

Immunomodulatory properties of *Ascaris suum* glycosphingolipids – phosphorylcholine and non-phosphorylcholine-dependent effects

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SUMMARY

Immunomodulatory properties of phosphorylcholine (PC)-containing glycosphingolipids from Ascaris suum were investigated utilizing immune cells from BALB/c mice. Proliferation of splenic B cells induced either via F(ab')₂ fragments of anti-murine Ig (anti-Ig) or LPS was significantly reduced when the glycosphingolipids were present in the culture medium. However whereas the LPS-mediated effect was dependent on the PC moiety of the glycosphingolipids, the result generated when using anti-Ig was not. Analysis of cell cycle status and mitochondrial potential indicated that the combination of the glycosphingolipids and anti-Ig reduced B cell proliferation, at least in part, by inducing apoptosis. Consistent with the observed suppression of B cell activation/cell cycle progression, investigation of the effect of glycosphingolipid pre-exposure on mitogenic B cell signal transduction pathways activated by anti-Ig, revealed a PC-independent inhibitory effect on dual (thr/tyr) phosphorylation and activation of ErkMAPK kinase. The glycosphingolipids were also investigated for their inhibitory effect on LPS/IFN- γ induced Th1/pro-inflammatory cytokine production by peritoneal macrophages. It was found that IL-12 p40 production was inhibited and in an apparently PC-dependent manner. Overall these data indicate that PC-containing glycosphingolipids of A. suum appear to have at least two immunomodulatory constituents – PC and an as yet unknown component.

Keywords apoptosis, *Ascaris*, glycosphingolipid, immunomodulation, phosphorylcholine

INTRODUCTION

It is reasonable to postulate that the longevity of parasitic nematodes is dependent on molecular secretions that interact with the host immune system. Consistent with this, we have found that the rodent filarial nematode *Acanthocheilonema viteae* secretes a protein, ES-62 (1), that possesses many immunomodulatory properties (2). For example, ES-62 is found to reduce murine lymphocyte proliferation and polarize immune responses towards an anti-inflammatory phenotype.

In the course of studying ES-62, we elucidated that it contained a post-translational modification, the covalent addition of phosphorylcholine (PC) to an N-type glycan (reviewed in (3)). We have found this PC group to be a major contributor to the immunomodulatory effects of ES-62 (reviewed in (2,4)). The addition of PC to carbohydrate appears to be a feature of the nematode phylum, extending to a number of proteins and in addition, glycosphingolipids (reviewed in (3–5)). The immunomodulatory properties of these latter molecules are largely unexplored, but recently we have described two PC-containing glycosphingolipids of *Ascaris suum* that induce secretion of TNF- α , IL-1 and IL-6 from human peripheral blood mononuclear cells (6). That this activity was due to PC is shown by its loss when PC is removed from the molecules (6). In the present paper we further investigate the immunomodulatory properties of the PC-containing glycosphingolipids, specifically repeating a number of the experiments we undertook with ES-62. We show that *Ascaris* glycosphingolipids inhibit B cell receptor (BCR)-driven cell proliferation induced by anti-Ig but in a PC-independent manner. Although these effects on proliferation are mirrored by suppression of mitogenic ErkMAPK kinase signals, at least some of the inhibition of proliferation appears to reflect induction of apoptosis resulting from crosstalk between *Ascaris* glycosphingolipid- and BCR-derived signals. In contrast, *Ascaris* glycosphingolipids inhibit LPS-induced B cell proliferation in a PC-dependent

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manner. Such disruption of LPS signalling is not simply B cell-specific, as release of IL-12 from macrophages stimulated with LPS plus IFN- γ is also inhibited by glycosphingolipids utilizing a PC-dependent mechanism. Thus *Ascaris* glycosphingolipids can modulate both innate and adaptive host immune responses by PC-dependent and -independent mechanisms.

MATERIALS AND METHODS

Parasite materials

A. suum glycosphingolipids (6,7) and *A. viteae* ES-62 (8) were both prepared as described previously. Removal of PC from the glycosphingolipids was achieved using HF (6).

B cell preparation

B cells (>98% sIg⁺) were purified from murine BALB/c spleens as described previously (9) by negative selection using anti-CD43-coated magnetic beads according to the manufacturer's instructions (Miltenyi Biotec Ltd, Surrey, UK). Briefly, single cell suspensions of splenocytes were centrifuged (400 g) through Ficoll cushions and the enriched lymphocyte fraction harvested from the Ficoll-aqueous interface. The cells were washed and resuspended (2×10^8 cells/mL) in PBS containing 5% BSA and 2 mM EDTA and incubated with anti-CD43-coated beads (100 μ L/ 2×10^8 cells) for 20 min at 4°C. The cells were then loaded onto a MACS negative selection column and the B cells eluted, washed and resuspended in RPMI 1640 medium containing 5% FCS (Life Technologies, Paisley, UK).

B cell proliferation assay

Cells were cultured in triplicate at 2×10^5 cells/200 μ L in flat bottom microtitre wells in RPMI 1640 Glutamax I (Life Technologies) supplemented with 5% heat-inactivated FCS, 50 μ M 2-mercaptoethanol (Sigma, Dorset, UK), 2 mM glutamine, 1 mM sodium pyruvate, 50 μ M non-essential amino acids, 100 U/mL penicillin and 100 μ g/mL streptomycin (Life Technologies). F(ab')₂ fragments of anti-Ig (Jackson Immunoresearch Laboratories Inc., Stratech Scientific Ltd, Beds., UK) and LPS (Sigma) were present at 50 μ g/mL and *Ascaris* glycosphingolipids at 1 and 10 μ g/mL. 0.0185 MBq [³H]-thymidine (specific activity: 185 GBq/mmol) (Amersham Pharmacia Biotech, Bucks. UK) was added at 44 h, and cultures were harvested 4 h later with an automated cell harvester and counted for incorporated label (8).

Flow cytometry analysis of DNA content and cell cycle analysis

Cells were analysed for propidium iodide (PI) incorporation as described previously (10,11). At least 10^4 stained cells were analysed for PI fluorescence on a Becton Dickinson FACScalibur (Oxford, UK).

Flow cytometry analysis of mitochondrial potential

Incorporation of the cationic lipophilic dye DiOC₆(3) into mitochondria is proportional to the mitochondrial transmembrane potential, $\Delta\psi$ m, with low mitochondrial potential reflecting commitment to apoptosis (10). Briefly, cells were prepared as previously described (10) and resuspended at a concentration of 2×10^5 cells/mL. Cells were incubated for 30 min with 50 nM DiOC₆(3) (Molecular Probes, Eugene, OR, USA) and then washed once in PBS, pH 7.2. At least 10^4 stained cells were analysed using a Becton Dickinson FACScan using LYSIS II software for analysis.

Measurement of ErkMAPKinase activation

This was undertaken as described previously (9). Briefly, resting B cells were pre-incubated with *Ascaris* glycosphingolipids (5 μ g/mL) for 4 h and then stimulated for 0, 1, 5, 10 or 30 min. The cells were then lysed and the lysates resolved by SDS-PAGE. Phosphorylation of Erk was assessed by Western blotting using an anti-dually phosphorylated Erk monoclonal antibody (NEB, Hitchin, UK). An HRP anti-Ig enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used as indicator system.

Production of cytokines by macrophages

Thioglycollate-elicited peritoneal macrophages were prepared from BALB/c mice as described previously (12). They were cultured in 96-well plates with *Ascaris* glycosphingolipids (2 μ g/mL) for 18 h before stimulation with 100 U/mL IFN- γ + 100 ng/mL *Salmonella minnesota* LPS (Sigma). Culture supernatants were collected 24 h later and cytokines measured by ELISA as described previously (13). Antibodies against cytokines were obtained from PharMingen (San Diego, CA).

RESULTS

Ascaris glycosphingolipids interfere with murine B cell proliferation

Murine resting splenic B cells were incubated with *Ascaris* glycosphingolipids $\pm$ PC and either F(ab')₂ fragments of anti-Ig (anti-Ig) or LPS, and incorporation of [³H]-thymidine

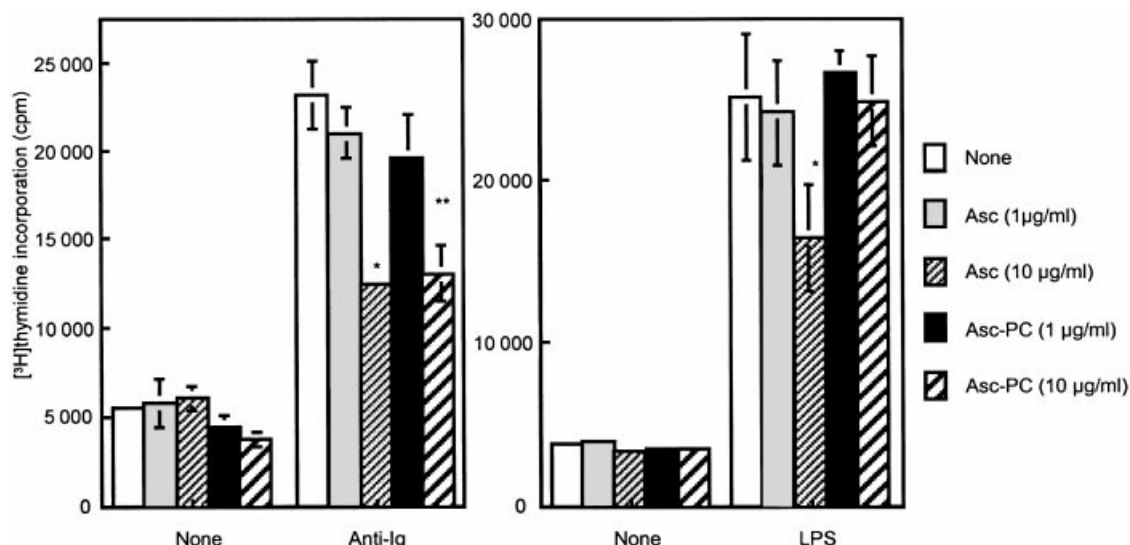


Figure 1 *Ascaris* glycosphingolipids inhibit anti-Ig- and LPS-induced DNA synthesis of B cells. Splenic B cell cultures were stimulated for 48 h with (Fab')₂ fragments of anti-Ig (50 µg/mL) or LPS (50 µg/mL) in the presence of native (Asc) or PC-free (Asc-PC) *Ascaris* glycosphingolipids at the indicated concentrations. Culture wells were pulsed with [³H]thymidine (0.5 µCi/well) 4 h prior to harvesting and thymidine-incorporation was assessed by scintillation counting. Data are expressed as mean ± SD (*n* = 3) and are representative of three independent experiments. Significant differences for anti-Ig cultures are illustrated as **P* < 0.01, ***P* < 0.006 compared with control levels. Significant differences for LPS cultures are illustrated as **P* < 0.05 compared with control levels and *P* < 0.03 compared with PC-free levels.

was measured. As expected, both polyclonal stimuli resulted in a substantial increase in incorporation (Figure 1). The presence of *Ascaris* glycosphingolipids significantly reduced this proliferation at the higher concentration employed (10 µg/mL). This reduction was found to be dependent on the PC moiety in the case of LPS stimulation but not when employing anti-Ig.

***Ascaris* glycosphingolipids inhibit anti-Ig induced B cell proliferation by causing increased apoptosis**

Resting B cells were stimulated with anti-Ig in the presence of *Ascaris* glycosphingolipids and then stained with propidium iodide to provide cell cycle analysis by DNA content (10). The combination of the glycosphingolipids and anti-Ig was found to increase the number of cells in the subdiploid phase of the cell cycle and correspondingly reduce those in mitogenic phases (Figure 2). The increase in the level of subdiploid cells is an indication of induction of apoptosis, and this was supported by a reduction in mitochondrial potential within these cells (Figure 3). Unlike *Ascaris* glycosphingolipids, the ability of ES-62 to interfere with B cell proliferation induced by anti-Ig is considered to be heavily reliant on its PC moiety (8). We have also previously found that the effect of ES-62 in reducing proliferation is not dependent on increased apoptosis (14). The results of the present study (Figure 1) therefore suggest that ES-62 and

Ascaris glycosphingolipids may act by different mechanisms and hence the *A. viteae* glycoprotein was re-examined. As expected, and unlike the *Ascaris* glycosphingolipids, it did not cause increased apoptosis (Figures 2 and 3).

***Ascaris* glycosphingolipids prime B cells for loss of ErkMAPKinase activity induced by anti-Ig**

The ability of ES-62 to interfere with B cell activation induced by anti-Ig can be correlated with changes in signal transduction molecules critical for cell proliferation (14). For example, a reduction in activation of ErkMAPKinase is consistently noted and this is considered to be dependent on PC in the sense that it can be mimicked by PC alone (2,4,9,15). Since *Ascaris* glycosphingolipids also inhibited B cell mitogenesis, we investigated their ability to prime for a subsequent reduction in ErkMAPKinase phosphorylation induced by anti-Ig. The *Ascaris* glycosphingolipids caused decreased Erk phosphorylation but this was not dependent on the presence of PC (Figure 4).

Pre-exposure to *Ascaris* glycosphingolipids modulates cytokine production in peritoneal macrophages induced by LPS/IFN-γ

ES-62 has also previously been shown to inhibit Th1/pro-inflammatory cytokine production induced by LPS/IFN-γ in

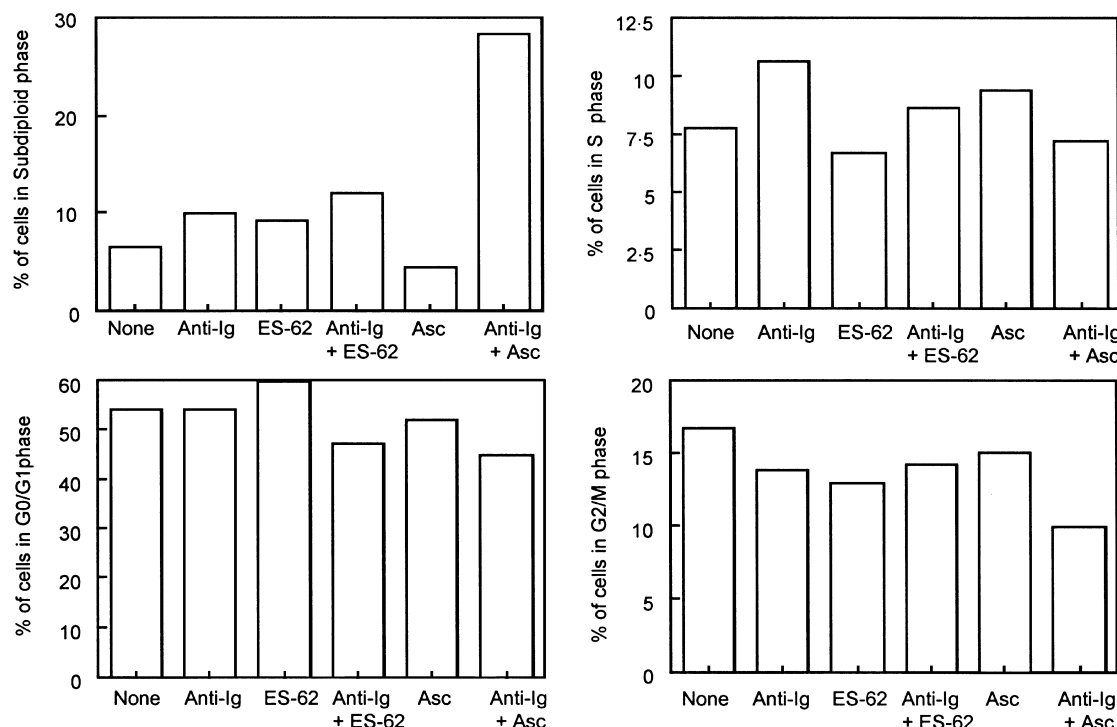


Figure 2 Co-culture with anti-Ig and *Ascaris* glycosphingolipids induces B cell apoptosis. Splenic B cells (10^6) were stimulated for 20 h with anti-Ig (50 μ g/mL) in the presence or absence of ES-62 (2 μ g/mL) or *Ascaris* glycosphingolipids (Asc; 5 μ g/mL) before determining apoptosis and cell cycle status, DNA content by propidium iodide staining and FACS analysis as described in Materials and Methods.

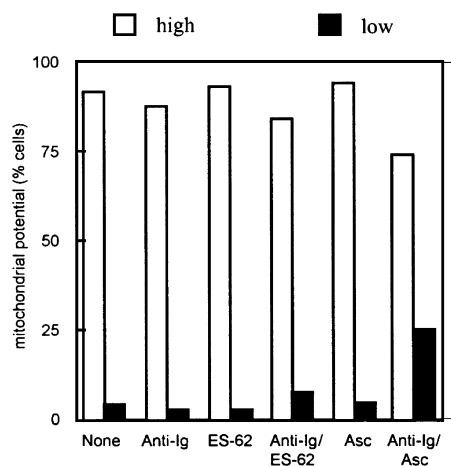


Figure 3 Co-culture with anti-Ig and *Ascaris* glycosphingolipids induces disruption of mitochondrial potential in B cells. Splenic B cells (10^6) were stimulated for 20 h with anti-Ig (50 μ g/mL) in the presence or absence of ES-62 (2 μ g/mL) or *Ascaris* glycosphingolipids (5 μ g/mL) before determining mitochondrial potential. Mitochondrial potential was determined by DiOC₆(3) staining and FACS analysis as described in Materials and Methods. Data represent DiOC₆(3) high (non-apoptotic) or low (apoptotic) cell populations as determined on a logarithmic FL-1 axis and expressed as a percentage of the total number of cells analysed.

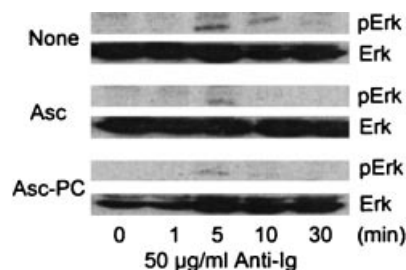


Figure 4 *Ascaris* glycosphingolipids inhibit anti-Ig-induced ErkMAPK activation in B cells. Whole cell lysates were prepared from B cells pre-incubated with either native *Ascaris* glycosphingolipids (Asc; 5 μ g/mL), PC-free *Ascaris* glycosphingolipids (Asc-PC; 5 μ g/mL), or media alone for 4 h before being stimulated with 50 μ g/mL anti-Ig for the time indicated at 37°C. Lysates were probed for activation of Erk1 and Erk2 by Western Blot analysis of dual phosphorylation (pTpY) of ErkMAPKs. Equivalence of protein loading was assessed by an antibody that recognizes total Erk expression.

murine peritoneal macrophages (12). We therefore tested the *Ascaris* glycosphingolipids in this assay and found that IL-12 p40 levels were reduced ($35 \pm 8\%$ control) in four separate experiments (Figure 5). The inhibition required preincubation with the *Ascaris* glycosphingolipids, because unlike the

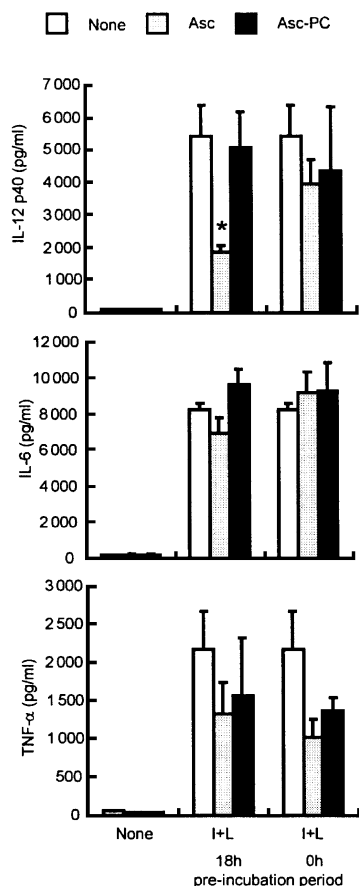


Figure 5 *Ascaris* glycosphingolipids inhibit IL-12 secretion from macrophages in a PC-dependent manner. Peritoneal macrophages were pre-incubated overnight in media, native *Ascaris* glycosphingolipids (Asc; 5 µg/mL) or PC-free *Ascaris* glycosphingolipids (Asc-PC; 5 µg/mL) before stimulation with LPS (100 ng/mL) plus IFN-γ (100 U/mL) in the presence or absence of *Ascaris* glycosphingolipids for a further 24 h. Supernatants were retrieved and measured for the production of IL-12 p40, IL-6 and TNF-α. Results are displayed as pg/mL of cytokine produced ± SD ($n = 3$) and are representative of at least three independent experiments. Significance is illustrated by * $P < 0.02$ compared with control levels. I + L = IFN-γ + LPS.

situation with ES-62 (12), no inhibition was observed in simultaneous co-cultures. The glycosphingolipids were less effective when PC was removed ($72 \pm 23\%$ control) suggesting a dominant role for this moiety and, in fact, PC-free material never produced a statistically significant reduction (Figure 5). The *Ascaris* glycosphingolipids had no significant effect on IL-6 production by LPS/IFN-γ ($78 \pm 22\%$ control and $98 \pm 20\%$ control for the native and PC-free material, respectively, $n = 3$) and no *reproducibly* significant effect on TNF-α ($65 \pm 6\%$ control and $87 \pm 24\%$ control for the native and PC-free material, respectively, $n = 3$). Moreover, again unlike ES-62 (12), the glycosphingolipids on

their own failed to induce macrophage production of the three cytokines during 42 h culture.

DISCUSSION

Previous work in our laboratories has shown that two PC-containing 'molecules', the filarial nematode glycoprotein ES-62 (reviewed in (2,14)) and a glycosphingolipid fraction of *A. suum* (6), are immunomodulatory. The latter was found by bioassay to cause release of the proinflammatory cytokines, IL-1, IL-6 and TNF-α when co-cultured with human PBMC. That this effect was likely to be due to the PC moiety was revealed when the selected glycosphingolipid, components A and C, lost their biological activity following removal of PC by HF treatment (6). Whereas these experiments targeting human PBMC represent our only investigation of the immunomodulatory properties of the glycosphingolipids to date, ES-62 has been subject to analysis for almost a decade (reviewed in (2,14)). Based mainly on studies on mice, it has been shown to affect a number of cells of the immune system, with an overall tendency to suppress pro-inflammatory immune reactivity and hence produce a phenotype that is essentially anti-inflammatory. Although only one study has been conducted on PC-depleted ES-62 (on how removal of PC influences antibody responses to other epitopes present on this molecule (16)), many of ES-62's effects can be mimicked by PC alone or conjugated to BSA (reviewed in (4,17)). Taking all of this into account, it was assumed that the glycosphingolipids would mirror ES-62 with respect to their immunomodulatory properties and hence we set out to test the PC-containing *Ascaris* glycosphingolipids in some of the assays in which ES-62 had previously scored positive.

As predicted, PC-containing *Ascaris* glycosphingolipids were found to inhibit activation of resting splenic B cells induced via the antigen receptor. This form of immunomodulation represents the first ever observed with ES-62 and was considered to be due to PC as it could be mimicked by PC-conjugated to BSA or even PC alone (8). We were therefore surprised to find that in the present study, inhibition of anti-Ig-mediated B cell proliferation could also be inhibited by PC-free glycosphingolipids. This result indicates that the glycosphingolipids contain a non-PC immunomodulatory component. Additionally of interest, however, was the finding that the PC-depleted glycosphingolipids inhibited B cell proliferation at least as well as the native *Ascaris* material. This result suggests that either the PC component of the glycosphingolipids is not active in this system or that the two immunomodulatory components are active in a way that is not additive.

Previously we have shown that the ability of ES-62 to inhibit anti-Ig-mediated B cell proliferation is not due to

apoptosis (E. H. Wilson, M. R. Deehan & E. Katz, in preparation). In the present study, the inhibitory effect induced by the glycosphingolipids was however, associated with increased apoptosis. This latter result underscores the idea initially invoked by the difference in PC-dependence, that ES-62 and the glycosphingolipids inhibit B cell proliferation by different mechanisms. Nevertheless, the key mitogenic signalling target, ErkMAPKinase is affected in both cases, presumably reflecting the induction of growth arrest (11) by both agents. However, it would appear that the two parasite molecules, in spite of both mediating inhibition of this pivotal transducer of cellular proliferation in B cells, ultimately induce distinct effects by subsequent differential modulation of putative downstream regulators of cell survival/apoptosis.

Of the two non-PC components of glycosphingolipids – carbohydrate and ceramide, the latter is perhaps more likely to be the immunomodulatory moiety. This is because ceramide has been shown to be an apoptotic agent in many cell types, including B cells (10) and references therein. The finding that treatment with *Ascaris* glycosphingolipids on their own does not induce apoptosis, but that it requires co-stimulation with anti-Ig, however, suggests that crosstalk between signals elicited by the ceramide moiety and the BCR is required for induction of cell death, presumably via an activation-induced cell death mechanism.

The PC-containing *Ascaris* glycosphingolipids were also found to inhibit LPS-induced activation of B cells. Although more difficult to show than anti-Ig-mediated inhibition (8), we had also observed this result with ES-62 (E. H. Wilson, M. R. Deehan & E. Katz, in preparation). We had not, when using the filarial nematode product however, investigated its PC-dependence. In the present work the effect of the *Ascaris* glycosphingolipids was shown to be PC-dependent. This would suggest that the inhibitory effect results from either PC targeting the LPS receptor or from crosstalk following ligation of the LPS and PC receptors. BiaCore and Far Western analysis has indicated that ES-62 binds to lymphocyte and monocyte membrane fractions with receptor-like affinity in a PC-dependent manner (MMH & WH, unpublished results). This binding could reflect the PC-receptor previously identified on B cells (18). Alternatively, as PC has been conserved by pathogens throughout evolution and can be detected in a wide range of prokaryotic and eukaryotic pathogens (4), it is possible that PC-containing pathogen products are recognized via binding of PC to pattern-recognition receptors, such as the recently identified Toll-like receptors (TLRs). TLRs have been proposed to initiate and direct innate and adaptive immune responses by recognizing conserved 'Danger signals' such as LPS on pathogens (19–21).

We have previously shown that with respect to cytokine release, the initial response of mouse peritoneal macro-

phages to ES-62 is to demonstrate a low and transient release of IL-12, IL-6 and TNF- α , before becoming refractory to further release induced by LPS/IFN- γ (12). Consistent with the idea that TLRs may act as a potential PC/ES-62 receptor, recent studies have shown that signalling via TLR2 suppresses LPS-stimulated generation of IL-12, IL-6 and TNF- α (22) in a manner reminiscent of ES-62-induced modulation of macrophage function. We could only mirror part of the ES-62-induced results with the *Ascaris* glycosphingolipids, specifically, we found strong inhibition of LPS/IFN- γ -induced IL-12, but lesser effects on TNF- α and no suppression of IL-6 secretion. Moreover, although there was evidence that the inhibitory effects on IL-12 were PC-dependent, the putative effects on TNF- α were relatively PC-independent. The failure to see any induction of cytokine production by the glycosphingolipids was also surprising, given that we have previously shown them to cause secretion of pro-inflammatory cytokines from human PBMC, as determined by bioassay (6). This presumably reflects differences in the host species and/or cell type analysed, since differences in cytokine induction by the glycosphingolipids were observed at the mRNA level when different human cell lines were investigated (GL, unpublished results).

In summary, we have found glycosphingolipids of *A. suum* to have a number of immunomodulatory properties, but not all of these are due to the PC moiety. Our next step will be to establish the nature of the non-PC immunomodulatory moiety within the molecules and to establish its relative importance with respect to PC.

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