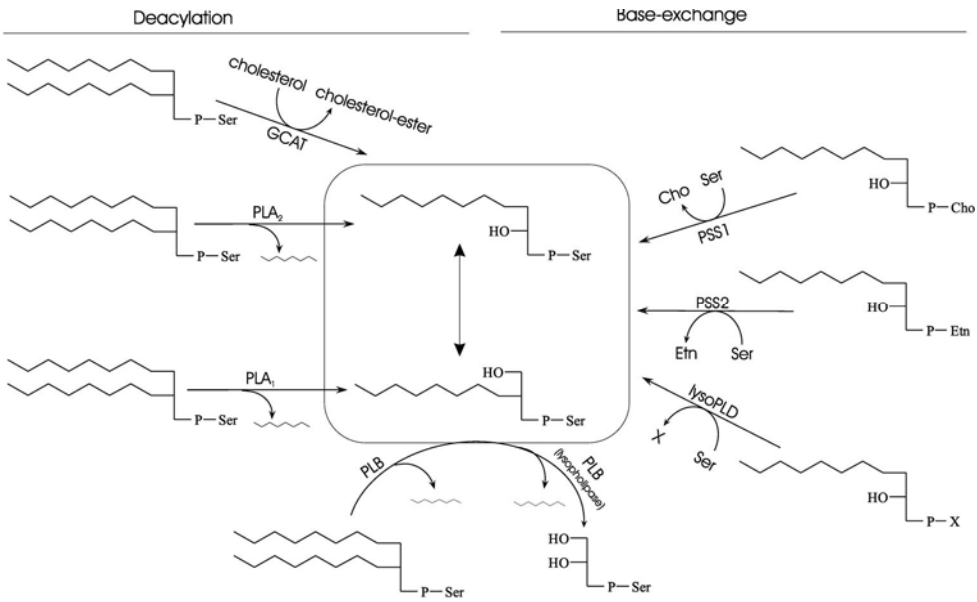

Figure 1: Theoretical biosynthetic pathways for the synthesis of lysoGPSer by schistosomes

In eukaryotes GPSer *in vivo* is mainly synthesized from glycerol-phosphatidylcholine (GPCho) or glycerophosphatidylethanolamine (GPEtn) via the exchange of the choline or ethanolamine head-group by

serine, reactions catalysed by GPSer synthase 1 or 2, respectively (PSS1 or PSS2, see Figure 1) [7]. However, to our knowledge, these enzymes have a high substrate specificity and no indications have been reported that lysoGPCho and lysoGPEtn are substrates for these GPSer synthases. Therefore, it is not likely that schistosomes synthesize lysoGPSer using GPSer synthases.

Another enzyme that might catalyse base-exchange for the synthesis of lysoGPSer from lysoGPCho or lysoGPEtn is phospholipase D (PLD). Phospholipase D is present in many organisms and this enzyme catalyses the hydrolysis of the head-group of phospholipids, which results in the formation of phosphatidic acid (GPA). However, under specific (in vitro) conditions, most PLD-enzymes are also able to catalyze head-group exchange, also known as transphosphatidylation. This transphosphatidylation potential of PLDs is widely exploited for phosphatidylation transformation *in vitro*, including the trans-phosphatidylation of GPCho to GPSer. Such a method has been used in chapter four to synthesize specific GPSer species from commercially available GPCho species. Although homologues of PLD can be identified in the genome of schistosomes, as for instance *S. japonicum* contains a gene that is similar to a gene encoding PLD in *Drosophila melanogaster*, no evidence has yet been reported that PLD enzymes can facilitate transphosphatidylation reactions *in vivo* [8]. Furthermore, in order to synthesize lysoGPSer from lysoGPCho, the schistosomal PLD would also need the capacity to use lysophospholipids as substrate and thus function as a lysoPLD [9], that is also capable to catalyze transphosphatidylation. Therefore, formation of lysoGPSer *in vivo* by transphosphatidylation of lysophospholipids by PLD is not likely.

In addition, when the phospholipid-species composition of tegumental membranes (Chapter 3) is taken into account, the use of base-exchange to synthesize lysoGPSer from other lysophospholipids seems unlikely. In this case, it would be expected that schistosomes contain a similar composition of lysoGPCho and lysoGPEtn species when compared to the lysoGPSer species, since lysoGPCho and lysoGPEtn species are used substrates for the formation of lysoGPSer. However, we could only detect lysoGPEtn(20:1) and some lysoGPCho(20:1), but no lyso-GPCho(22:4), or lysoGPEtn(22:4). Therefore, it is unlikely that lysoPGSer is synthesized in schistosomes from other lysoGPCho or lysoGPEtn by head-group exchange *in vivo*.

Acyltransferases are enzymes that catalyze the transfer of acyl chains from one substance to another. For instance, Glycerophospholipid Cholesterol AcylTransferase (GCAT) transfers an acyl chain from a diacylphospholid to cholesterol, resulting in the formation of cholesterol ester and a lysophospholipid [10]. The presence of an acyltransferase with high homology to GCAT, LCAT (Lecithin Cholesterol AcylTransferase)-like lysophospholipase, has been demonstrated in adult schistosomes [11], but the kinetic properties and the substrate specificity of this enzyme have not been determined yet. However, LCAT has a higher affinity for GPCho than for other phospholipids [12], and therefore, GPSer is not likely to function as a major substrate for this enzyme.

Deacylation of diacylGPSer to monoacylGPSer can be facilitated by two enzyme classes: the phospholipases (PL) and the acyltransferases. Four distinct phospholipases are known to catalyze the hydrolysis of acyl-chains from phospholipids: PLA_1, PLA_2, PLB and lysophospholipase. PLA_1 hydrolyzes the acyl chain from the *sn*-1 position, whereas PLA_2 hydrolyzes the acyl-chain from the *sn*-2 position. On the other hand, PLB subsequently hydrolyzes both acyl chains from a phospholipid, and lysophospholipase hydrolyzes the acyl chain from lysophospholipids. In the genome of *S. mansoni* genes encoding homologues of lysophospholipase have been identified, but their substrate specificity has not been investigated yet [13]. In addition, in adult *S. japonicum* worms PLA_2 activity has been detected, but the gene encoding this enzyme has not been identified yet [14]. Thus, phospholipases seem to be present in adult schistosomes. Hydrolysis of acyl chains from diacyl-GPSer species present in the tegument could result in the formation of the detected lysoGPSer species in the tegument, because the tegument is enriched in the GPSer(20:1/18:0) and GPSer(18:1/22:4) species, which could produce lysoGPSer(20:1) and lysoGPSer(22:4) by the action of PLA_2 and PLA_1, respectively.

Although the *S. mansoni* genome project is nearly completed, not for all enzymes of (lyso)phospholipid metabolism gene homologues could be detected, like for instance phospholipase A_1. However, many genes of schistosomes are not yet functionally annotated, and many of the encoded proteins show no significant homology to any other proteins present in the database of other species than schistosomes. Therefore, schistosomes contain many unknown or unrelated proteins, which could encode enzymes involved in lysoGPSer formation.

LysoGPSer transfer from the parasite to the host

The tegumental outer-surface of schistosomes is a unique double-membrane structure and although we have shown that lysoGPSer is enriched in the tegument compared to the whole worm, it is not yet known in which leaflet of the double membrane the lysoGPSer is enriched. When the lysoGPSer is formed in the outer leaflet of the outer membrane, it might just simply diffuse from the membrane, as it is well soluble in water.

In newly transformed schistosomula, it has been demonstrated that a variety of surface proteins and lipids are lost at a high rate from the tegumental membranes. The tegumental membranes are continuously renewed from membranous bodies. However, the exact turnover-rate of these membranes is debated, as several studies reported half-life values for tegumental proteins or glycoconjugates ranging from a few hours to several days [15-18]. Studies on the turnover of the phospholipids demonstrated that tegumental lipids have a shorter half-life than those in the worm body, and that lysophospholipids are excreted as degradation products into the environment [19]. Two mechanisms for the high turnover rate have been proposed. Firstly, sloughing of membrane-sheets has been proposed, whereby tegumental lipids, including lysophospholipids, will be excreted by the parasite into the blood vessel of the host [19]. Secondly, fast deacylation followed by re-acylation of lipids in the tegumental membrane has been proposed [20]. The latter theory is supported by a pulse-chase experiment with labeled fatty acids, in which the labeled fatty acids are rapidly incorporated in tegumental phospholipids and subsequently released into the medium [20].

After lysoGPSer has been excreted from the tegumental membrane of the schistosome into the blood of the host, it could bind in part to albumin, which is known for its capacity to bind acyl chains [21]. Bound to albumin or soluble in the blood, lysoGPSer could then interact with the TLR-complex on immune cells present in blood.

LysoGPSer and TLR2 activation

In chapter four, we show that four synthesized lysoGPSer species, lysoGPSer(18:1), lysoGPSer(18:3), lysoGPSer(20:1) and lysoGPSer-(24:1) induce TLR2 activation in a dose dependent manner. We also show that the serine head group is essential for TLR2 activation and that diacylGPSer has no TLR2 activating capacity. Furthermore, we showed

that lysoGPSer species with saturated acyl chains with 16 to 20 carbon atoms have a very poor TLR2 activating capacity, whereas long chain GPSer species have a potent TLR2 activating capacity. Both lysoGPSer species from schistosomal eggs and from schistosomal adult worms activate TLR2. Previously it has been shown that schistosomal lysoGPSer from both worms and eggs affect, via TLR2, dendritic cells such that mature dendritic cells gain the ability to induce the development of IL-10 producing regulatory T cells. It remains unclear whether lysoGPSer from both lifecycle stages contributes to the induced regulatory T-cell response during the chronic stage of the disease.
It is known that TLR2 forms heterodimer complexes with either TLR1 or TLR6, depending on the ligand. In general, triacylated lipopeptides (such as PAM_3CSK_4) activate TLR2 via a heterodimer with TLR1, while diacylated lipopeptides (such as MALP2) activate TLR2 via heterodimers with TLR6. Not many monoacylated compounds activate TLR2, but lauric acid, a fatty acid, activates TLR2 via a heterodimer with TLR6. Preliminary data of the activation of TLR2 in TLR2-, TLR1- or TLR6-knockout mice suggests that also lysoGPSer activates TLR2 via a heterodimer with TLR6, but more experiments with knock-out mice or antibodies against these receptors are needed to confirm this finding.
In chapter five, we studied the effect of two TLR2 activating schistosomal lipid fractions, one containing lysoGPSer plus diacylGPSer and the other fraction highly enriched in lysoGPSer. Immune activation by the lipid fractions was analyzed in schoolchildren living in an area in Gabon where schistosomiasis is endemic. The effect of these schistosomal lipid fractions was compared with the known TLR2 ligands PAM_3CSK_4 and MALP-2. In whole blood cell cultures stimulated with MALP-2 or PAM_3CSK_4, it was shown that these TLR2 ligands had preferential IL-10 activating capacity and seem therefore anti-inflammatory. The fraction containing lysoGPSer plus diacylGPSer had a strong TNF-α producing capacity and seems therefore more pro-inflammatory. LysoGPSer alone was more neutral with respect to pro- and anti-inflammatory responses. When the same TLR2 activating components were tested on whole blood of Gabonese children and analyzed for Th1 and Th2 cytokines, the schistosomal lipid fraction containing lysoGPSer plus diacylGPSer showed a strong Th2 inducing activity compared to the other TLR2 ligands. Therefore, it seems that activation of TLR2 ligands can lead to various effects, depending on co-activating ligands.

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Samenvatting

Schistosomen en het immuunsysteem van de gastheer
De parasieten van de schistosoma familie zoals *Schistosoma mansoni* en *Schistosoma haematobium* veroorzaken de ziekte schistosomiasis, ook wel bekend onder de naam bilharzia. Vooral in de tropische ontwikkelingslanden is deze ziekte een groot probleem, op de eerste plaats voor de gezondheid van mens en dier, maar daardoor ook op sociaal en economisch gebied.
De levenscyclus van schistosomen is complex doordat ze twee gastheren hebben. Naast zoogdieren, zoals de mens, heeft de parasiet ook een tropische zoetwaterslak nodig voor zijn levenscyclus (zie figuur 1 van hoofdstuk 1). De volwassen schistosomen leven als wormpaartjes in aderen in de buurt van de darmen (*S. mansoni*) of in de buurt van de blaas (*S. haematobium*). Hoewel de parasieten in het bloed omgeven zijn door cellen van het immuunsysteem van de gastheer, lijkt het afweersysteem van de gastheer de parasieten nauwelijks op te merken als (gevaarlijke) indringers, waardoor ze decennia lang kunnen overleven in hun gastheer. De wormpaartjes produceren veel eieren, die vervolgens via de ontlasting of urine (afhankelijk van het type schistosoom) de gastheer verlaten om zich verder te ontwikkelen (zie de levenscyclus, figuur 1, hoofdstuk 1). Een klein deel van de eieren verlaat het lichaam niet en komt in het lichaam van de gastheer vast te zitten, bijvoorbeeld in de lever. De eieren zijn zeer immunogeen wat betekent dat de eieren door het immuunsysteem worden opgemerkt en dat de afweer tegen deze eieren op gang komt. De respons van het immuunsysteem op een infectie met schistosomen verloopt in fases. Tijdens de eerste fase komt een respons opgang waarbij T helper cellen van het type 1 een belangrijke rol spelen. Deze zogehete Th1 respons zorgt ervoor dat indringers worden gedood en opgeruimd door macrofagen. Direct nadat de schistosomen beginnen met eieren leggen, verandert de immuunrespons en worden vooral T helper cellen van het type 2 belangrijk. Deze Th2 respons zorgt voor de productie van antilichamen tegen de indringer. In de laatste fase, wanneer de ziekte chronisch wordt, zijn vooral T helper cellen van het regulatoire type belangrijk. Deze regulatoire T helper cellen zorgen voor een onderdrukking van de immuunrespons van de gastheer. Via het uitscheiden van immunomodulatoren lijkt de parasiet zelf een belangrijke rol te spelen in het ontstaan van de regulatoire T helper cel

respons. Lysofosfatidylserine (lysoPS), een fosfolipid van schistosomen, is zo'n stof met een immunomodulatoire functie.

Fosfolipiden van schistosomen

Fosfolipiden zijn de bouwstenen van membranen. Fosfolipiden bestaan uit een kopgroep, een fosfaatgroep, een glycerol ruggengraat en een of twee vetzuurstaarten (zie figuur 2 van hoofdstuk 1). De fosfolipiden kunnen op basis van hun kopgroep worden ingedeeld in verschillende klassen. Deze klassen worden weer verder onderverdeeld in species, dit gebeurt op basis van de aanwezige vetzuurstaarten. De samenstelling van fosfolipiden in een membraan is verschillend per type membraan. Schistosomen hebben een karakteristieke dubbele membraan als buitenste oppervlakte-laag, het tegument. Deze tegumentstructuur is in de natuur tot nu toe alleen bekend bij parasieten die in de bloedbaan van de gastheer leven. De fosfolipiden van het tegument worden vaak vernieuwd en daarom hebben schistosomen veel fosfolipiden nodig. Echter, schistosomen kunnen de benodigde vetzuurstaarten niet zelf synthetiseren en zullen deze dus moeten opnemen uit hun directe omgeving, het bloed van de gastheer. Wel kunnen ze de opgenomen vetzuurstaarten modificeren. Omdat ze worden opgenomen van de gastheer, komen veel fosfolipiden die in de gastheer voorkomen, ook voor in de schistosomen. Daarnaast zijn er wel een paar grote verschillen, zoals de aanwezigheid van lysofosfatidylserine. Deze fosfolipidklasse komt nauwelijks voor in de gastheer maar wel in schistosomen. Tijdens onderzoek uitgevoerd in de jaren voorafgaande aan het werk beschreven in dit proefschrift, is gevonden dat lysofosfatidylserine van schistosomen het immuunsysteem van de gastheer kan beïnvloeden via het binden en activeren van een receptor die aanwezig is op cellen van het immuunsysteem. Deze receptor is de "Toll-like receptor 2" (TLR2). Deze receptor behoort tot een groep receptoren die fragmenten van pathogenen kan herkennen, waardoor vervolgens het immuunsysteem van de gastheer geactiveerd wordt. Dit kunnen moleculen van parasieten zijn, maar ook van virussen of bacteriën. Lysofosfatidylserine activeert TLR2 en door deze activatie worden regulatoire T cellen geïnduceerd. Via lysofosfatidylserine (lysoPS) kunnen de schistosomen de immuunrespons van de gastheer onderdrukken.

Een nieuwe methode om de fosfolipidensamenstelling van schistosomen te onderzoeken.

Omdat er geen goede scheidingsmethode bestond voor de detectie en isolatie van lysoPS en ander lysofosfolipiden, hebben we een nieuwe methode ontwikkeld. Hierin wordt gebruik gemaakt van vloeistof-chromatografie direct gekoppeld aan massaspectrometrie. Deze methode wordt beschreven in hoofdstuk 2. Identificatie door massa-spectrometrie berust op de mogelijkheid een molecuul een negatieve of positieve lading te geven en vervolgens het gewicht (de massa) van dit geladen molecuul vast te stellen. Tevens kan een geladen molecuul in fragmenten worden gebroken om ook van de geladen fragmenten de massa vast te stellen. Doordat moleculen niet willekeurig breken, maar in karakteristieke fragmenten (het fragmentatiepatroon), is het mogelijk om op deze manier een molecuul te identificeren. Voor elk van de verschillende fosfolipidklassen is een specifiek fragment bekend, wat gebruikt kan worden bij de identificatie van de verschillende species. Het is mogelijk dat twee verschillende moleculen in een complex lipid monster dezelfde massa en hetzelfde fragmentatiepatroon hebben. Deze moleculen zijn dan ook moeilijk van elkaar te onderscheiden met massaspectrometrie. Echter, vaak kunnen deze moleculen wel van elkaar gescheiden worden met vloeistofchromatografie. Een combinatie van vloeistofchromatografie en massaspectrometrie maakt het dus mogelijk om deze moleculen toch te identificeren. Dit kan belangrijk zijn als deze moleculen bijvoorbeeld zeer verschillende biologische activiteit hebben.

In hoofdstuk 3 wordt de hierboven beschreven methode gebruikt om de fosfolipidensamenstelling van de hele *S. mansoni* worm te vergelijken met de fosfolipidensamenstelling van de buitenste laag van de worm, het tegument. Ook worden deze twee fosfolipidensamenstellingen vergeleken met de fosfolipidensamenstelling van de natuurlijke leefomgeving van de worm, het bloed van de gastheer. Het blijkt dat de fosfolipidensamenstelling van het tegument sterk afwijkt van dat van de hele worm en van het bloed. Zo zijn fosfolipiden met langere vetzuurstaarten verrijkt aanwezig in het tegument en bevatten deze vetzuurstaarten ook meer dubbele bindingen. Bovendien blijken de lysofosfolipiden, lysoPS en lysoPE, sterk verrijkt te zijn in het tegument.

Immunologische activiteit van lysoPS

Om te kunnen testen welke lysoPS species Toll-like receptor 2 kunnen activeren, zijn er enkele lysoPS species gesynthetiseerd. In hoofdstuk 4 is beschreven dat al deze gesynthetiseerde lysoPS species TLR2 activeren. Aan de andere kant bleken sterk gelijkende moleculen zoals fosfatidylserine (met een vetzuurstaart meer dan lysoPS) en lysofosfatidylcholine (met een andere kopgroep dan lysoPS) TLR2 niet te activeren. Het is dus voor de TLR2 activatie belangrijk dat het molecuul een enkele vetzuurstaart heeft en een serine groep als kopgroep. Omdat niet alle lysoPS species synthetiseerbaar zijn, is er ook gekeken naar de potentie van verschillende lysoPS species uit eieren van *S. mansoni* om TLR2 te activeren. Deze lysoPS species zijn verdeeld in verschillende fracties met behulp van de methode beschreven in hoofdstuk 2. Helaas kunnen niet alle species los van elkaar worden opgevangen en daarom is van een groep lysoPS species de potentie om TLR2 te activeren gemeten. Tevens zijn de verschillende fracties met waterstofgas behandeld, waardoor alle dubbele bindingen in de vetzuurstaarten zijn verzadigd zodat enkele bindingen ontstaan. Ook van deze behandelde fracties hebben we de potentie om TLR2 te activeren gemeten. Door de resultaten van de verschillende fracties te vergelijken, kunnen we concluderen dat lysoPS species met lange vetzuurstaarten (22 of meer C atomen), zeer potent TLR2 activeren. LysoPS species met vetzuurstaarten zonder dubbele bindingen en met minder dan 22 C atomen, hebben een lage potentie om TLR2 te activeren.

In hoofdstuk 5 zijn lysoPS species uit *S. mansoni* wormen getest op immunologische activiteit. Er is hierbij gekeken naar het verschil tussen immunologische activiteit van lysoPS alleen of lysoPS samen met phosphatidylserine (PS, met een vetzuurstaart meer dan lysoPS). Verder is deze activiteit vergeleken met twee commercieel verkrijgbare TLR2 activatoren, PAM en MALP. Om de immunologische activiteit van de moleculen te analyseren, werd er bloed afgenomen van kinderen die leven in Gabon. In Gabon is er qua hygiene, voeding en ziektes een duidelijk verschil tussen kinderen die leven in dorpen en kinderen die leven op het platteland. De kinderen op het platteland zijn veel vaker geïnfecteerd met parasieten, zoals *S. mansoni*, dan kinderen die leven in de dorpen. Als lysoPS of een ander molecuul wordt toegevoegd aan het bloed dan zullen de cellen in het bloed hier een reactie op geven. De bloedcellen weerspiegelen het immuunsysteem van het kind waaruit ze komen. Het blijkt dat lysoPS, als het alleen wordt toegevoegd, een

andere immunologische reactie oproept dan wanneer lysoPS samen met PS wordt toegevoegd. Verder geven verschillende TLR2 activatoren niet allemaal dezelfde immunologische respons, hoewel ze wel dezelfde immuunreceptor activeren. Ook blijkt dat het immuunsysteem van kinderen die op het platteland wonen anders reageert dan van kinderen die in de stad wonen.

Conclusie

Voordat het onderzoek beschreven in dit proefschrift werd uitgevoerd, was bekend dat lysoPS species uit *S. mansoni* TLR2 kunnen activeren en via deze receptor het immuunsysteem van de gastheer kunnen beïnvloeden. Na het onderzoek beschreven in dit proefschrift, is nu bekend dat deze lysoPS species zich in de buitenste laag van de parasiet bevinden en dus in contact kunnen komen met de cellen van het immuunsysteem van de gastheer. Bovendien is nu bekend dat vooral de lysoPS species met lange vetzuurstaarten een hoge potentie hebben tot activeren van de TLR2. Deze lysoPS species komen juist veel voor bij de parasiet *S. mansoni*. Ook is nu bekend dat de reactie van het immuunsysteem van de gastheer op lysoPS sterk afhangt van eventuele gelijktijdige blootstelling aan andere moleculen. Bovendien lijkt het immuunsysteem onderscheid te maken tussen verschillende activatoren van TLR2.

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Curriculum Vitae

Kim Retra werd geboren op 13 augustus 1977 te Alkmaar. In 1995 behaalde zij het HAVO diploma, in 1997 gevolgd door het VWO diploma, beide aan het Coornhert Lyceum te Haarlem. In 1997 begon zij met de studie farmacochemie aan de Vrije Universiteit (VU) te Amsterdam. Zij verrichtte haar bijvakstage bij de sectie bio-analytische chemie van de afdeling scheikunde van de Faculteit der exacte wetenschappen aan de VU. Onder begeleiding van Drs. N. Visser, Dr. H. Lingeman en Prof. Dr. H. Irth werkte zij aan de opzuivering van AMP, ADP en ATP met behulp van vaste fase extractie (SPE) en capillaire electroforese (CE). Haar hoofdvakstage verrichtte zij bij de sectie moleculaire farmacologie van de afdeling farmacochemie van de Faculteit der exacte wetenschappen aan de VU. Onder begeleiding van Dr. M. Hoffmann en Prof. Dr. R. Leurs werkte zij aan de interactie tussen zes verschillende isovormen van de histamine H3 receptor. Hierna schreef zij onder begeleiding van Prof. Dr. R. Leurs een scriptie over de betrokkenheid van chemokines en chemokinereceptoren in de ontwikkeling van atherosclerose. In 2002 behaalde zij haar doctoraal diploma farmacochemie. Datzelfde jaar startte zij ook haar promotieonderzoek bij de afdeling biochemie en celbiologie van de Faculteit diergeneeskunde aan de Universiteit Utrecht. Onder begeleiding van Prof. Dr. A.G.M. Tielens, Prof. Dr. M. Yazdanbakhsh, Dr. J.J. van Hellemond en Dr. D. van der Kleij werkte zij aan lysofosfatidylserine van schistosomen en de immunomodulerende rol van dit molecuul. De resultaten van het onderzoek staan beschreven in dit proefschrift.

List of publications and manuscripts

van der Kleij, D.; van den Biggelaar, A.H.; Kruize, Y.C.; Retra, K.; Fillie, Y.; Schmitz, M.; Kremsner, P.G.; Tielens, A.G.M.; Yazdanbakhsh, M. Responses to Toll-like receptor ligands in children living in areas where schistosome infections are endemic. (2004) *J. Infect. Dis.* 189 (6): 1044-51

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Retra, K.; Blijerveld, O.B.; van Gestel, R.; Tielens, A.G.M; van Hellemond, J.J.; Brouwers, J.F. A simple and universal method for the separation and identification of phospholipid molecular species. *Submitted*

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Retra, K.; Schmitz, M.; Yazdanbakhsh, M.; Tielens, A.G.M.; Brouwers, J.F.; van Hellemond, J.J. The phosphatidylserine and lysophospholipid species composition of the tegumental outer-surface membranes of *Schistosoma mansoni*. *In preparation*

Retra, K.; van Riet; E., Houweling, M.; Brouwers, J.F.; van der Kleij, D.; van Hellemond, J.J.; Yazdanbakhsh, M.; Tielens, A.G.M. Activation of Toll-like receptor 2 by synthetic and schistosomal lysophosphatidylserine. *In preparation*

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Abbreviations

BMP	bismonoacylphosphatidic acid
ELISA	enzyme-linked immunosorbent assay
FFA	free fatty acids
GCAT	glycerophospholipid:cholesterol acyl transferase
GPA	glycerophosphatidic acid
GPCho	glycerophosphocholine
GPCR	G protein-coupled receptor
GPEtn	glycerophosphoethanolamine
GPGro	glycerophosphoglycerol
GPIns	glycerophosphoinositol
GPSer	glycerophosphoserine
HEK293	human embryonic kidney cell line 293
HPLC	high performance liquid chromatography
IgE	immunoglobulin E
IL	interleukin
INF	interferon
LBPA	lysobisphosphatidic acid
LCAT	lecithin:cholesterol acyl transferase
LC-MS	liquid chromatography on-line coupled to mass spectrometry
lysoGPSer	lysophosphatidylserine (monoacylglycerophosphoserine)
MRM	multiple reaction mode
PAMP	pathogen-associated molecular patterns
PLA_2	phospholipase A2
PLB	phospholipase B
PLD	phospholipase D
PSS	phosphatidylserine synthase
S. mansoni	*Schistosoma mansoni*
TGF	transforming growth factor
Th	T helper cells
TIC	total ion count
TLR	Toll-like receptor
TNF	tumor necrosis factor