The phosphorycholine moiety of the filarial nematode immunomodulator ES-62 is responsible for its anti-inflammatory action in arthritis

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ABSTRACT
Objective: In countries where parasitic infections are endemic, autoimmune disease is relatively rare, leading to the hypothesis that parasite-derived immunomodulators may protect against its development. Consistent with this, we have previously demonstrated that ES-62, a 62 kDa phosphorylcholine (PC)-containing glycoprotein that is secreted by filarial nematodes, can exert anti-inflammatory action in the murine collagen-induced arthritis (CIA) model and human rheumatoid arthritis-derived synovial tissue cultures. As a first step to developing ES-62-based drugs, the aim of this study was to determine whether the PC-moiety of ES-62 was responsible for its anti-inflammatory actions.

Methods: We compared the anti-inflammatory activity of a PC-free form of recombinant ES-62 (rES-62) and a synthetic PC-ovalbumin conjugate (OVA-PC) with that of native ES-62 in the CIA model and synovial tissues from patients with rheumatoid arthritis.

Results: The anti-inflammatory actions of ES-62 in CIA appear to be dependent on the PC moiety as indicated by the reduction in severity of disease and also suppression of collagen-specific T helper 1 cytokine production observed when testing OVA-PC, but not rES-62. Interestingly, the anti-inflammatory activity of PC did not correlate with a reduction in anti-collagen IgG2a levels. Also, the ES-62-mediated suppression of interferon-γ from human patient tissues could be mimicked by OVA-PC but not rES-62 or ovalbumin.

Conclusions: In countries where filariasis is endemic the reduced detection of inflammatory diseases, such as rheumatoid arthritis may be because of the anti-inflammatory action of the PC moieties of ES-62. PC may thus provide the starting point for the development of novel, safe immunomodulatory therapies.

In countries where parasitic infections are endemic, autoimmune disease is relatively rare.1 It has therefore been suggested that parasitic infection, and in particular products derived from helminths, may protect us from the development of autoimmunity.2 We have previously identified and characterised ES-62, an anti-inflammatory phosphorylcholine (PC)-containing glycoprotein that is secreted by the rodent filarial nematode Acanthocheilonema viteae.2,4 ES-62 is also produced by filarial nematodes that parasitise humans5 and hence may play a role in preventing the massive inflammatory responses (eg, elephantiasis) that the worms are capable of inducing. We therefore hypothesised that ES-62 had the potential to inhibit inflammatory responses arising from aberrant immune responses as seen in autoimmune diseases, such as rheumatoid arthritis (RA). Consistent with this, we have recently shown that ES-62 can delay the onset and reduce the severity of inflammation exhibited in the murine collagen-induced arthritis (CIA) model.5 These actions of ES-62 were manifested by a reduction in antigen-specific T helper (Th) 1-type cytokine production and immunoglobulin levels, with no compensatory increase in Th2 responses. Crucially, ES-62 was also found to suppress CIA severity and progression when administration was delayed until after clinically evident disease onset6 and the therapeutic potential of ES-62 in arthritic inflammation was further demonstrated using samples from human RA.5

PC has been characterised as an immunomodulatory moiety utilised by a number of pathogens6 and consistent with this, several of the immunomodulatory effects of ES-62 are known to be facilitated by the PC moiety that is attached to one of its N-type glycans.6,7 We now show that the anti-inflammatory effects of ES-62, in both the CIA model and human patient samples, are dependent on the PC moiety and indeed, can be mimicked by synthetic PC-containing molecules. Identification of this key structural component of ES-62 as an anti-inflammatory agent provides the first step towards addressing the development of ES-62-based drugs for treating autoimmunity. This is a particularly attractive idea as the evolution of the host–parasite relationship has produced in ES-62, a molecule that is immunomodulatory but at the same time “safe”, in leaving defences against infection, intact.

METHODS AND MATERIALS

Animals
Male DBA/1 mice obtained from Harlan Olac (Bicester, UK) were used at 8–10 weeks of age and maintained at the Joint Animal Facilities, University of Glasgow. All animals were cared for in accordance to the Home Office, UK animal guidelines.

ES-62 and phosphorylcholine conjugated to ovalbumin preparations
Purified, endotoxin-free ES-62 from the rodent filarial nematode A. viteae was prepared as described previously.2 Sequence comparison of
human filarial homologues (*Brugia malayi*) of ES-62 reveals >70% homology at the amino acid level and the presence of conserved sites for post-translational modification with PC-N-glycans.9 PC conjugated to ovalbumin (OVA-PC) and OVA were synthesised as described previously.10 Recombinant ES-62 lacking PC (rES-62) was prepared following expression in *Pichia pastoris* as described previously.11 Absence of endotoxin from ES-62, rES-62 and PC-materials was confirmed using an Endosafe Kit (Charles River Laboratories, Kent, UK).

**Induction and assessment of collagen-induced arthritis**

Male DBA/1 mice received 200 μg of bovine type II collagen (CII, Sigma, Poole, UK) in Freund’s complete adjuvant (Difco, Detroit, MI, USA) by intradermal injection (day 0). Collagen (200 μg in phosphate-buffered saline (PBS)) was given again on day 21 by intraperitoneal injection. Mice were monitored daily for signs of arthritis for which severity scores were derived as follows: 0 = normal, 1 = erythema, 2 = erythema plus swelling, 3 = extension/loss function, and total score = sum of four limbs. For the prophyactic protocol, mice were treated with 2 μg ES-62 or other molecules subcutaneously on day –2, day 0 and day 21. For the therapeutic studies, mice were treated daily with ES-62 or other molecules, 2 μg subcutaneously for 14 days commencing 1 day after CIA was clinically detectable. Control mice received PBS alone at the same time points.

**Ex vivo culture conditions**

Draining lymph node cells and splenocytes were cultured at 2×10^6/ml for up to 96 h in RPMI medium, supplemented as described previously.12 Cells were stimulated with CII (50 μg/ml), F(ab’)_2 fragments of anti-immunoglobulin antibodies (50 μg/ml) or concanavalin A (5 μg/ml). For proliferation assays, [³H]-thymidine (Amersham, Little Chalfont, UK) was added for the last 18 h of culture while supernatants from parallel triplicate cultures were stored at −70°C until estimation of cytokine content by ELISA or LumineX.

**Human studies**

Samples were derived with approval from the Glasgow Royal Infirmary Ethical Committee. Patients with RA fulfilled American College of Rheumatology diagnostic criteria. Primary synovial membrane cultures were obtained as previously described by collagenase (Worthington, Lakewood, NJ, USA)/DNAse (Sigma-Aldrich, Gillingham, Dorset, UK) digestion of synovial membrane samples obtained at knee arthroplasty.14 Peripheral blood and synovial fluid were obtained from patients with RA and mononuclear cells prepared in Lymphoprep by density gradient centrifugation. Synovial membrane or synovial fluid cultures were at 2×10^6/ml for up to 96 h in RPMI medium, supplemented as described previously.12 Cells were stimulated with CII (5 μg/ml), F(ab’)_2 fragments of anti-immunoglobulin antibodies (50 μg/ml) or concanavalin A (5 μg/ml). For proliferation assays, [³H]-thymidine (Amersham, Little Chalfont, UK) was added for the last 18 h of culture while supernatants from parallel triplicate cultures were stored at −70°C until estimation of cytokine content by ELISA or LumineX.

**Cytokine and immunoglobulin analysis**

Tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-5, IL-6 and IL-10 were detected by OptEIA ELISA kits and IL-12p70 and transforming growth factor (TGF)-β were analysed using antibody pairs (all BD PharMingen, San Diego, CA, USA) or LumineX assays according to the manufacturer’s instructions. Detection limits were as follows: IL-5, IL-6 and TNF-α all at 10 pg/ml; IL-10, IL-12 and IFN-γ at 20 pg/ml and TGF-β at 50 pg/ml. TGF-β samples were acidified to release the biologically active form prior to measurement as described by BD PharMingen. Human TNF-α and IL-6 (Biosource, Nivelles, Belgium) were assayed by ELISA with limits of detection of 30 pg/ml. For LumineX analysis, Bioplex Manager software with five parametric curve fitting was used for data analysis (BioRad, Hemel Hempstead, UK). Unlike ELISA that provides a single OD measurement per well, LumineX provides a value based on the mean of at least 100 measurements per sample. Anti-collagen II antibody titres of individual sera were detected by ELISA: serum samples were titrated on ELISA plates coated with 20 μg/ml type II collagen and then developed with biotin-conjugated anti-mouse IgG1 or IgG2a (Pharmingen), followed by conjugated avidin peroxidase (Sigma) and developed with tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD).

**RESULTS**

The role of phosphorylcholine in the anti-inflammatory effects of ES-62 in the collagen-induced arthritis model

We have previously shown that while ES-62 does not reduce the incidence of CIA, it suppresses the severity of disease.8 To investigate whether the PC moiety is responsible for this, rES-62 lacking PC was used to treat mice in the prophylactic and therapeutic treatment regimens. In contrast to native ES-62, prophylactic treatment with rES-62 did not significantly inhibit the severity of CIA (fig 1A). Nevertheless, like ES-62, rES-62 treatment significantly reduced levels of CII-specific IgG2a, without a compensatory modulation of CII-specific IgG1, IgG3 or IgM (fig 1B and results not shown). Therapeutic treatment of mice with rES-62 after the onset of inflammation did not significantly modulate the incidence or severity of arthritis, or the profiles of collagen-specific IgG1, IgG2a, IgG3 or IgM, relative to PBS-treated mice (results not shown).

These results support the proposal that PC may mediate the effects of ES-62 observed in the CIA model. However, rES-62 is not simply a PC-free copy of native ES-62: it also contains higher levels of mannos and has been shown to exhibit differential secondary and tertiary structure.13 To investigate whether PC, independently of the rest of ES-62, could inhibit inflammation exhibited in the CIA model, we treated mice with PC conjugated to an irrelevant protein, ovalbumin (OVA-PC). As a negative control for OVA-PC, a group of mice were treated with sham-conjugated ovalbumin (OVA). Prophylactic treatment of mice with OVA-PC significantly reduced the severity of inflammation, when compared with the OVA treatment group (fig 1C), suggesting that PC was independently capable of anti-inflammatory action in this arthritis model. However, serum samples from OVA and OVA-PC-treated CIA model mice exhibited no significant differences in their levels of CII-specific IgG1, IgG2a and IgG3, indicating that, unlike ES-62, PC did not specifically modulate antigen-specific IgG (results not shown). Therapeutic treatment of mice with OVA-PC, but not OVA, after the onset of clinically detectable arthritis also prevented progression of the inflammation (fig 1D). Again, analysis of serum immunoglobulins revealed no significant differences in the levels of CII-specific IgG1, IgG2a, IgG3 and IgM between OVA- and OVA-PC-treated mice (results not shown).

ES-62 does not modulate the serum factor profile of collagen-induced arthritis model mice

Serum samples from the PBS group mice (day 38) were found to contain measurable quantities of the pro-inflammatory cytokines, IL-1α, IL-12 and IL-2. Similarly, KC, MIP-1α, IP-10 and MIG, inflammatory chemokines that recruit neutrophils16 17 and are important markers of TNF-α18 and IFN-γ-mediated inflammation19 20 were also detected, along with fibroblast growth
factor-basic (FGF-2) a potent stimulator of cellular proliferation and angiogenesis. Prophylactic or therapeutic treatment of mice with ES-62 or OVA-PC, however, did not induce any significant effects on the serum levels of these inflammatory mediators (results not shown).

Investigation of the role of phosphorylcholine in ES-62-mediated modulation of immune cell responses

We have previously demonstrated that the anti-inflammatory actions of ES-62 in CIA correlate with suppression of Th1 responses in draining lymph node cells (DLN) challenged with antigen (CII), but not mitogen (concanavalin A), ex vivo. To further address the role of PC, we assessed the effects of ES-62, rES-62 and OVA-PC on immune system cell number and subset distribution following induction of CIA in vivo. These studies showed that none of the treatments significantly altered the mean number of total lymph node or splenic cell populations after induction of CIA. Moreover, relative proportions of B220+ B cells, CD4+ T helper cells and CD8+ cytotoxic T cells were not modulated by any of these treatments (results not shown). Consistent with this, in subsequent ex vivo cultures, such DLN and splenic cells were as responsive to stimulation with the mitogen, concanavalin A as those from PBS or OVA-treated mice (results not shown). In contrast, ES-62- and rES-62-treated splenic cells were hyporesponsive to stimulation via the BCR (anti-immunoglobulin). Moreover, ES-62- and OVA-PC-, but not rES-62- or OVA-treated cells were refractory to antigen (CII) stimulation of T cells, results consistent with our previous findings on the in vivo action of ES-62 in normal and CIA mice (fig 1E,F and results not shown). Reflecting these findings, the ex vivo responses of DLN and splenic cultures from CIA mice to concanavalin A in producing a wide range (TNF-α, IL-12, IL-6, IL-2, IFN-γ, IL-17, IL-5, IL-13 and granulocyte-macrophage colony-stimulating factor) of cytokines were not modulated by previous in vivo exposure to ES-62, rES-62 or OVA-PC (results not shown). However, ES-62

**Figure 1** OVA-PC, but not rES-62, reduces severity of collagen-induced arthritis. (A–C,E,F) DBA/1 mice were immunised on days 0 and 21 with collagen, and were treated with ES-62 (n = 9), rES-62 (n = 9), OVA-PC (n = 9), OVA (n = 9) or PBS (n = 9) on days –2, 0 and 21. Clinical score was monitored daily. For mean articular index (A, C), data are expressed as mean (SEM). ***p<0.001; ES-62 compared with rES-62 (and PBS) and **p<0.01 OVA compared with OVA-PC. (B) Collagen-specific IgG2a levels in serum samples from PBS-, ES-62- and rES-62-treated mice were measured by ELISA. Data are expressed as mean concentration (pg/ml) (SEM) from individual mice (n = 9/group); *p<0.05 rES-62 or ES-62 versus PBS. (D) DBA/1 mice were treated daily, 1 day after detectable clinical collagen-induced arthritis with OVA-PC (n = 5) or OVA (n = 6). Mean articular index was monitored daily and data are expressed as mean (SEM) and ***p<0.001 for OVA compared with OVA-PC. Draining lymph nodes and spleens from the prophylactic protocols were obtained for each treatment group after they were killed and cellular proliferative responses assessed by [³H]thymidine uptake. Spleen cells (E) were stimulated with media or F(ab')² fragments of anti-immunoglobulin antibodies (50 μg/ml) for 72 h and draining lymph node cells (F) were stimulated with media or collagen (F; CII, 50 μg/ml) for 72 h. Data are plotted as the mean values from the indicated number of individual mice (of triplicate determinations) from each treatment group. (E) *p<0.05 for rES-62 versus PBS and ***p<0.001 for ES-62 versus PBS, and (F) ***p<0.001 for ES-62 or OVA-PC versus PBS and OVA (Student’s t-test). All data are representative of at least three independent experiments. PBS, phosphate-buffered saline; PC, phosphorylcholine; OVA-PC, PC–ovalbumin conjugate.
Figure 2  Comparison of ex vivo collagen-stimulated cytokine responses from CIA model mice treated with ES-62, OVA-PC and rES-62. DBA/1 mice were immunised on days 0 and 21 with collagen, and were treated with ES-62, rES-62, OVA-PC, OVA or PBS as indicated on days –2, 0 and 21. Splenic cells were obtained from naive mice and from each treatment group at day 33 and stimulated with media or collagen (CII, 50 μg/ml) for 96 h and cytokine secretion assessed by ELISA. Data are plotted as the mean of triplicate samples from the indicated number of individual mice in each of the treatment groups; the bar represents the mean of mean values for the group. Data are representative of three independent experiments. *p < 0.05 for ES-62 versus PBS and ***p < 0.001 for OVA-PC versus OVA (Student's t-test). CIA, collagen-induced arthritis; TNF, tumour necrosis factor; PBS, phosphate-buffered saline; TGF, transforming growth factor; IL, interleukin; PC, phosphorylcholine; OVA-PC, PC–ovalbumin conjugate.
significantly inhibited collagen-specific TNF-α and IL-12 responses but this was not the case with rES-62 (fig 2A,B). Moreover, exposure to OVA-PC in vivo also led to significantly reduced antigen-specific IL-12, but this was not noted with TNF-α, responses (fig 2E,F), suggesting that PC played a key role in the former immunomodulatory effect of ES-62. Interestingly, pre-exposure to ES-62, but not rES-62, in vivo leads to enhanced spontaneous production of the regulatory cytokines IL-10 and TGF-β by splenocytes from CIA mice (fig 2C,D). However, although some increases in spontaneous and CII-induced IL-10 and TGF-β responses were observed in cells derived from OVA-PC relative to OVA-treated CIA mice (fig 2G,H), these were generally not significant.

Role of phosphorylcholine in the therapeutic potential of ES-62 in rheumatoid arthritis in humans

Our previous studies showed that in addition to suppressing CIA in mice, ES-62 exhibited anti-inflammatory activity in peripheral blood mononuclear cell and synovial fluid and membrane cultures from human patients with RA. In these studies, we showed that ES-62 could inhibit LPS-induced pro-inflammatory cytokine production from peripheral blood mononuclear cells and synovial cells and also from macrophages activated by T cells in a cell contact-dependent manner.5 Here, we demonstrate that the anti-inflammatory action of ES-62 is lost when a PC-deficient recombinant homologue of ES-62 (rES-62) is employed. This potentially important role of PC in ES-62-mediated inhibition of rheumatological inflammation is corroborated by the finding that OVA-PC, is capable of mimicking the anti-inflammatory actions of ES-62 in both the CIA model and in human patient samples.

DISCUSSION

We have previously established that ES-62 can inhibit Th1-type immune responses in the CIA model of inflammatory autoimmune RA in human RA patient blood and synovial samples.5 Here, we demonstrate that the anti-inflammatory action of ES-62 is lost when a PC-deficient recombinant homologue of ES-62 (rES-62) is employed. This potentially important role of PC in ES-62-mediated inhibition of rheumatological inflammation is corroborated by the finding that OVA-PC, is capable of mimicking the anti-inflammatory actions of ES-62 in both the CIA model and in human patient samples.

Ultimately, inflammation and pathology in CIA occurs as a result of developing a chronic collagen (CII)-specific, Th1-type immune response. Previously, it was demonstrated that ES-62-mediated inhibition of inflammation in the CIA model was associated with reduced antigen-specific IgG2a, a Th1-promoting antibody subclass, with no modulation of IgG1, IgG3 and IgM levels. Interestingly, the anti-inflammatory action of OVA-PC on CIA model mice was not associated with significant modulation of the level or nature of the serum IgG response, indicating that the inhibitory action of ES-62 on antigen-specific IgG2a was PC-independent. Furthermore, despite lacking anti-inflammatory action in the CIA model, treatment of mice with PC-deficient rES-62 induced inhibition of antigen-specific IgG2a production, similar to that induced by treatment with parasite-derived ES-62. Thus, inhibition of collagen-specific IgG2a by native and rES-62 indicates that this effect was mediated by a non-PC, ES-62-specific, component(s) and presumably reflects the ability of both ES-62 and rES-62 to inhibit BCR-mediated B cell proliferation. The apparent inconsistency in anti-inflammatory action and IgG2a inhibition indicated that the serum
antibody profile did not reflect the inflammatory status, in terms of cytokine production and arthritic score, of the mice in the CIA model. Although at first sight this was surprising given that IgG2a levels are generally considered to correspond to inflammation severity in CIA, some other studies have argued against a correlation between IgG2a levels and CIA incidence in mice and have demonstrated maintenance of a Th1 immune response, despite absence of an IgG2a antibody response.22 23 In relation to this, we also failed to find a correlation between the antigen-specific splenic IL-12 response and serum IgG2a levels with respect to the various stimulants. Although TNF-α was not detected in serum samples from CIA model mice, it is possible that TNF-α was still acting locally, at the inflammatory site for example, inducing production of KC and MIP-1α at this location, but not circulating in the bloodstream. Consistent with this, DLN and splenocytes from CIA mice exposed to ES-62 but not rES-62, in vivo showed reduced capacity to generate TNF-α in recall responses to collagen ex vivo. Similarly, although serum IL-10 levels were not induced by ES-62 treatment, ex vivo DLN and splenocyte cultures from ES-62-treated mice exhibited enhanced spontaneous IL-10 responses suggesting that this cytokine, which has been demonstrated to act in an anti-inflammatory manner on synovial cells,24 particularly in terms of regulating TGF-β was not implicated by serum analysis, spontaneous production of this regulatory cytokine was found in ES-62-treated DLN responses. Surprisingly, none of these effects on cytokines was observed at a statistically significant level when employing OVA-PC in spite of its anti-inflammatory activity, but as mentioned above a similar failure to modulate the IgG response, despite absence of an IgG2a antibody response.22 23 In contrast, we also failed to find a correlation between IgG2a levels and CIA incidence in mice and have demonstrated maintenance of a Th1 immune response, despite absence of an IgG2a antibody response.22 23 In relation to this, we also failed to find a correlation between the antigen-specific splenic IL-12 response and serum IgG2a levels with respect to the various stimulants.

In conclusion, our findings suggest that PC largely mimics the anti-inflammatory action of ES-62 in CIA, in particular being associated with the reduction of antigen-specific Th1 cytokine production. However, not all of the effects of ES-62 on this model required PC as the ES-62-mediated inhibition of CIA-IgG2a production was PC-independent, suggesting that this particular action on antibody levels is not directly associated with, or necessary for, reduction of inflammation. PC-containing homologues of A. viteae ES-62 are produced by human filarial nematode parasites. Thus, the reduced incidence of inflammatory autoimmune diseases, such as RA, in countries where filariasis is endemic could be attributed to the anti-inflammatory action of these molecules.

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