



# Mini Review: Structure and Function of Nematode Phosphorylcholine-Containing Glycoconjugates

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An unusual aspect of the biology of nematodes is the covalent attachment of phosphorylcholine (PC) to carbohydrate in glycoconjugates. Investigation of the structure of these molecules by ever-increasingly sophisticated analytical procedures has revealed that PC is generally in phosphodiester linkage with C6 of *N*-acetylglucosamine (GlcNAc) in both *N*-type glycans and glycosphingolipids. Up to five PC groups have been detected in the former, being located on both antenna and core GlcNAc. The PC donor for transfer to carbohydrate appears to be phosphatidylcholine but the enzyme responsible for transfer remains to be identified. Work primarily involving the PC-containing *Acanthocheilonema viteae* secreted product ES-62, has shown that the PC attached to nematode *N*-glycans possesses a range of immunomodulatory properties, subverting for example, pro-inflammatory signalling in various immune system cell-types including lymphocytes, mast cells, dendritic cells and macrophages. This has led to the generation of PC-based ES-62 small molecule analogues (SMAs), which mirror the parent molecule in preventing the initiation or progression of disease in mouse models of a number of human conditions associated with aberrant inflammatory responses. These include rheumatoid arthritis, systemic lupus erythematosus and lung and skin allergy such that the SMAs are considered to have widespread therapeutic potential.

**Keywords:** anti-inflammatory drug development, ES-62, glycoconjugate, immunomodulation, nematode, phosphorylcholine

## INTRODUCTION

During the human-infectious stage of the helminthic life cycle, parasites engage in a series of actions designed to evade the host immune response (1). Initially, defensive manoeuvres such as tegument moulting and membrane turnover were described (2, 3), but it is now understood that parasites can further promote their longevity by directly manipulating host systems, *via* the release of biologically active excretory/secretory molecules (E/S). The evolution of molecular parasitology in recent years has enabled an increasingly precise characterisation of these molecules.



(Hex<sub>3</sub>HexNAc<sub>5</sub>Fuc<sub>0-1</sub>PC<sub>1-3</sub>), with attachment occurring on non-reducing GlcNAc or LacDiNAc motifs. This suggests that such PC-*N*-glycans may be conserved throughout the phylum (26, 27), although the PC-containing structures in free-living nematodes are tri-antennary rather than tetra-antennary (tetra-antennary *N*-glycans don't appear to be formed in free living species) as observed for ES-62. Moreover, PC-substituted glycosphingolipids have been reported in embryonic-stage *C. elegans*, as well as in *A. viteae*, *O. volvulus*, *O. ochengi*, *Litomosoides sigmodontis* and *A. suum* species (28–30).

## BIOSYNTHETIC PATHWAY OF PC-GLYCANS

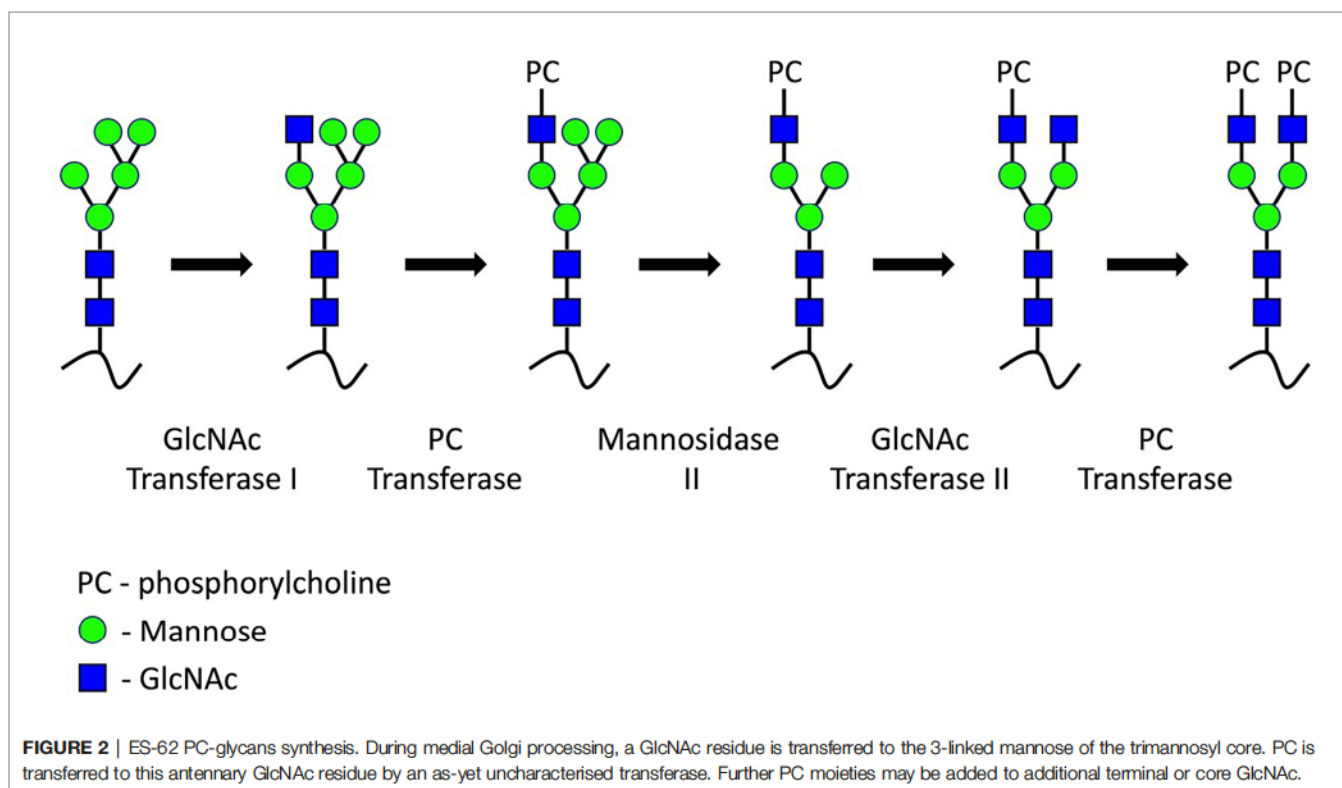
Employment of a combination of intracellular trafficking inhibitors, oligosaccharide processing inhibitors, pulse-chase radiolabelling experiments and FAB-MS analysis has permitted characterisation of the intracellular processing events which attach PC to newly produced glycans in *A. viteae* and *C. elegans* (23, 30–33). It is within the medial Golgi that the process of PC attachment to maturing *N*-glycans occurs (Figure 2). Initially, an antennary GlcNAc residue is attached to the glycan structure by GlcNAc Transferase I. PC is subsequently transferred from a donor, likely phosphatidylcholine, via a C-6 phosphodiester linkage of the antenna GlcNAc residue. Two mannose sugars are then trimmed from the core by Mannosidase II. Next, GlcNAc Transferase II adds a second terminal GlcNAc residue and then

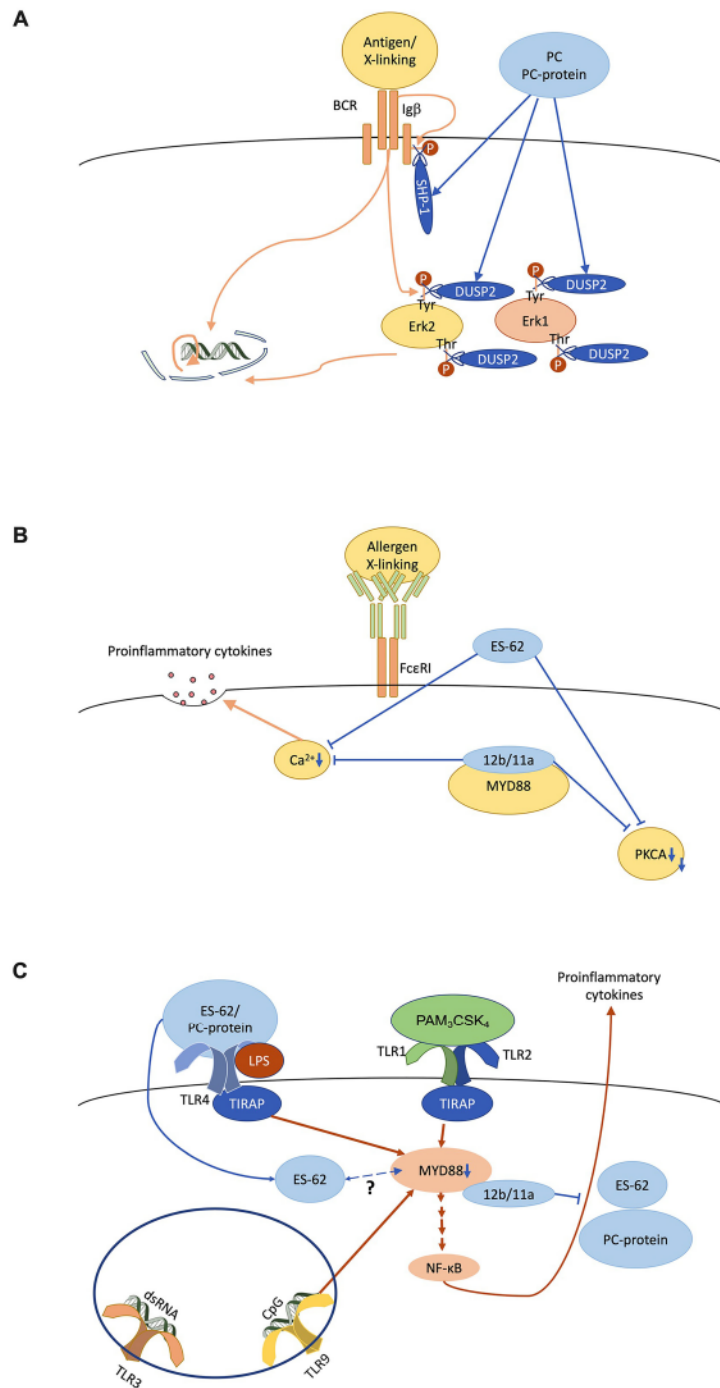
additional PC may be added. As yet, the PC-transferase enzyme involved in this process remains uncharacterised.

## FUNCTION OF NEMATODE PC-GLYCANS: STUDIES *IN VITRO*

PC on nematode glycoproteins has immunomodulatory properties as first shown by its inhibition of lymphocyte proliferation (34, 35). The mechanisms behind such activity have been extensively studied using ES-62 and to a lesser degree, PC alone or conjugated to proteins such as bovine serum albumin (BSA), or small molecule analogues (SMAs) of ES-62 based on its PC moiety (Figure 3A) (36). For example, the PC-containing molecules inhibit B cell receptor (BCR)-stimulated phosphoinositide-3-kinase (PI3K) and protein kinase C activities (35–37), as well as reducing the phosphorylation of Igβ and the adaptor Shc1 resulting in reduced Erk1/2 MAPK activation (37, 38). This appears to reflect that ES-62 promotes recruitment of the protein tyrosine phosphatase, SHP-1 resulting in rapid dephosphorylation of these BCR-stimulated substrates (38). Furthermore, ES-62 terminates ongoing Erk1/2 activity upon BCR stimulation by recruitment of dual specificity phosphatase Dusp2, which dephosphorylates their threonine and tyrosine activation motifs.

Such immunomodulatory actions have also been observed with innate immune system cells. Thus, like ES-62, PC-based SMAs 11a and 12b inhibit FcεRI-mediated signalling and degranulation, as well as proinflammatory cytokine secretion





**FIGURE 3** | ES-62/phosphorylcholine (PC) desensitises immune system cell signalling *in vitro*. **(A)** The B cell antigen receptor (BCR) signals via ITAM-containing Igα/β accessory molecules to recruit proliferative signalling pathways like the ERK MAPK cascade in a tyrosine kinase-dependent manner. PC/ES-62 desensitises the BCR both by recruiting SHP-1 to dephosphorylate Igβ and uncouple downstream signalling and by inducing DUSP2 dephosphorylation of the TEY activation motif of ERK1/2. **(B)** FcεRI-mediated degranulation requires calcium and PKCα signalling whilst, at least in some mast cell subsets, cytokine secretion also requires crosstalk with MyD88 signalling and all of these elements are targeted by PC/ES-62. **(C)** TLR4 homodimer and TLR1/2 heterodimer located at the cell surface on dendritic cells and macrophages signal through the adaptor proteins TIRAP and MyD88 in response to LPS and bacterial lipopeptide (e.g., PAM<sub>3</sub>CSK<sub>4</sub>) respectively. TLR9, which responds to unmethylated cytosine-phosphate-guanine (CpG) DNA, also signals through MyD88. MyD88 stimulates NF-κB regulated proinflammatory cytokine production. ES-62 and PC-conjugated proteins subvert TLR4-MyD88 signalling to reduce proinflammatory cytokine secretion in response to TLR1/2, TLR4 and TLR9 ligation. Small molecule analogues 11a and 12b interact directly with MyD88 thereby similarly inhibiting cytokine production. TLR3 signalling, which is MyD88-independent, is not affected by these anti-inflammatory agents. ES-62 is internalised by macrophages and may also directly interact with MyD88 via its PC moieties.

(**Figure 3B**) (39–42). This can in part be explained by reduced calcium mobilisation and protein kinase C alpha (PKC $\alpha$ ) protein expression, actions also reproduced by pre-treatment with PC-BSA (39–41). Moreover, antigen-presenting cells (APCs) like dendritic cells and macrophages, that educate adaptive immunity are also desensitised in response to ES-62 and PC (**Figure 3C**) (43, 44). Thus here, PC-ovalbumin and SMAs (11a, 12b) inhibit NF- $\kappa$ B mediated pro-inflammatory cytokine secretion stimulated *via* MyD88-dependent toll-like receptors (TLRs; TLR4, LPS; TLR9, CpG; TLR1/2 heterodimer, PAM<sub>3</sub>CSK<sub>4</sub>) (43–50). ES-62 also suppresses the synergistic NF- $\kappa$ B-mediated pro-inflammatory cytokine secretion observed in response to these stimuli when tested in combination with interferon gamma (IFN $\gamma$ ) (44, 51). In contrast, TLR3 signalling, which is independent of MyD88, is unaffected by PC-ovalbumin or ES-62 (43, 44, 52–54). This selectivity reflects that MyD88 protein expression is reduced in the presence of ES-62 or SMAs, 11a & 12b (50, 55–58) and as this downregulation is also recapitulated in certain mast cell subsets (41) and lymphocytes (50, 55–59), it provides a unifying primary target for PC/ES-62 action in limiting chronic inflammatory responses.

PC, either as part of ES-62 or attached to ovalbumin, appears to “signal” through TLR4 (43, 44). Unlike LPS, the most defined TLR4 ligand, PC maintains its activity in the context of a proline to histidine substitution at position 712 on the receptor (43, 44, 60, 61). This residue sits within a cytoplasmic domain required for interaction with the signalling adaptor Mal (itself required for TLR4 interaction with MyD88), which suggests PC signals through a different mechanism to LPS (62, 63). Indeed, SMAs 11a and 12b interact directly with the TIR domain of MyD88, inhibiting homodimer formation (56). As ES-62 is internalised in both mast cells and macrophages, it is possible that the parent molecule also interacts with MyD88 through its PC moieties (40, 64).

Complement is an innate mechanism that recognises microbial PAMPs and facilitates microbicidal activity; it is also intensely activated by PC, which has bound to C-reactive protein (65–68). PC directly attached to proteins also activates complement *in vitro*; but complement activation drops considerably when PC is attached at the end of either synthetic flexible linkers, or importantly, to glycans such as in ES-62 (69, 70). This functionality may benefit parasitic worms by sequestering components of the complement cascade, resulting in a low complement activation state.

## PC IN MURINE MODELS OF DISEASE

PC on the glycans of ES-62 is also active *in vivo*, for example, it alters the subclass of antibody produced in response to ES-62 in mice, in that removal of PC results in the generation of IgG2a antibodies that are absent when the intact molecule is employed (71). Moreover, exploring the potential of a potent anti-inflammatory molecule like ES-62 as a biologic intervention in human disease has been dependent upon robust pre-clinical evaluation using *in vivo* experimentation. In fact, ES-62, and

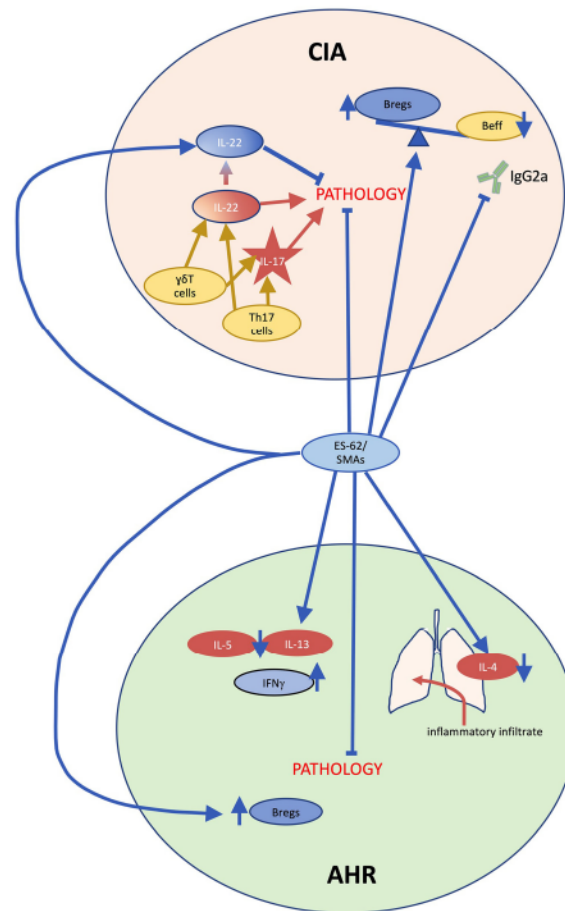
the drug-like SMAs represent the only stand-alone nematode PC-products that have been tested *in vivo*, although the suppression of rodent arthritis by *A. suum* extract is suspected to be attributed to a homologous PC-bearing molecule (72). These treatments have been demonstrated to be safely tolerated throughout the summarised experiments below, with potency illustrated in the resolution of a range of inflammatory conditions, irrespective of phenotype.

## Arthritis

The performance of ES-62 has been most thoroughly described in the collagen-induced arthritis (CIA) model of rheumatoid arthritis, where it has displayed a potent ability to protect against disease development when delivered prophylactically, and to ameliorate established CIA when administered therapeutically (73–76). Induction of CIA in DBA/1 mice instigates articular inflammation dominated by Th17 cells (77, 78) and clinical indicators of arthritic disease, including paw thickness, erythema and loss of function, are reduced or absent in ES-62-treated mice (**Figure 4**), with histological analyses confirming protection against the synovial hyperplasia and cartilage erosion traditionally seen in this condition (73). In comparison to vehicle controls, ES-62-treated mice demonstrate a skewing towards IL-10-producing regulatory B cell (Breg) phenotypes, with a downregulation in effector cells bearing CD80, CD86 and TLR4/MyD88, and a dramatic curtailing of IL-17 production by Th17 and  $\gamma\delta$ T cells (58, 73). Further molecular studies have proposed that in resolving CIA, ES-62 targets signalling cascades which drive effector T cell migration and activation. Somewhat unexpectedly, suppression of IL-22 neutralised ES-62’s joint-protective effects, with the data suggesting dual pathogenic and protective roles in the initiation and established phases of disease for this cytokine: indeed, exposure to ES-62 upregulated protective IL-22 production by  $\gamma\delta$  T cells (75, 79). Most recently, it has become clear that the gut-bone marrow axis of immune regulation plays a key role in ES-62’s anti-inflammatory actions, with normalisation of gut microbiota being associated with promotion of Bregs and suppression of osteoclast differentiation (76, 80). Treatment in the CIA model with PC-BSA, SMA 11a or SMA 12b also significantly suppressed arthritic pathology (50, 55). Whilst SMA 11a appeared to mimic the suppression of IL-17-polarised inflammation, interestingly, SMA 12b instead appeared to act predominantly *via* reduction of IL-12p40 and IL-1 $\beta$ : thus, these SMAs appeared to target differential downstream effectors of MyD88-NF- $\kappa$ B signalling.

## Systemic Lupus Erythematosus (SLE)

ES-62 has displayed efficacy in two separate models of SLE-induced pathology. In characterising the renal components of disease progression, MRL/*Lpr* mice receiving ES-62 demonstrated a significant and consistent reduction in proteinuria, coupled with a resistance to arthritogenesis and anti-nuclear antibody (ANA) production, evidenced at termination (81). Protection against autoimmune inflammation was again associated with increased IL-10-producing Bregs in the kidneys and spleen. PC-based SMAs also proved effective in this model, with both inhibiting IL-6 responses but whilst 12b was



**FIGURE 4** | ES-62/PC-mechanism of action in collagen-induced arthritis and ovalbumin-induced airway hyperresponsiveness. In collagen induced arthritis (CIA), during the initiation phase,  $\gamma\delta$ T cells and T helper (Th)17 cells secrete interleukin (IL)-17 and IL-22 causing joint pathology. By contrast, during established disease IL-22 acts to limit pathology, indicating that this cytokine plays dual pathogenic and protective roles in CIA. Treatment with ES-62 or small molecule analogue (SMA) 11a inhibits IL-17 production and ES-62 promotes protective IL-22 production as well as restoring regulatory B cell (Breg) responses to counter pathogenic effector B cell (Beff) responses including collagen-specific IgG2a production, thereby substantially reducing arthritis. In ovalbumin-induced airway hyperresponsiveness (AHR), inflammatory immune system cells such as eosinophils and neutrophils infiltrate the lungs, generating IL-4 production, and a Th2 anti-inflammatory immunological phenotype is evident in immune cells in draining lymph nodes as the cells secrete IL-5 and IL-13 upon re-stimulation *ex vivo*. ES-62 and SMA administration reduce the infiltration of inflammatory immune cells and the former has also been shown to inhibit the *ex vivo* production of IL-5 and IL-13. Moreover, ES-62-activity in this model has been found to be dependent on IFN- $\gamma$  and in addition, similar to CIA, the helminth product increases IL-10-producing Bregs.

more effective in preventing ANA deposition, 11a was more potent in suppressing proteinuria (59).

In the *Gld.apoE<sup>-/-</sup>* model of accelerated atherosclerosis in lupus (82), mice maintained on a Western-style high cholesterol diet were continuously delivered ES-62 by osmotic pump to mimic serum levels during active infection (83). At termination, examination of aortae revealed a dramatic ES-62-mediated reduction in lesion area (approximately 60%), which was associated with a decrease in collagen deposition and macrophage frequency. Cholesterol levels and lymphadenopathy remained unchanged. Distinct, but non-significant, trends in glomerular cellularity and proteinuria were also detected. Of note, the therapeutic effects in ES-62-treated mice were not attributable to generation of antibodies to ES-62's PC moieties despite recent findings that such antibodies, which cross-react with

PC-containing oxidised low-density lipoproteins, may prevent atherosclerosis by inhibiting lipoprotein uptake by macrophages (see below).

### Asthma

Inflammatory damage in ovalbumin (OVA)-induced airway hyperresponsiveness, a mouse model of asthma, is ablated by prophylactic administration of ES-62 (40, 84). Administration of ES-62 robustly inhibited eosinophil, neutrophil, and lymphocyte infiltration upon inflammatory challenge (Figure 4). This is associated with a suppression of Th2-like responses, with significant reductions in IL-4 in lung tissue at termination, and downregulation of IL-13 and IL-5 and upregulation of IFN $\gamma$  production in *ex vivo* draining lymph node cultures. Increased expression of the regulatory T cell marker FOXP3 was not

observed in ES-62-treated mice, in contrast with the mechanism reported for other helminth molecules in suppressing the OVA-induced asthma model (85, 86). Subcutaneous treatment with SMAs 11a or 12b induced a similarly protective phenotype, associated with a distinct reduction in lung neutrophil and mast cell populations and in the chronic model of asthma, such protection was associated with a decrease in lung MyD88 levels and an increase in splenic Bregs (87). There was again no indication of any elevation in FOXP3 or Treg responses.

### Fibrotic Lung Disease

SMAs 12b and 11a have also performed strongly in the LPS- and bleomycin-induced models of fibrotic lung disease, reducing collagen deposition, structural corruption and cell infiltration to levels comparable with the dexamethasone control (88). Two additional SMAs derived from 11a (16b and AIK-29/62) were also tested, and displayed a similar potency in reduction of collagen deposition and lung tissue weight at termination.

### Dermatitis

The effect of ES-62 on mast cell function was also assessed in the oxazolone sensitisation model of skin hypersensitivity (40). Treated mice displayed a 60–70% reduction in ear swelling, which correlated with an absence of TNF $\alpha$ , IL-3, and IL-6 mRNA. Degranulation of mast cells captured from sensitised and challenged mice treated with ES-62 was also inhibited *ex vivo*, although no reduction in Fc $\epsilon$ RI expression was detectable, implying an interference in the activation cascade in the generation of these effects. Treatment with SMAs 11a and 12b similarly suppresses ear thickening in this model, with reductions of up to 82% being measured in comparison to the negative control (50). Interestingly, no reduction in TNF $\alpha$  or IL-6 mRNA was detected, suggesting that in this model the SMAs may show some differences in mechanism to native ES-62.

### Obesity-Accelerated Ageing

In this most recent study, Crowe et al. (89) report the effects of ES-62 administration on ageing-induced ill health, in the context of mice being fed a high calorie 'Westernised' diet throughout life. ES-62 was shown to protect against ageing-related liver fibrosis, with collagen deposition severely limited in both male and female ES-62-mice up to 500 days of age. Treatment with a combined dose of SMAs 11a and 12b also showed protection against liver fibrosis, particularly in female mice, whilst male mice were noted to experience reduced metabolic dysregulation over a time course of some 340 days (90). Interestingly, in male, but not female mice, treatment with ES-62 extended the median lifespan, inhibited late-ageing weight loss, and significantly reduced age-related ileal and colonic erosion, whilst normalising gut microbiota.

### Type 1 Diabetes, Multiple Sclerosis and Colitis

It should be noted that ES-62 and its PC-based SMAs do not resolve all chronic inflammatory responses, failing to afford protection in mouse models of Type 1 diabetes, multiple sclerosis and colitis (91).

## SERENDIPITOUS INDUCTION OF ANTI-INFLAMMATORY ANTIBODIES

Anti-PC antibodies have been found to reduce inflammation in ischemic mouse models and are plentiful in human subjects at low risk of atherosclerosis (92–94). In addition, in the context of rheumatic disease, an inverse correlation between anti-PC antibodies and organ damage and disease activity in SLE patients has been demonstrated (95, 96). Although the exact mechanism(s) underlying the protective effects of anti-PC antibodies is still under investigation, anti-inflammatory and cardioprotective benefits of anti-PC antibodies in SLE and also Sjögren's syndrome and mixed connective tissue disease are associated with regulatory T cell polarisation, oxidised low density lipoprotein uptake inhibition and enhanced apoptotic cell clearance (96–99). Interestingly, IgM anti-PC antibodies are greatly increased in response to ES-62 in murine models of lupus and in high calorie diet (HCD)-fed mice (82, 89). These antibodies were not cross-reactive to oxidised low density lipoprotein nor conformed to the T15 idiotype (characteristic of most of the protective anti-PC antibodies reported) (82, 93, 100, 101). Nevertheless, machine learning identified anti-PC IgM levels as the best predictor of effective ES-62 treatment in HCD-fed mice (89). Thus, overall, it would perhaps be premature to rule out a protective role for ES-62-induced anti-PC antibodies in terms of protecting the host against obesity-accelerated ageing at this stage. Also of interest, natural IgM antibodies appear to promote a regulatory phenotype of B cells, which then reduce inflammation *in vivo* (102). Natural antibodies are secreted by B1 B cells independently of T cell stimulation, have limited diversity and recognise multiple antigens (103–107). Indeed, ES-62 stimulates B1 B cell activation *in vivo* and increases regulatory B cell numbers in several *in vivo* models (74, 76, 81, 87, 108).

## CONCLUSION AND FUTURE DIRECTIONS

Increasingly sophisticated analytical procedures are being applied to the elucidation of the structure of nematode PC-containing glycoconjugates. This has revealed a general uniformity across the nematode phylum and also that these novel carbohydrate structures may have not one, but multiple, PC groups attached. PC-containing nematode glycoconjugates possess immunomodulatory properties such that they are potentially therapeutic but full exploitation of this with respect to PC-containing glycoproteins such as ES-62 is handicapped by an inability to produce fully active recombinant forms, as no convenient protein expression system exists that encodes the (unidentified) requisite PC transferase enzyme (109–112). PC-based SMAs can alternatively circumvent this problem and may represent the form in which active nematode molecules make it to the clinic. At the same time, three issues which remain to be resolved are: (i) increased understanding of the molecular complexity underlying the range of immunomodulatory activities of secreted PC-containing proteins like ES-62. For example, we have learned of how ES-62 is reliant on expression

of TLR4 for PC-dependent activity against macrophages and dendritic cells (46), but it appears that additional receptors may exist in cells such as lymphocytes with which ES-62 can interact in a PC-dependent manner (66). Moreover, ES-62's modulation of immune cell function can be associated with effects on multiple cell signalling pathways, e.g., in mast cells, ES-62 impacts on cross talk amongst TLR4-, ST2- and FcεRI-dependent pathways (113), highlighting the level of complexity that may need to be dissected. (ii) a greater understanding of the function of PC-glycoconjugates found as internal rather than secreted products of nematodes – this is unlikely to relate to immunomodulation because as described earlier the structures are found within free-living species in addition to parasitic species. One possibility relates to growth and development. Consistent with this, chemical blockage of production of PC-containing glycosphingolipids by targeting enzymes upstream of PC addition impairs embryonic development in *C. elegans*, although a direct role for PC has not been shown (114). (iii) the identity of the aforementioned nematode enzyme which transfers PC to carbohydrate – as such

structures are absent from mammals, this enzyme if identified could offer a potential novel drug target. Interestingly, we have shown previously that enzymes acting upstream of the PC transferase in *C. elegans* can be knocked down by RNAi and that this results in reduced transfer of PC to proteins (115). We believe this approach could be employed in investigating the identity of the PC transferase when applied to genes of likely related function based on sequence homology in nematode genomes. Similarly, CRISPR (112) knockdown of potential related genes offers a more recent but similar approach, as do proteomics approaches focusing on the site of PC transfer, the Golgi. Of benefit to these analyses should be an *in vitro* assay of PC transferase activity developed by Cipollo and colleagues (33).

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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