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Review

Modulation of the host immune system by phosphorylcholine-containing glycoproteins secreted by parasitic filarial nematodes

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Abstract

Phosphorylcholine (PC) is increasingly becoming recognised as a carbohydrate-associated component of a wide variety of procaryotic and eucaryotic pathogens. Studies employing nematode PC-containing molecules indicate that it possesses a plethora of immunomodulatory activities. ES-62 is a PC-containing glycoprotein, which is secreted by the rodent filarial nematode *Acanthocheilonema viteae* and which provides a model system for the dissection of the mechanisms of immune evasion induced by related PC-containing glycoproteins expressed by human filarial nematodes. At concentrations equivalent to those found for PC-containing molecules in the bloodstream of parasitised humans, ES-62 is able to inhibit antigen receptor-stimulated proliferation of B and T lymphocytes in vitro and in vivo. The active component of ES-62 appears to be PC, as PC conjugated to albumin or even PC alone broadly mimic the results obtained with ES-62. PC-induced impaired lymphocyte responsiveness appears to reflect uncoupling of the antigen receptors from key intracellular proliferative signalling events such as the phosphoinositide 3-kinase, protein kinase C and Ras mitogen-activating protein kinase pathways. Although PC-ES-62 can desensitise B and T cells, not all cells are affected, and in fact it is still possible to generate an antibody response to the molecule. Dissection of this response indicates that it is of the TH-2 type. This appears to reflect the ability of ES-62 to direct the polarity of the T cell response by suppressing the production of proinflammatory cytokines, inducing the induction of anti-inflammatory cytokines and by driving the maturation of dendritic cells that direct TH-2 T cell responses.

Keywords: Cytokine; Immunomodulation; Lymphocyte; Nematode; Phosphorylcholine; Signal transduction

1. Introduction

1.1. Filarial nematode parasites and evasion/modulation of the host immune system

Filarial nematodes are arthropod-transmitted parasites of vertebrates including humans. Of the eight species, which are known to infect humans, three, *Wuchereria bancrofti*, *Brugia malayi* and *Onchocerca volvulus*, represent major causes of morbidity in the Tropics [1]. It is currently estimated that in the region of 150 million people are infected with one or more of these three worms and a significant proportion of these individuals suffer debilitating health problems. These include elephantiasis, severe and chronic skin lesions and several forms of eye damage, all of which can lead to blindness.

Infection of humans (or animals) with filarial nem-

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atodes is long term with individual worms surviving for in excess of 5 years [2]. The consensus of opinion amongst workers in this field is that such longevity reflects suppression or modulation of the host immune system and indeed 'defects' in immune responsiveness have been revealed in infected individuals [3,4]. The exact nature of these defects is uncertain: there appears to be a lack of uniformity in findings from the many studies undertaken to define them. Nevertheless, in general the defects incorporate impairment of lymphocyte proliferation and bias in production of both cytokines and antibodies. With respect to cytokines the bias can be shown in reduced IFN-γ and increased IL-10. For antibodies, there are imbalances in IgG subclasses: greatly elevated IgG4 (an antibody of little value in eliminating pathogens due to an inability to activate complement or bind with high affinity to phagocytic cells), and decreases in other IgG subclasses. Overall therefore, the picture is of an immune response demonstrating a somewhat suppressed, anti-inflammatory phenotype which is often classified as 'TH-2' ('TH' is derived from a category of T lymphocyte referred to as 'helper'). It has been speculated that such a phenotype is conducive to both parasite survival and host health (the latter by limiting pathology resulting from an aggressive immune response). At present, the mechanism underlying its induction is probably the most frequently addressed question in research into the immunology of filariasis.

1.2. *Phosphorylcholine: an immunomodulator during filarial nematode infection?*

Phosphorylcholine (PC) is an abundant component of eucaryotes, both vertebrates and invertebrates, where it is found as the polar head group of the phospholipids, phosphatidylcholine and sphingomyelin. However, in certain bacteria, fungi and lower invertebrates including filarial nematodes, PC has been found in a different association, specifically, attached to carbohydrate (reviewed in [5]). In filarial nematodes, PC has been found attached to both glycolipids [6] and glycoproteins (reviewed in [7]). Some of the latter are secreted by the worms and can indeed be detected in the bloodstream of infected individuals [8]. A major puzzle in filarial nematode research is the role of PC on these secreted proteins.

PC is a chemical group known to a generation of immunologists due to its potent immunogenicity making it a suitable antigen for use in experiments investigating the generation and diversity of the antibody response [9,10]. This potent immunogenicity also dictates that it can act as a dominant hapten during nematode infections. Thus many human filariasis patients have significant anti-PC responses [11–13], strong anti-PC responses appear rapidly during experimental infections of animals [14–16] and indeed the latter may dominate the antibody response to the parasite. Likewise, PC appears to be *a*, if not *the*, major antigenic determinant of certain species of bacteria, e.g., *Streptococcus pneumoniae* [17,18]. However, occasionally during the last 25 years, a paper has appeared in the literature, which questions the idea that PC's effect on the immune system is simply to activate it. In 1976, for example, Mitchell and Lewers [14] showed that conjugation of PC to some molecules reduced their immunogenicity. In 1991, Sloan and colleagues [19] demonstrated that successful immunotolerance against PC was associated with increases in antibodies to other epitopes in *Fasciola hepatica*-infected mice. In 1998 Bordmann and coworkers [20] indicated that exposure of mice to PC resulted in TH-2 cytokine production. Such effects on the immune system are to some degree consistent with what is observed during filarial nematode infection. For this reason we explored the idea that the function of PC on secreted nematode proteins is to modulate the host immune response.

2. Effect of phosphorylcholine on lymphocyte signal transduction pathways

2.1. *Inhibition of lymphocyte proliferation by PC on ES-62*

It has been known for almost two decades that a percentage of B lymphocytes (murine and human) expresses a non-immunoglobulin receptor for PC [21]. Interaction of this receptor with PC-containing *S. pneumoniae* results in the polyclonal secretion of antibody from the cells [22]. It may therefore be predicted that interaction of PC-containing molecules of filarial nematodes with B lymphocytes would also result in polyclonal antibody production. This in

fact may well be the case as incubation of ES-62, a well-characterised PC-containing molecule secreted by the rodent filarial nematode *Acanthocheilonema viteae* [23], with small resting spleen-derived murine B lymphocytes results in cell activation as measured by DNA synthesis [23]. However, this effect of ES-62 was noted when 'high' concentrations were employed (25–50 μg ml^{-1}). Interestingly, at concentrations 10–100-fold less, i.e., within the range at which PC-containing molecules can be found in the human bloodstream [24], ES-62 does not cause polyclonal stimulation. Rather, it acts to prevent (by up to 60%) proliferation of B lymphocytes associated with ligation of the antigen receptor [23]. This effect of ES-62 is almost certainly due to PC as PC conjugated to bovine serum albumin (BSA) or even PC alone can mimic it. Furthermore, weekly injections of PC–BSA (10 μg) into mice was found to reduce the ability of recovered B lymphocytes to be activated via the antigen receptor [25]. These latter inhibitory effects of ES-62/PC on subsequent activation of B lymphocytes suggests that exposure to low levels of PC may actually anergise B cells. This will be discussed in some detail later.

It was also found that ES-62 was able to inhibit polyclonal activation via the antigen receptor of the human T cell line Jurkat [26] and again this effect appears to be mainly due to PC [25]. These results are consistent with an earlier finding that PC-containing molecules isolated from a whole worm extract of the human filarial nematode *Brugia malayi* could inhibit activation of human T lymphocytes induced by the mitogen phytohaemagglutinin [27]. Inhibition of T lymphocyte proliferation during filarial nematode infection has been a much more frequently documented phenomenon than the same effect on its B lymphocyte counterpart. However, we are only currently beginning to investigate whether PC contributes to this in vivo.

2.2. Mechanism of action of ES-62/PC

We have undertaken considerable effort in attempting to elucidate how PC inhibits lymphocyte activation associated with ligation of the antigen receptor. Revealingly, although the concentrations of ES-62 which interfere with activation of B lymphocytes induce no activation of the cells per se as measured by DNA synthesis, they affect a number of signalling elements associated with the transduction of cellular activation and proliferation. Thus, pretreatment with the parasite molecule has been found to: (1) induce tyrosine phosphorylation and activation of the protein tyrosine kinases (PTKs) Lyn, Syk and Blk but not Fyn [28]; (2) phosphorylate and activate the Erk2 isoform of mitogen-activated protein (MAP) kinase [28]; (3) downregulate the total level and activity of protein kinase C (PKC) [23]; and (4) modulate the expression of a number of PKC isoforms (for example, whereas α, β, ι/λ, δ and ζ are downregulated, expression of γ and ε is upregulated) [29]. That ES-62-induced modulation of these signal transducers requires PC is demonstrated by the fact that the effects on the PTKs, Erk2 and overall PKC levels at least, are known to be mimicked by PC [23,28]. Importantly, we have also found that pre-exposure of B cells to ES-62 at the low concentrations serves to almost completely desensitise the cells to subsequent activation via the B cell receptor (BCR) of the phosphoinositide 3-kinase (PI-3-kinase) and Ras-MAPK pathways [28]. Thus, uncoupling of the BCR from these crucial proliferative pathways could provide a molecular mechanism for the ES-62-mediated inhibition of BCR-driven proliferation of B lymphocytes. More recently [26], we have found that the ES-62-induced rendering of Jurkat cells anergic to cellular activation via the T-cell antigen receptor (TCR) is associated with disruption of TCR coupling to the phospholipase D, PKC, PI-3-kinase and Ras-MAPK signalling cascades. As with murine B cells, however [23], the PLC-mediated generation of inositol phosphates is not affected. Again, as for inhibition of BCR signalling, PC appears to be the active component of the parasite molecule. Thus, culture with PC or PC–BSA has similar effects to ES-62 on the coupling of the TCR to tyrosine kinase activation (ZAP-70, Lck and Fyn) and the PLC, Ras and MAP kinase signalling cascades [25].

In B lymphocytes, coupling of the antigen receptors to Erk MAP kinase is PTK-dependent [30]. Following ligation of the BCR, Lyn, tyrosine phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the accessory transducing molecules Ig-α and Ig-β. This leads to the recruitment and activation of additional PTKs (such as Syk, Blk and

Fyn) and also signalling molecules (PLC-γ, RasGAP) and adaptors (Shc, Grb2) in a SH2 and SH3 domain-dependent manner. Thus, Shc binds to the phosphorylated ITAMs and in turn is phosphorylated by Syk permitting recruitment of the Grb2Sos complexes required for activation of Ras at the plasma membrane. Following Sos-driven guanine nucleotide exchange and generation of the GTP-bound form of Ras, Ras binds and derepresses Raf-ser/thr kinase, triggering stimulation of MEK (MAP kinase kinase) and consequent activation of MAP kinase. Although we have found that ES-62 profoundly suppresses BCR-stimulated tyrosine events, it does not appear to uncouple the BCR from MAP kinase activation by disrupting activation of the BCR-associated protein tyrosine kinases such as Lyn, Syk, Blk or Fyn [28]. Conversely, we have found that ES-62/PC appears to target two major negative regulatory sites in the control of BCR coupling to the Ras MAP kinase cascade. Specifically, it induces the activation of SHP-1 tyrosine phosphatase and the MAP kinase phosphatase, Pac-1 (M.R. Deehan, W. Harnett and M.M. Harnett, unpublished data). Activation of the former results in dephosphorylation of ITAMs on Ig-β and hence loss of recruitment of other signalling molecules; activation of the latter results in dephosphorylation and hence inactivation of MAP kinase. Fig. 1 summarises known effects of ES-62/PC on signal transduction via the BCR.

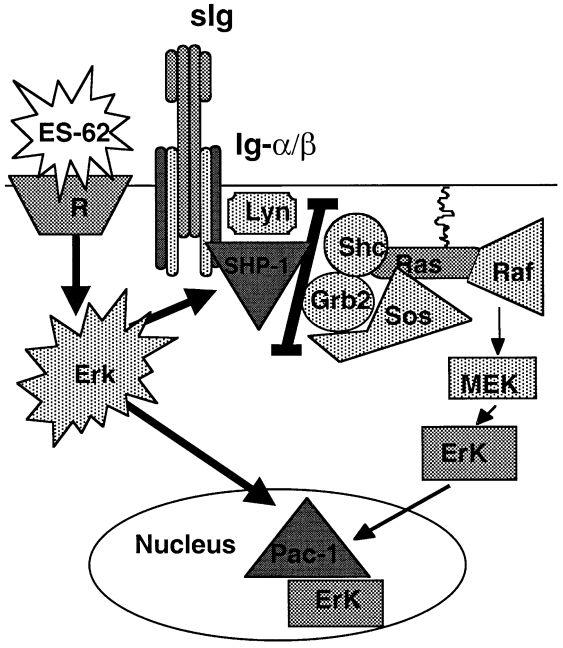

Fig. 1. ES-62 uncoupling of the BCR-RasErkMAP kinase cascade. Following ligation of the BCR the kinase, Lyn, tyrosine phosphorylates the ITAMs on the accessory transducing molecules Ig-α and Ig-β. The Ras adaptor protein, Shc, binds to the phosphorylated ITAMs and in turn is phosphorylated leading to the recruitment of the Grb2Sos complexes (Grb2 is an adaptor protein which binds Sos, a guanine nucleotide exchange factor) required for activation of the GTPase, Ras. Active Ras initiates the Erk MAP kinase cascade by binding and activating the ser/thr kinase, Raf, leading to stimulation of the thr/tyr kinase MEK and consequent activation and nuclear translocation of the ser/thr kinase Erk. ES-62/PC signalling appears to target two major negative regulatory sites in the control of BCR coupling to the Ras MAP kinase cascade. Firstly, ES-62 signalling promotes the BCR activation of SHP-1 tyrosine phosphatase to prevent initiation of BCR signalling by maintaining the ITAMs in a resting, dephosphorylated state and hence prevents recruitment of the ShcGrb2Sos complexes required to activate Ras. Secondly, ES-62 signalling promotes the BCR-driven association of the nuclear MAP kinase dual (thr/tyr) phosphatase, Pac-1, with Erk to terminate any ongoing Erk signals. This dual-pronged mechanism results in a rapid and profound desensitisation of BCR coupling to the RasErkMAP kinase cascade.

How then is PC able to exert these effects on lymphocyte signal transduction pathways? Examination of the literature reveals clear evidence from a number of studies for PC playing a role in cellular proliferation. For example, many human tumours have elevated levels of PC [31] and Ras-transformed cell lines produce increased levels, which are necessary for cell proliferation [32]. Furthermore, it has recently been found that PC can exert mitogenic and co-mitogenic effects on fibroblast cell lines in vitro [33] and this is associated with activation of MAP kinase [34]. Thus, since it has been shown for B lymphocytes that ES-62 does not itself induce the generation of cellular PC [28], it is conceivable that the PC component of ES-62 could result in partial activation of B cells which renders them desensitised to subsequent activation via the BCR. However, evidence has been produced indicating that the mitogenic effects of PC can occur not only intracellularly but also following extracellular interaction with PC [35]. With respect to ES-62, we cannot at this stage state whether the PC moiety acts at the cell surface or following internalisation. The ability of PC to activate certain protein tyrosine kinases, which are associated with receptors found at the plasma membrane [28], may be an argument in favour of the former. It is also worth considering, however, that it may be possible for ES-62 to become

readily internalised by lymphocytes, perhaps following interaction with a specific receptor. Certainly, the PAF receptor is utilised by PC-containing *S. pneumoniae* to enter human endothelial cells during infection [36] and it is known that B lymphocytes express PAF receptors at the plasma membrane [37] (whether the PAF receptor is the uncharacterised receptor referred to earlier [21], awaits elucidation). Regardless of where ES-62 acts, what is interesting about the activation of MAP kinase induced by the parasite product is that it appears to take place in the absence of Ras activation [28]. Recent studies in our laboratories indicate that ES-62/PC actually blocks Ras activation by preventing association between Shc and the Ras guanine nucleotide exchange factor Sos (M.R. Deehan, W. Harnett and M.M. Harnett, unpublished data). The alternative mechanism by which MAP kinase is activated remains to be established but whatever it is, as mentioned earlier, it clearly fails to result in cell proliferation.

3. Effect of phosphorylcholine on antibody and cytokine responses

The observation that the effect of PC on B lymphocytes in vitro varies with respect to concentration suggests that its effects in vivo may be phenotypically diverse. Thus, it is possible to predict that infected individuals exposed to low levels of PC might have suppressed antibody responses to parasite molecules whereas those exposed to high levels might be subject to increased polyclonal production of immunoglobulin. Review of the literature as it currently stands suggests we have some way to go before we have fully characterised the effects of filarial nematode infection on host antibody production but certainly there is evidence for both types of individual existing [38–40]. The situation is complicated, however, by the finding referred to earlier that people harbouring filarial nematodes often show a bias in antibody class/subclass production. In particular, it is frequently noted that IgG4 and IgE levels (both specific and non-specific) are greatly elevated whereas levels of IgG1, IgG2 and IgG3 may be reduced (reviewed in [41,42]). Intriguingly, we have recently discovered that the combination of IL-4 and ES-62, the latter in concentrations that render B cells anergic to activation via the antigen receptor, actually synergises to produce B lymphocyte activation in vitro [25]. This may relate to our earlier finding that IL-4 can overcome the downregulatory effects of ES-62 on PKC-α and ι/λ expression in B cells [29] as these PKC isoforms are considered to transduce key activation signals [43,44]. Since IgG4 and IgE are promoted by IL-4, a similar synergistic activation occurring in vivo might offer an explanation for the increased production of these types of antibody. Having said all of this, however, it is important to note that we currently have no real proof that PC is modulating antibody responses during filarial nematode infection. Nevertheless, we have recently obtained some evidence confirming that the presence of PC on ES-62 modulates the antibody response to other epitopes on the molecule [45]. However, as shown below, the mechanism responsible for this effect does not seem to be dependent on IL-4.

In spite of its apparent ability to desensitise lymphocytes, jirds naturally infected with *A. viteae* mount an IgG antibody response to ES-62 [46]. BALB/c mice subjected to subcutaneous exposure to ES-62 also mount an antibody response [25]. When this is examined with respect to the TH-1 and TH-2 signature IgG subclasses – IgG2a and IgG1 respectively – it is found that only the latter is produced [25]. Thus, ES-62 induces a TH-2 antibody response. That this is dependent on IL-4 was shown by its absence in the IL-4 knockout (KO) mouse [45]. ES-62 induces the production of a little IL-12 by murine spleen cells (W. Harnett, unpublished data). This cytokine is essential for induction of the TH-1 phenotype but induction does not occur in the presence of IL-4 as the latter cytokine inhibits TH-1 cell development by downregulating the β-chain of the IL-12 receptor [47]. It might therefore be predicted that the IL-4 KO mouse would make a compensatory IgG2a response as has been observed in the response to adult *B. malayi* [48]. No IgG2a response to ES-62 is detected, however [45], indicating that such a response is not being 'blocked' by IL-4. Thus either ES-62 simply does not induce a TH-1 response or it is being inhibited in some other way.

ES-62 lacking PC can be produced by culturing *A. viteae* in the presence of 1-deoxymannojirimycin (dMM) [49] or hemicholinium-3 (HC-3) [50]. The former is a mannose analogue which inhibits an ol-

igosaccharide processing step on glycoproteins [51] which is necessary for the generation of the substrate for PC addition to ES-62 [49,52]. The latter is a choline kinase inhibitor and hence prevents synthesis of PC [53]. When PC-free ES-62 prepared in either way was injected into BALB/c mice, subsequent ELISA analysis of recovered serum samples found that there was no significant effect on the previously noted [25] IgG1 antibody response to non-PC epitopes of the parasite product [45] (Fig. 2). Unlike the results obtained with normal ES-62, however, the PC-free material was able to induce a substantial IgG2a response [45] (Fig. 2). This implicates a role for PC in blocking the IgG2a response. We thus investigated whether the addition of PC to BSA would inhibit any IgG2a antibody response associated with it. Although it was found that the BALB/c IgG2a response to BSA was relatively weak, the presence of PC did indeed appear to be inhibiting it [45].

It has previously been reported that the PC moiety of filarial nematodes can induce IL-10 production in B1 cells [54]. IL-10 can downregulate production of IFN-γ production, the cytokine necessary for antibody class switching to IgG2a in mice [55,56]. Thus, to determine whether PC was blocking production of IgG2a antibodies by promoting production of IL-10, the antibody response to normal ES-62 in IL-10 KO mice was investigated. When ES-62 was injected into these mice, an IgG2a response to the parasite molecule was generated [45]. This result therefore implicates PC-induced IL-10 as playing a role in determining the nature of the IgG subclass response to ES-62.

As might be expected from the results of the IL-10 KO mouse study, ES-62 is able to induce production of IL-10 in naive BALB/c spleen cells [25]. Although we have not investigated whether this is due to the PC component of the molecule, this would be predicted from the work of Hoerauf and colleagues, who showed that PC causes release of this cytokine from B1 cells [54,57]. However, we have also found, as mentioned earlier, that ES-62 promotes the release from naive spleen cells of IL-12 and, in addition, IFN-γ [25], two cytokines associated with the TH-1 axis of the immune response. Again, we have not investigated whether this is due to PC. We are describing these results, however, in the context of data obtained by Lochnit and colleagues [58] which demonstrated that PC on glycolipids of *Ascaris suum* induces human peripheral blood mononuclear cells to release the proinflammatory cytokines TNFα, IL-1 and IL-6. Thus although the immunomodulatory relevance of these observations is uncertain (the glycolipids are internal worm components and hence may not have an opportunity to interact with the host immune system), PC clearly has the potential to be more than simply an inducer of cytokines involved in promoting TH-2 responses. Having said this, however, we have recently found that although ES-62 can induce a low level production of IL-12 in macrophages, it also blocks the subsequent substantial production of this cytokine (and also IL-6 and TNFα, but not nitric oxide) induced by LPS/IFN-γ (H. Goodridge et al., unpublished data).

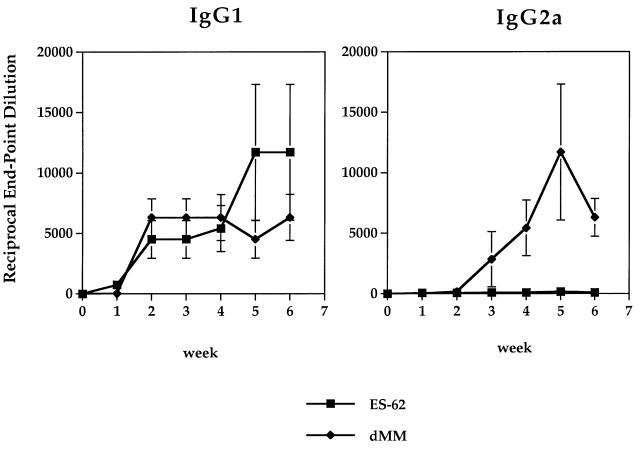

Fig. 2. Comparison of IgG1 and IgG2a antibody levels against ES-62 in BALB/c mice exposed to either native ES-62 or ES-62 synthesised in the presence of dMM. Groups of three 6–8-week-old female BALB/c mice were given a total of four subcutaneous injections of purified parasite material (3 μg per injection) each 1 week apart. Serum samples were collected on a weekly basis and assayed by ELISA. Identical results were obtained when HC-3 was employed to produce PC-free ES-62.

It has already been alluded to that the phenotype of an acquired immune response is considered to reflect the early cytokine environment in which naive $CD4^+$ T cells interact with antigen. Again, it has been suggested, for example, that early exposure to IL-4 can push an immune response in a TH-2 direction [59]. We therefore investigated (by ELISA) whether ES-62 was able to spontaneously induce IL-4 secretion in naive murine spleen cells (48 h exposure). Ironically, given that the molecule induces a TH-2 antibody response and seems to be able to

induce the release of a number of other cytokines, IL-4 was not detected [25]. It was noted, however, that spleen cells from mice that had been pre-exposed to ES-62 produced IL-4. This 'established' TH-2 phenotype is consistent with the antibody data.

More recently, we have investigated the ability of ES-62 to prime immune responses via dendritic cells [60]. These are specialised antigen presenting cells required for the priming and activation of $CD4^+$ T cells and as such, could potentially direct the subsequent differentiation of T cell function [61]. We employed a well characterised in vitro TH cell assay in which $CD4^+$ T cells from the DO.11.10 transgenic mouse express a T cell receptor that is specific for an ovalbumin peptide [62]. When these naive $CD4^+$ T cells are cultured with bone marrow-derived dendritic cells in the presence of the peptide they secrete both IFN-γ and IL-4. However, we found that if the dendritic cells were precultured in the presence of ES-62, then there was an increase in the amount of IL-4 produced and a decrease in the amount of IFN-γ [60]. Thus ES-62 is found to induce the maturation of dendritic cells with the capacity to induce TH-2 responses. The next step will be to investigate whether this is due to PC. We also need to investigate the molecular mechanism underlying this effect – our initial observations suggest that it is not due to IL-10 or upregulation of known costimulatory molecules on the dendritic cell surface.

4. Concluding remarks and future prospects

PC has been suggested as meeting the requirements for a general vaccine targetted against mucosal pathogens [63] and indeed anti-PC antibodies can protect against various bacterial infections (*S. pneumoniae*, *Salmonella typhimurium*) (reviewed in [5,63]). Furthermore, filarial nematode infective larvae, a stage possibly unique in expressing PC on their surface, may also be susceptible to host antibody-mediated immunity [57]. However, it is becoming increasingly apparent that PC has a number of immunomodulatory properties, which dictates that caution should be exercised when considering its use in vaccination. By secreting PC-containing molecules, adult filarial nematodes appear to have utilised these immunomodulatory properties to manipulate the host immune system in certain ways and hence aid their survival in the parasitised host. What could be of interest therefore would be to explore the use of PC not for vaccination, but for manipulating immune responses in ways analogous to those being demonstrated by the parasites. One wonders for example whether molecules such as PC, by pushing immune responses in an anti-inflammatory, TH-2 direction, might be of value in the treatment of autoimmune diseases such as rheumatoid arthritis. Clearly, however, the fact that PC appears to have a plethora of immunomodulatory activities, some of which in fact appear to be proinflammatory, dictates that much more investigation has to be carried out on this intriguing molecule, prior to considering its use in therapies.

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