# Helminth infections induce immunomodulation : consequences and mechanisms

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# Antibody responses to *Ascaris*-derived proteins and glycolipids: the role of phosphorylcholine

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## Summary

In addition to proteins, glycolipids can be targets of antibody responses and contribute to host pathogen interaction. Following the structural analysis of *Ascaris lumbricoides* derived glycolipids, the antibody responses of a group of children with no, light and heavy infections were analysed. The role of the phosphorylcholine moiety, present on *Ascaris* glycoproteins and glycolipids, in antibody reactivity of these infected individuals was determined.
Children carrying heavy infections showed highest IgG reactivity to glycolipids compared to lightly or non-infected children. Substantial IgG antibody reactivity to both (glyco)proteins and glycolipids was found to be directed to the phosphorylcholine moiety as determined by either removal of this group or a competition assay. This was most pronounced for glycolipids, where removal of the phosphorylcholine moieties by hydrofluoric acid treatment abrogated IgG antibody reactivity. Measurement of IgG4 and IgE isotypes showed no IgG4 reactivity to *Ascaris* glycolipids, but raised IgE responses were detected in subjects with light or no *Ascaris* infections, suggesting that IgE responses to glycolipids may play a role in controlling parasite burden. Differences found in antibody profiles to glycolipids and (glyco)proteins, indicates that these different classes of compounds may have distinct roles in shaping of and interacting with humoral immune responses.

## Introduction

*Ascaris* infections affect one quarter of the population worldwide [110], causing low mortality, but considerable morbidity. Helminth infections, including ascariasis, are generally long lasting, occasionally causing severe pathology, however, major clinical symptoms are often absent. This is due to the dual effect of helminth infections on the immune system; helminths skew the host immune response towards a T helper 2 cytokine profile that induces B cells to switch to IgE antibody production but they are also associated with immune hyporesponsiveness, in part mediated by regulatory T cells, TGF-β and IL-10 [6]. It is known that IL-10 is involved in upregulation of the IgG4 isotype, thereby influencing the IgE/IgG4 balance, which has been shown to be important in immunity to helminth infections, including ascariasis [51, 111-113]. The current study was designed to compare antibody responses to both (glyco)proteins and glycolipids in well-characterized groups of children with different infection levels; a heavily infected group was compared to a lightly infected group and to a group of endemic controls who were free of infection.
Investigations of antibody profiles during infection have focused mainly on proteins and their carbohydrate conjugates [114, 115]. However antibody

reactivity to lipids and their conjugates has been reported, for example in schistosomiasis [116, 117], indicating that not only proteins but also lipids can be targets of humoral immune responses. Moreover, in schistosomes it has been shown that immunogenic carbohydrate epitopes can be shared between proteins and lipids [118, 119] and mediate antibody binding.

In this study we were particularly interested in phosphorylcholine (PC), a small hapten, often linked to carbohydrate epitopes. PC is a major antigenic determinant in a wide variety of organisms, varying from Gram positive and –negative bacteria, to protozoa and several intestinal nematodes [120-122]. It has been shown that PC is not only involved in physiological functions of micro-organisms (e.g. normal growth and cell division in *Streptococcus pneumoniae*), but also in modulation of the immune response of the host in favour of either the host or the pathogen. PC on *S. pneumoniae* can mediate the invasion of endothelial cells via interaction with platelet-activating factor, which can result in severe morbidity like pneumonia [123]. It has also been shown that antibodies against PC can help the host to eradicate *S. pneumoniae* [124]. Moreover, PC moieties on glycosphingolipids of *Ascaris suum* were shown to be able to modulate B cell, macrophage and T cell activation [125, 126]. PC-substituted molecules have been found to be present in large amounts in *Ascaris* worms with widespread anatomical location in the worm, including the epicuticle, indicating these components will be surface exposed and available to the host immune system [121]. Given the diverse functions of the PC moiety in host-parasite interaction [120], the development of antibodies to PC might play an important role in *Ascaris* infections.

Although glycolipids from *A. suum* have been characterized in detail, no information exists on the composition of glycolipids present in *A. lumbricoides*, the parasite of humans. In this study we have characterized these glycolipids and we have analysed IgG, IgG4 and IgE antibody responses to glycolipids, in a well-defined population residing in Indonesia where *A. lumbricoides* infections are prevalent. The antibody reactivity of children with both heavy and light infections to *Ascaris* derived glycolipids and (glyco)proteins was investigated and the contribution of PC moieties to these reactivities was determined.

## Materials and methods

### Study population

Banked plasma samples from Indonesian schoolchildren attending schools in different areas of the city of Makassar (Sulawesi) were studied. In one school (S.D. Cambaya) *A. lumbricoides* was highly endemic (76%) while in the second school (S.D. Mangkura) low prevalence of *A. lumbricoides*

infection was found (6.2%). The intensity of infection in S.D. Mangkura (geometric mean of 715 eggs per gram of faeces) was significantly lower than in S.D. Cambaya (geometric mean of 9,239 eggs per gram of faeces). Plasma samples were selected based on availability. For the non-infected group, children with coinfections with other intestinal parasites were excluded to prevent cross-reactivity. As indicated in table 2.1, we used plasma from 22 heavily infected children from S.D. Cambaya, 17 lightly infected children from S.D. Mangkura and 22 uninfected children from S.D. Mangkura. In addition, plasma from 14 European donors without a history of intestinal helminth infections was assayed for antibody responses. Informed consent was obtained from the parents of each child and blood withdrawal was approved by the ethics committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

**Table 2.1.** Characteristics of study population

| | *Ascaris* infection intensity | | |
|---|---|---|---|
| | High | Low | Not infected |
| Male / female | 11/11 | 8/9 | 11/11 |
| Median age, years | 9 | 9 | 9.5 |
| Range | 8-11 | 8-11 | 8-11 |
| *Ascaris* infection | + | + | - |
| Geometric mean (epg) | 10084* | 609 | 0 |
| Range | 288-87600 | 48-43704 | - |

**p<0.0001 compared to infected group with low level infections*
*epg; number of eggs per gram of faeces.*

### *Ascaris* protein isolation

Proteins were extracted from *A. lumbricoides* worms that were collected in Indonesia from infected subjects. The worms were freeze-dried for two days and were then homogenized in phosphate-buffered saline (PBS) containing 1% (v/v) n-octyl β-D-glucopyranoside (Sigma) untill a homogeneous suspension was obtained, whilst keeping the mix on ice. The suspension was sonicated 6 times for 25 seconds at 20-second intervals. Samples were spun at 18,000 g and clear supernatants were collected. The supernatants were dialysed overnight against PBS to remove detergent and filtered (0.45 μm). Protein concentration was measured using a BCA-determination as described by Smith *et al* [127].

### *Ascaris* lipid isolation

Glycolipids were extracted from *A. lumbricoides* (collected from Indonesian infected subjects) and *A. suum* (collected from pigs in a Dutch abattoir). Per extraction, lipids from 4 grams of homogenized *Ascaris* worms were extracted according to the method described by Bligh and Dyer [128]. The organic phase was dried by rotary evaporation, dissolved in 10 ml

chloroform and the total lipid extract was applied to a 20 ml column of the anion exchanger TEAE cellulose (Serva, Heidelberg, Germany) that was converted to the hydroxyl form. Lipids were eluted as described by Rouser *et al* [129]. The ceramide-containing fraction was eluted from the column as the third fraction (TEAE fraction 3) with a mixture of 67% chloroform and 33% methanol (v/v).





**Hydrofluoric acid treatment for lipids**
Lipids were transferred to a plastic vial and dried under nitrogen. Samples were dissolved in 50 μl of hydrofluoric acid (HF) and sonicated for 7 minutes. After incubation O/N at 4°C, HF was evaporated under a stream of nitrogen. Methanol (50 μl) was added and evaporated 3 times to remove traces of HF [130]. The lipids were transferred to glass vials and were stored at -20°C.

**Mass spectrometry**
Matrix assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) of both *A. lumbricoides* and *A. suum* TEAE fraction 3 before and after HF treatment was performed in the reflector positive-ion mode on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using 6-aza-2-thiothymine as a matrix (5 mg/ml in water; Sigma). Fragment-ion spectra were acquired with laser-induced fragmentation as outlined previously [131].

**ELISA**
For glycolipids, polysorp microtitration plates (Nunc, Denmark) were coated with 0.3% (IgG4 and IgE) or 0.05% (IgG) of the ceramide containing lipid fraction derived from 4 grams of worm, dissolved in methanol. Each well was coated either with 25 μl of lipid in methanol or with methanol only as a control, and the plates were air-dried overnight at room temperature.

For proteins, maxisorp microtitration plates (Nunc, Denmark) were coated overnight at room temperature with 100 μl 5 μg/ml (for IgG4 and IgE) and 0.83 μg/ml (for IgG) *Ascaris lumbricoides* proteins in PBS. As controls, wells were incubated with PBS only.

The following incubations were at 37°C while shaking, with a volume of 100 μl per well, unless stated otherwise. Between each incubation, plates were washed 4 times with 0.5 mM PBS / 0.01% (v/v) Tween-20. Plates were blocked by incubation with 200 μl of blocking solution 0.07% (w/v) bovine non-fat dry milk in PBS for an hour. Plasma samples as well as detection antibodies were diluted in blocking solution. Plates were incubated with plasma solutions (1/100, 60 minutes for IgG or 1/20, 90 minutes for IgG4 and IgE), followed by incubation with biotin-conjugated IgG (goat-anti-

human total IgG, 1/10,000, 60 min. Sanquin, Amsterdam, The Netherlands), biotin-conjugated IgE (goat-anti-human IgE, 1/1000, 90 min. Vector, Burlingame, CA) or HRP-conjugated IgG4 (goat-anti-human IgG4, 1/3000, 90 min. Vector, Burlingame, CA). The plates for determination of IgG and IgE antibody reactivity were in addition incubated for 1 hour with streptavidin-HRP (1/10,000 Sanquin, Amsterdam, The Netherlands). Assays were developed at room temperature with 3,3',5,5' tetramethylbenzidin (TMB, Sigma) as substrate. Reactions were stopped by adding 1.8 M H2SO4, and absorbance was read at 450 nm in an automated plate reader. For analysis, optical density (OD) values of each plasma samples from the control-coated wells (PBS or methanol) were subtracted from OD values of antigen-coated wells.

**Competition with phoshorylcholine**
To compete for antibody binding with the coated proteins or lipids, phosphorylcholine (Sigma) was diluted in blocking solution and added at a final concentration of 10 mM to the coated wells before adding plasma.

**Statistical analysis**
The Mann-Whitney test was used to compare data between the different patient groups. For comparison of the data with or without application of competition with PC or HF treatment, the Wilcoxon matched pairs test was used. The relationship between antibody levels and total IgE was examined using a Spearman correlation test.

## Results

**Structural characterization of glycolipids by mass spectrometry**
Isolated lipids of *A. lumbricoides* were fractionated on TEAE cellulose according to Rouser [129]. TEAE fraction 3, containing the glycolipids, was analysed by MALDI-TOF-MS (figure 2.1A). The pattern of observed glycosphingolipid species was similar to that observed for *A. suum*, with conservation of oligosaccharide structures, phosphorylcholine substitution pattern, and ceramide structure [130, 132].

Based on the pronounced conservation of structural motifs indicated by the MALDI-TOF-MS data (figure 2.1A), the observed *A. lumbricoides* glycolipids, which also do occur in *A. suum*, were assigned as follows: the species at *m/z* 1000 corresponded to Man(β1-4)Glc(β1-1)ceramide, and the species at *m/z* 1203 to GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide [130]. For the latter glycolipid structure partial substitution with phosphorylcholine was found resulting in detection of PC-6GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide at *m/z* 1369 and *m/z* 1309 with the latter ion arising from specific fragmentation of the phosphorylcholine group, in accordance with

the described fragmentation behavior of PC-substituted biomolecules [132, 133]. The attachment of the PC moiety to the *N*-acetylhexosamine was shown by fragment ion analysis (figure 2.1C). This smallest PC-containing glycolipid of *A. lumbricoides* is also shared with *A. suum* [132]. In addition, a Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide was detected at *m/z* 1567, which may carry a phosphorylcholine substituent on the GlcNAc residue, as confirmed by fragment ion analysis of the species at *m/z* 1734 (figure 2.1B).

Larger structures were found to contain PC throughout and exhibited one or two additional hexose residues (*m/z* 1896 and *m/z* 2058, respectively) or one additional HexNAc unit (*m/z* 1937). The first two compositions are in accordance with the structures Gal(β1-3)Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide and Gal(β1-6)[Gal(β1-3)]Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide which both are also found in *A. suum* [132]. No mass spectrometric evidence was obtained for alternative biosynthetic pathways like in *C. elegans*, which expresses complex neutral glycosphingolipids with methylated fucose residues [134].

As for the ceramide structures, the major ceramide dihexoside signals observed for *A. lumbricoides* at *m/z* 998 (not labeled) and *m/z* 1000 (figure 2.1A) are in accordance with the major ceramide compositions observed for neutral and PC-substituted glycosphingolipids of other nematodes, namely C17 sphingosin or sphinganin, which may exhibit a branched hydrocarbon chain, and is amide-linked predominantly to hydroxylated tetracosanoic acid [121, 130, 132, 135, 136]. Based on the MALDI-TOF-MS data, the ceramide composition of the larger glycosphingolipids seems to be largely identical to the ceramide composition of ceramide dihexoside.

Taken together, we found that *A. lumbricoides* glycolipids exhibited typical nematode-type glycolipid structures, which were partially substituted with PC. The similarity with glycolipids of *A. suum* was very high, for which detailed structural information is available in literature [130, 132]. As the latter one is more readily available in larger amounts, *A. suum* glycolipids were used in this study. In addition, we found no differences in antibody responses when lipids from either *A. lumbricoides* or *A. suum* were used in a pilot study in 2 independent experiments with 4 plasma samples, which confirms that *A. lumbricoides* and *A. suum* are highly related [137]. *A. suum* glycolipids were prepared in the same way. Analysis of the *A. suum* preparation by MALDI-TOF-MS indicated nematode-specific glycolipids of 2 to 5 monosaccharide residues, with Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide as the major PC-containing species (data not shown). In order to be able to assign the role of PC in antibody-binding studies, the PC-moiety was selectively removed by HF-treatment, which

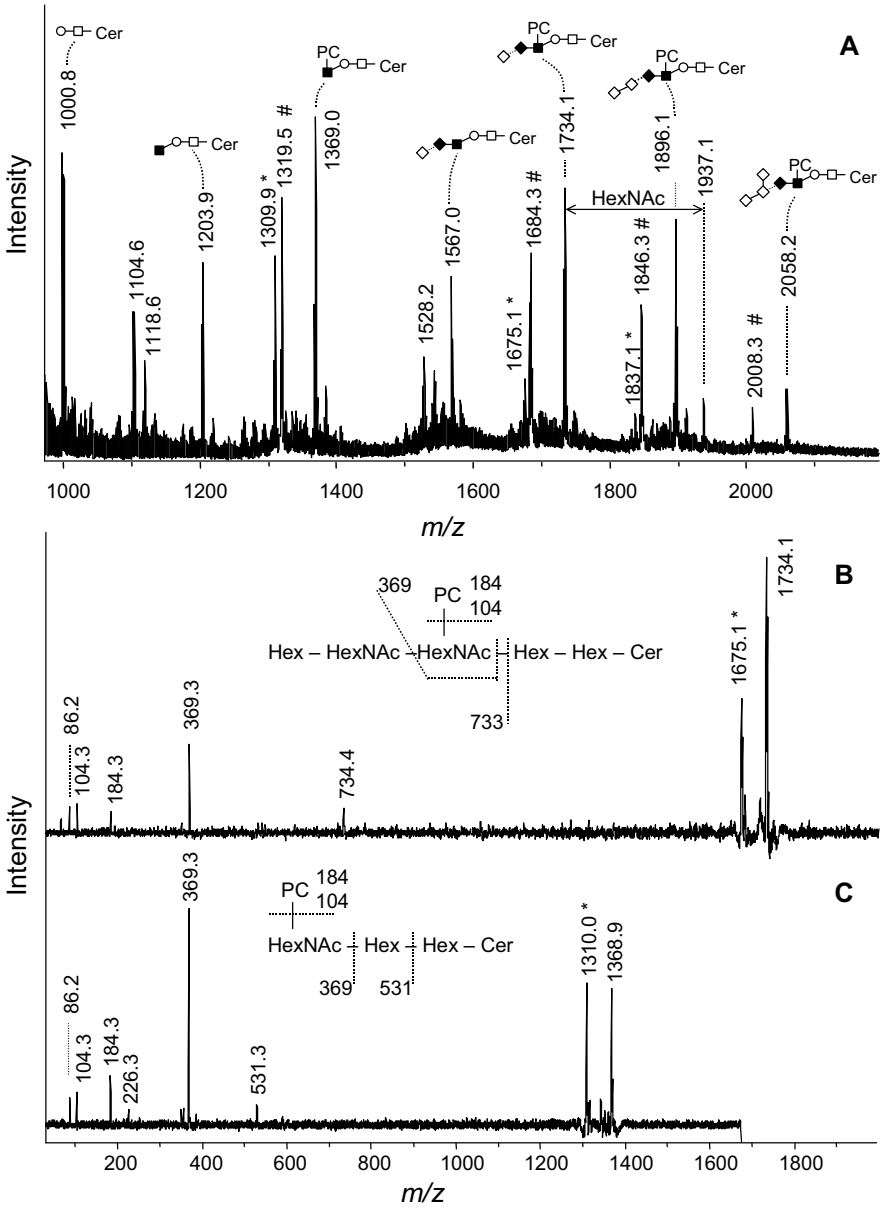


**Figure 2.1.** Mass spectrometric characterization of *A. lumbricoides* glycolipids.
((A) MALDI-TOF-MS of *A. lumbricoides* glycolipids in the positive-reflector mode. (B, C) MALDI-TOF/TOF-MS fragment ion spectra of two *A. lumbricoides* glycolipids. Fragment ions were registered for the two species at *m/z* 1734 (B) and *m/z* 1369 (C) observed in (A). The following symbols are used: open circle, mannose; open square, glucose; filled square, *N*-acetylglucosamine; open diamond, galactose; filled diamond, *N*-acetylgalactosamine. Lines between monosaccharide residues were placed as follows: continued line, β-linkage; dashed line, α-linkage; vertical line, 6-linkage; horizontal line, 4-linkage; oblique line, 3-linkage; Hex, hexose; HexNAc, *N*-acetylhexosamine; Cer, ceramide; PC, phosphorylcholine; #, metastable fragment arising from PC-containing glycolipids; *, loss of trimethylamine from the choline group (Δm=59 Da).

either removes the total PC molecule, or the choline part only. HF-treatment was monitored by MALDI-TOF-MS, which indicated the specific removal of the PC substituent, resulting in a preparation of unsubstituted nematode-type glycolipids of 2 to 5 monosaccharide residues (data not shown).

**IgG antibody responses to *Ascaris* lipids**

The IgG reactivity to *Ascaris* glycolipids was measured in plasma samples of 61 subjects (table 2.1) from Makassar, Indonesia, as well as in plasma samples obtained from 14 European controls. *Ascaris* glycolipids were recognized by IgG antibodies and IgG reactivity increased with increasing infection intensity (figure 2.2) with antibody levels in heavily infected children being significantly higher than in the European controls (p=0.017). In order to determine the role of phosphorylcholine (PC) as an epitope for antibody recognition of *Ascaris* glycolipids, the same experiments were performed in the presence of free soluble PC. In the presence of soluble PC the antibody reactivity decreased in all Indonesian groups, with the decrease reaching statistical significance in the group of heavily infected children (figure 2.2). In addition to the competition assay, the PC moieties were removed from *Ascaris*-derived lipids by hydrofluoric acid (HF) treatment. HF treatment abolished antibody reactivity in all groups (figure 2.2), showing PC plays a major role in recognition of *Ascaris* glycolipids by IgG.

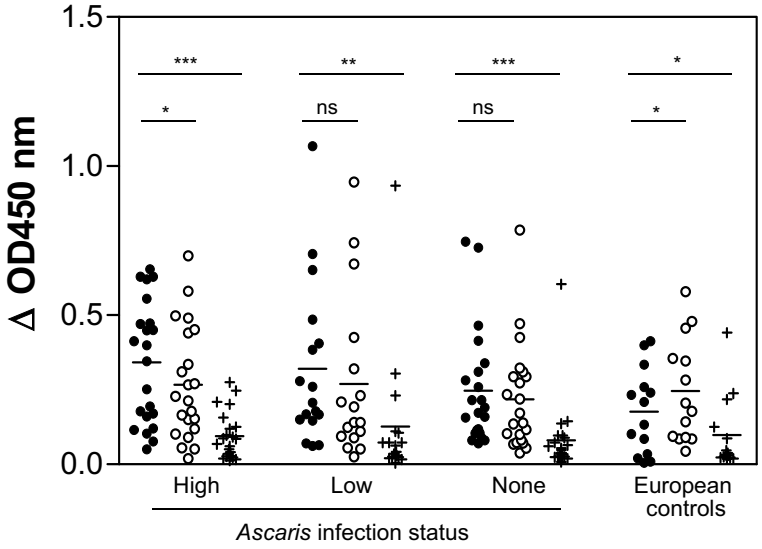


**Figure 2.2.** Reactivity of total IgG to *Ascaris* lipids. Antibody binding is plotted as ΔOD450 nm (OD coat with lipid – OD coat with methanol). Filled circles; no treatment, open circles; competition with PC, plus sign; HF treatment of the glycolipids. Within the groups antibody activity without treatment is compared to competition with PC (short line) or HF treatment (longer line) *: p<0.05, **: p <0.001, ***: p<0.0001, ns: not significant.

**Total IgG responses to *Ascaris* proteins**

In contrast to the IgG response to glycolipids, the highest IgG reactivity to *Ascaris* proteins was found in children with light infections; their antibody reactivity being significantly higher than endemic (p=0.016) or European (p=0.007) controls (figure 2.3). Although not statistically significant, children with high infection levels showed lower IgG reactivity to *Ascaris* proteins (p=0.11), suggesting that high intensity infections lead to diminished IgG responses to proteins.

Upon competition with soluble PC, IgG reactivity of the European controls was almost completely abolished (figure 2.3). The antibody responses of the infected groups and the endemic controls also showed a significant reduction in these competition experiments, indicating that PC plays a considerable role in antibody reactivity to *Ascaris* proteins. However, unlike European plasma samples, in Indonesian plasma samples substantial reactivity to *Ascaris* proteins remained after PC reactivity had been competed away. Again the highest IgG reactivity was seen in the lightly infected group, followed by those carrying intense infections (p=0.052). Both of the infected groups with light and heavy infections showed significantly higher remaining IgG reactivity than endemic (p=0.0003 and 0.03 respectively) and European (< 0.0001 and p=0.0005) controls. Proteins were not treated with HF in addition to the competition assay, as this treatment variably leads to precipitation of the proteins (Wuhrer, unpublished observation).

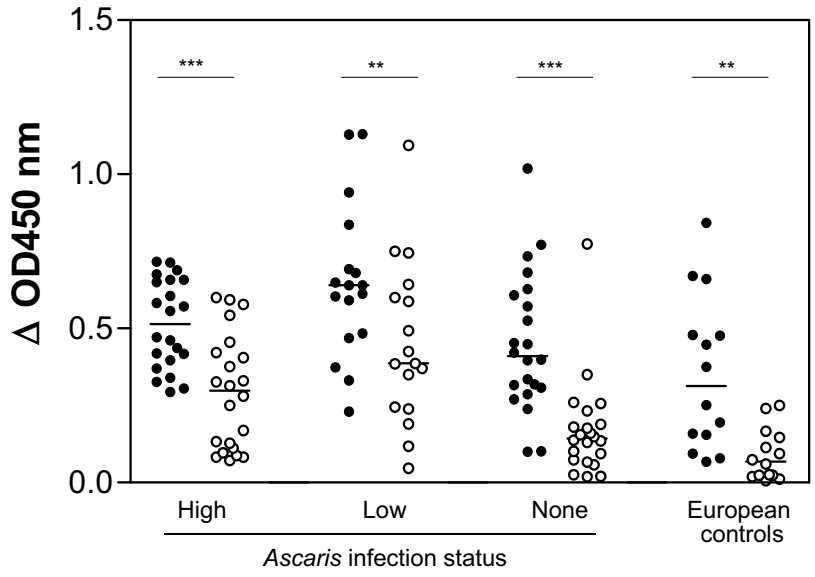


**Figure 2.3.** Reactivity of total IgG to *Ascaris* proteins. Antibody binding is plotted as ΔOD450 nm (OD coat with protein – OD coat with PBS). Filled circles; no treatment, open circles; competition with PC. Within the groups antibody activity without treatment is compared to competition with PC, indicated by a line. **: p <0.001, ***: p<0.0001.

**IgE and IgG4 antibody responses**

Since IgG4 and IgE are reported to be important antibody isotypes in helminth infections in the context of immunity, infection or reinfection, we analysed reactivity of these antibody isotypes against *Ascaris* glycolipids. With respect to IgG4 we found no responses in the European controls for either proteins or glycolipids. IgG4 antibody responses to proteins were most pronounced in the group of highly infected children ($p=0.0003$ and $p<0.0001$ compared to endemic or European controls respectively). There was a decreasing gradient of IgG4 antibodies to *Ascaris* proteins from the highly infected group to European controls (table 2.2). No IgG4 reactivity was found to *Ascaris* glycolipids in highly infected, non-infected and European control groups. Marginal levels of IgG4 to glycolipids were found in the lightly infected subjects. When examining IgE responses, highest levels of IgE to proteins were found in the group of heavily infected children (compared with endemic controls $p=0.042$), no significant differences were found between the other groups studied. IgE reactivity to glycolipids was weaker, but interestingly, individuals with light and low infections, showed higher IgE responses to *Ascaris* glycolipids compared with those with high intensity of *Ascaris* infections who had no detectable IgE reactivity to glycolipids. In addition, specific IgE reactivity to the proteins was positively correlated with total IgE ($rho = 0.39$, $p = 0.0022$) whereas no correlation was found between total IgE and IgE to *Ascaris* glycolipids.

**Table 2.2.** IgG4 and IgE antibody reactivity to *Ascaris* proteins and lipids (ΔOD values).

| | *Ascaris* infection status | | | |
|---|---|---|---|---|
| | High | Low | None | European controls |
| Protein | | | | |
| IgG4 | 0.137 | 0.073 | 0.028 | 0.010 |
| IQR | 0.049-0.397 | 0.024-0.377 | 0.010-0.047 | 0.010-0.010 |
| IgE | 0.247 | 0.226 | 0.195 | 0.213 |
| IQR | 0.183-0.314 | 0.162-0.286 | 0.160-0.218 | 0.167-0.275 |
| Lipid | | | | |
| IgG4 | 0.011 | 0.019 | 0.012 | 0.010 |
| IQR | 0.010-0.010 | 0.010-0.028 | 0.010-0.012 | 0.010-0.010 |
| IgE | 0.016 | 0.041 | 0.033 | 0.019 |
| IQR | 0.010-0.014 | 0.010-0.096 | 0.012-0.078 | 0.010-0.038 |

*Values below a ΔOD of 0.01 were regarded as negative and were given the value 0.01. IQR: Interquartile range.*

## Discussion

Mass spectrometric characterization of the glycolipids of *A. lumbricoides*, obtained from infected Indonesian children, revealed for the first time that typical nematode-type structures are present in this species. In particular, the similarity to glycolipids of *A. suum* [130, 132] is very high, as indicated by the obtained MALDI-TOF-MS and MALDI-TOF/TOF-MS data. Structural features of glycolipids including oligosaccharide backbone, substitution with PC, and ceramide composition are to a large extent shared between all the parasitic nematode species for which glycolipids have been characterized so far, including *A. suum* [130, 132], *O. volvulus* [136], and *A. vitae* [138]. Notably, also the non-parasitic nematode *C. elegans* exhibits these PC-substituted glycosphingolipid structures [135] and it has just recently been shown that they have a role in development and fertility [139], next to the established immunomodulatory role of PC-substituted glycolipids and/or glycoproteins in parasitic settings [120]. The characterization of *A. lumbricoides* glycolipids here in combination with antibody profile analysis has indicated that antibody reactivity to *Ascaris* glycolipids develops during infection and that the PC moiety, which is expressed abundantly, plays a prominent role therein.

Antibodies to glycolipids are known to play a role in infections with mycobacteria, where these antibodies have been used for diagnostic purposes [140]. The presence of antibodies to *Ascaris* glycolipids indicates that this might also be common to humoral responses in helminth infections, as antibodies to glycolipids in humans with schistosomiasis were found to play a role as well [116, 117]. Although antibodies directed to glycolipids could arise from cross-reactivity with PC present on proteins, it is also possible that antibodies develop directly to glycolipids. The pathway via which these responses are generated is still unknown, however CD1d, a nonclassical MHC molecule presenting lipids, has recently been shown to play a role in the production of *Borrelia hermsii* specific IgM by marginal zone B cells, that have high expression levels of the CD1d [141].

Interestingly, the responses to *Ascaris* glycolipids could be partially inhibited by competition with free PC, but almost completely by HF treatment. This difference might be accounted for by the fact that some antibodies will recognize only the PC moiety and can bind free PC whereas other antibodies that bind to PC, do so in the context of structures it is attached to and therefore will not be competed away by free PC alone [142]. The possibility that HF treatment destroys glycolipids was ruled out not only by mass spectrometry, but also functionally by showing intact binding of monoclonal antibodies to LacdiNAc (GalNac(β1-4)GlcNAcβ1-) structures present on glycolipids of *Ascaris* both before and after treatment with HF (data not shown).

When we examined the role of PC in antibody responses to proteins we found that competition with PC resulted in inhibition of IgG binding to *Ascaris* proteins in all groups. The effect was much stronger than seen for lipids, implying that although carbohydrate epitopes can be shared between proteins and lipids [118, 119], the epitope nature might be different. The IgG reactivity to *Ascaris* proteins was totally abrogated in European controls indicating that antibody responses to *Ascaris* proteins in European controls result from cross-reactivity with PC-epitopes present on other organisms like bacteria or fungi, to which European controls would be exposed to. As antibody reactivity in plasma from infected Indonesian subjects was only partially reduced, epitopes on *Ascaris* proteins other than PC would be expected to also be recognised by IgG in infected individuals.

The IgG responses to *Ascaris* proteins tended to be highest in lightly infected and lower in heavily infected subjects, which would either argue that these antibodies are involved in reducing worm burden or alternatively, as seen in filarial infections, that with intense infections antibody responses are downregulated [143]. The pattern of IgG reactivity to glycolipids was distinct and correlated with intensity of infection. As PC moieties were the main target of IgG reactivity to glycolipids, this indicates that anti-PC antibodies develop as a function of *Ascaris* infection intensity.

The finding that protein specific IgG4 levels were higher in infected children and, although not significantly, they tended to be even higher in the heavily infected children, supports the notion that IgG4 plays a role in susceptibility to infection and/or is associated with chronic infections, which is in agreement with findings of Palmer and Hagan [112, 144]. In contrast there was minimal IgG4 reactivity to *Ascaris* glycolipids which is in agreement with what has been reported for antibody responses to schistosomal glycolipids [117] and to filarial PC containing antigens [145], indicating that these PC-bearing molecules might suppress IgG4 levels. The mechanism behind this is still unknown, however, PC containing molecules might enhance IgG4 to IgE switching, or these molecules might induce direct IgM to IgE switching by preventing the class switch from IgM to IgG4.

In our study we found no clear differences in IgE antibodies to *Ascaris* proteins in the different groups, although elevated IgE levels to proteins were more often found in the highly infected group. In contrast, IgE to glycolipids seemed present in individuals with light or no infections and absent in those heavily infected with *A. lumbricoides* raising the possibility that such reactivity may contribute to keeping infection levels low. Interestingly, in schistosomiasis, pretreatment levels of IgE to adult worm glycolipids were negatively associated with reinfection, indicating a

possible role of these antibodies in controlling also schistosome infections.

To conclude, this study reports for the first time the structural characterization of glycolipids from *A. lumbricoides* and antibody reactivity to this class of molecules. The phosphorylcholine moiety was a major component of antibody reactivity to both *Ascaris* (glyco)proteins and glycolipids. However, IgG, IgG4 and IgE antibody reactivity profiles to *Ascaris* glycolipids and (glyco)proteins were different. This indicates that these different classes of molecules participate in overlapping, yet distinct, immune responses and when analysing host parasite interactions, not only (glyco)proteins but also glycolipids should be studied.

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