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The therapeutic potential of the filarial nematode-derived immunodulator, ES-62 in inflammatory disease

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Summary

The dramatic recent rise in the incidence of allergic or autoimmune inflammatory diseases in the West has been proposed to reflect the lack of appropriate priming of the immune response by infectious agents such as parasitic worms during childhood. Consistent with this, there is increasing evidence supporting an inverse relationship between worm infection and T helper type 1/17 (Th1/17)-based inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes and multiple sclerosis. Perhaps more surprisingly, given that such worms often induce strong Th2-type immune responses, there also appears to be an inverse correlation between parasite load and atopy. These findings therefore suggest that the co-evolution of helminths with hosts, which has resulted in the ability of worms to modulate inflammatory responses to promote parasite survival, has also produced the benefit of protecting the host from pathological lesions arising from aggressive proinflammatory responses to infection or, indeed, aberrant inflammatory responses underlying autoimmune and allergic disorders. By focusing upon the properties of the filarial nematode-derived immunomodulatory molecule, ES-62, in this review we shall discuss the potential of exploiting the immunomodulatory products of parasitic worms to identify and develop novel therapeutics for inflammation.

Keywords: allergy, autoimmune disease, ES-62, immunomodulation, inflammation, nematodes

Introduction

Filarial nematodes are parasitic worms that may protect from autoimmune and allergic inflammation. Although these worms are capable of inducing particularly severe pathology, including debilitating skin lesions, elephantiasis and blindness, the majority of infected individuals appear to be relatively asymptomatic, even when harbouring considerable parasite burdens [1,2]. Infection with such filarial nematodes is long term, with individual worms surviving for up to 10 years [2], and this has been proposed to be due to their ability to modulate and hence evade the host immune system. Indeed, analysis of those infected individuals free of pathology reveals an immunological phenotype that tends to be T helper type 2 (Th2)-polarized, anti-inflammatory and associated with regulatory components [3,4]. Similar Th2/regulatory strategies also appear to be utilized by gastrointestinal (GI) nematodes [5], indicating that these immune evasion mechanisms are a general feature of nematode infection which, in addition to promoting parasite survival, are conducive to host health by limiting development of pathological lesions resulting from aggressive, proinflammatory responses. This would provide a considerable advantage for humans, as although nematode infection can result in severe pathology the majority of infected people exhibit little evidence of an inflammatory response or overt tissue destruction/disruption [6].

Interestingly, such immunomodulation has also, in some cases, been reported to extend to the subsequent Th1/Th17-dependent responses of the infected individual to vaccines [7], suggesting that nematode-driven changes in immune-responsiveness could impact upon susceptibility to infection with other pathogens. Arising from this, there is currently great interest in the idea that the recent alarming increase in Th1/Th17-associated inflammatory autoimmune diseases in the industrialized but not developing world reflects increased hygiene, causing a reduction in infection with pathogens such as these parasitic worms (the 'hygiene hypothesis') [8]. Indeed, while it has long been suspected that autoimmune diseases may be less prevalent in areas

endemic for nematodes [9], there is now increasing epidemiological evidence for an inverse relationship between nematode infection and inflammatory disorders such as inflammatory bowel disease, type 1 diabetes, rheumatoid arthritis, multiple sclerosis and autoimmune liver disease [9–13]. Moreover, these human studies have been complemented recently by direct experimental evidence of the protection afforded by parasitic nematodes in animal models of inflammatory autoimmune diseases such as colitis and diabetes [14–17].

Intriguingly, given the Th2-associated phenotype of nematode infection [18], although there are cases in which no association or, indeed, increased susceptibility have been observed, the majority of studies suggest that nematode infection can also protect against allergic inflammation [7–9,18–20]. In human epidemiological studies such protection is evidenced by factors such as skin reactivity to allergens or wheezing and by comparing symptoms before and after anthelmintic treatment [7,9,19–21]. Again, these findings have been reinforced by direct experimental evidence showing protective effects of nematode infection in animal models of peanut and house dust mite (Der p1) allergy as well as asthma [22–26]. While the Th2 phenotype resulting from nematode infection could clearly provide a mechanism to explain the ability of the helminths to inhibit Th1/Th17-associated inflammation, it obviously cannot explain how nematodes suppress allergic diseases, which themselves are Th2-dependent [18]. However, both Th1 and Th2 responses may be restricted by regulatory responses [27], and perhaps consistent with this it has been proposed that the ability of nematodes such as *Heligmosomoides polygyrus* [25] to inhibit allergic airway inflammation and *Litomosoides sigmodontis* to inhibit airway hyper-reactivity but not lung inflammation [28] may be due to the generation of regulatory T cells (T_{regs}). Collectively, therefore, the idea that the immune evasion strategies developed to promote survival by such helminths can also protect against human allergic and autoimmune inflammatory disease has, not surprisingly, led to the consideration of the therapeutic potential of parasitic nematodes. Thus, within the past few years, certain species of these worms have been employed in clinical trials for the treatment of inflammatory diseases such as Crohn's disease [9,10,29] and asthma [20].

Although these clinical trials have met with some success due to the ethical problems and risks inherent in using potential pathogens as therapeutics, much interest has focused recently upon the identification of the parasite-derived molecules responsible for the immunomodulation in order to employ them (or small molecule derivatives) for the safe treatment of human disease. Some progress relating to a number of parasite-derived immunomodulators from several nematodes has been made recently on this front [30–36]. However, the most well-defined nematode-derived immunomodulatory molecule to date is ES-62, a phosphorylcholine (PC)-containing glycoprotein secreted by the rodent filarial nematode *Acanthocheilonema viteae* and found subsequently to have well-conserved orthologues in human filarial nematode parasites, including *Brugia malayi* and *Onchocerca volvulus* [31,32,37,38]. ES-62 exhibits a wide range of anti-inflammatory properties [37,39,40] and as a consequence of this, the molecule has been tested in mouse models of both autoimmune and allergic disease and has been found to be active against collagen-induced arthritis [41] and type I hypersensitivity in the skin and lungs [42]. For the rest of this review we shall focus, therefore, upon the anti-inflammatory actions of ES-62 and how we can exploit ES-62 to identify potential therapeutic targets for human inflammatory diseases.

ES-62 and its mechanism of action

ES-62 is a tetrameric glycoprotein (62 kDa subunits) that has PC-moieties attached via an *N*-type glycan and has highest sequence homology (37–38% identity) with members of the M28 peptidase family [38]. Although a function for the putative peptidase activity of ES-62 has not yet been demonstrated convincingly, many of its immunomodulatory effects have been attributed to the presence of PC [39,40]. These immunomodulatory actions include modulation of B cell proliferation and cytokine production, polarization of the responses of antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages towards an anti-inflammatory, hyporesponsive phenotype and desensitization of mast cell degranulation [31,32,39,40]. That the activity of ES-62 is dependent largely upon its PC-moiety has been demonstrated by studies in which normal and PC-free ES-62 were compared [43] and in which ES-62 was compared with PC-conjugated to proteins such as bovine serum albumin (BSA) or ovalbumin (OVA) [44,45]. These studies not only confirmed that PC-conjugates could indeed mimic ES-62-mediated modulation of B cell function and APC maturation *in vitro*, but could also induce much of the humoral and T cell effector polarization observed in response to ES-62 to *in vivo*.

PC is a pathogen-associated molecular pattern (PAMP) expressed by a diverse range of organisms, including bacteria, fungi and protozoa, as well as filarial and gastrointestinal nematodes [46]. Although it enables detection of pathogens by the host (for example via antibodies or C-reactive protein), it can also function to promote pathogen survival via modulation of the host immune response. Consistent, therefore, with the suggestion that the PC-PAMP is responsible for many of the observed immunomodulatory effects, ES-62 activity is dependent upon the presence of Toll-like receptor (TLR)-4 on the target cell. This was demonstrated by the finding that macrophages and DCs from TLR-4 (but not TLR-2 or TLR-6) knock-out (KO) mice were found to be unresponsive to ES-62 [47], and also by studies showing that the modulatory effects of ES-62 on mast cells were abrogated following knock-down of TLR-4 levels by the use of an anti-sense oligonucleotide approach [42]. However, ES-62

appears to signal via TLR-4 in a non-classical manner as, unlike lipopolysaccharide (LPS), ES-62 is fully active against APC derived from HeJ mice that have a mutation in the Toll-interleukin-1 receptor (TIR) domain of TLR-4 [45,47].

A key feature of such ES-62 action in target cells appears to be its ability to induce down-regulation of the signalling enzyme, protein kinase C (PKC)-α. This was first shown in B lymphocytes [48,49], where PKC-α is important for proliferation, and hence such down-regulation contributed to the observed ES-62-mediated inhibition of B cell proliferation. Subsequently it was found that ES-62 caused a similar degradation of PKC-α in mast cells; thus, complexing of ES-62 and TLR4 at the plasma membrane results in sequestration of PKC-α followed by caveolae/lipid raft-dependent internalization and non-proteosomal degradation at the perinuclear region of the cell [42]. As PKC-α is necessary for the phosphatidylcholine-specific phospholipase D (PLD)-coupled, sphingosine kinase-mediated, calcium mobilization that is required for mast cell degranulation, such down-regulation of PKC-α provides the molecular mechanism underpinning ES-62-mediated desensitization of FcεRI-coupled mast cell responses [42]. By contrast, sequestration and degradation of PKC-α does not occur when ES-62 is substituted by LPS, and indeed such LPS/TLR-4 signalling enhances FcεRI-mediated mast cell responses, findings again highlighting the non-canonical nature of ES-62/TLR-4 signalling by which ES-62 subverts the host immune response.

Therapeutic potential of ES-62

Rheumatoid arthritis (RA) has long been associated with an aberrant Th1 immunological phenotype, in which excess proinflammatory cytokine production within the inflamed RA synovial membrane contributes directly to cartilage/bone erosion through matrix metalloproteinase production and dysregulated chondrocyte/osteoclast function [50,51]. However, recent identification of the interleukin (IL)-17 inflammatory axis has led to a reassessment of the pathophysiology of RA as being representative of IL-17/Th17- rather than Th1-dependent inflammation [50,51]. Nevertheless, in the pre-IL-17 era, as we had previously found ES-62 to dampen aggressive Th1-associated proinflammatory responses [52], we investigated the prophylactic effect of ES-62 on the development of collagen-induced arthritis (CIA) in mice [41]. ES-62 suppressed the severity of developing CIA significantly and, as predicted, these prophylactic effects correlated with the inhibition of collagen-specific proinflammatory/Th1 cytokine production [tumour necrosis factor (TNF)-α, IL-6 and interferon (IFN)-γ] and suppression of collagen-specific immunoglobulin (Ig)G_{2a} antibodies but without inducing a corresponding increase in Th2-associated responses. Importantly, therapeutic administration of ES-62 after onset of established joint pathology also resulted in significant reduction in arthritis progression both in terms of the number of subsequently recruited arthritic joints and also progression of articular inflammation and destruction [41,44]. Notably, many of these protective effects could be mimicked by PC-OVA but not by PC-free rES-62 [44]. To investigate further the therapeutic potential of ES-62 in humans, we performed parallel studies on primary cultures from RA synovial fluid and membranes that showed significant suppression of LPS-induced TNF-α and IL-6 in the presence of ES-62. Moreover, peripheral blood mononuclear cells (PBMCs) and synovial fluid and membrane cultures from patients with RA, stimulated with the cytokines IL-12, IL-15 and IL-18 to mimic the proinflammatory milieu of the joint [50], were found to exhibit reduced capacity to secrete the signature Th1 cytokine, IFN-γ, when pre-exposed to ES-62 or OVA-PC but not rES-62 or OVA [44]. Collectively, these findings indicated clearly that ES-62 could modify critical arthritogenic proinflammatory pathways in disease-relevant tissues [50].

With respect to allergic inflammation, ES-62 was found to be protective in mouse models of OVA-induced airway hypersensitivity and immediate-type hypersensitivity (ITH) to oxazolone in the skin [42]. In the airway hypersensitivity model, ES-62 was found to reduce peribronchial inflammation and mucosal hyperplasia, inhibit eosinophila and prevent release of IL-4, the signature cytokine required for development of airway inflammation. In the skin ITH model, ES-62-mediated reduction of ear swelling correlated with ES-62 directly suppressing FcεRI-mediated mast cell degranulation and inflammatory mediator release.

ES-62 has now therefore been demonstrated to exhibit anti-inflammatory potential in both Th1- and Th2-polarized models of inflammatory disease: the precise mechanisms involved have not been defined, but unlike the situation with suppression of airway hyper-reactivity by infection with *H. polygyrus* [25] and *L. sigmodontis* [28], ES-62 does not appear to act via the induction of T_{regs} [53]. Moreover, ES-62 does not appear to be merely immunosuppressive, as it has failed to modulate the Th1 responses associated with the Bacillus Calmette–Guérin (BCG) vaccine component purified protein derivative (PPD) [54], as well as those associated with other filarial nematode antigens such as *Onchocerca volvulus*-fatty acid and retinol-binding-1 (rOv-FAR-1) and recombinant *Onchocerca volvulus*-activation associated secreted protein-1 (Ov-ASP-1) [55]. Similarly, ES-62 did not suppress the Th1-like pathology associated with *Toxoplasma gondii* [56] and *Plasmodium chabaudi* [57], nor did it modulate the responses to *Leishmania major* in either susceptible or resistant strains of mice (Xu, Harnett & Harnett; unpublished results). Collectively, these results suggest that ES-62 may be effective against a particular type of inflammation, which in the light of the recent reassessment of CIA/RA as being representative of IL-17/Th17- rather than Th1-dependent inflammation [50,58] might suggest that ES-62 can only target Th-1 (rheumatoid arthritis)- and Th-2 (allergy/asthma)-type inflammatory disorders that are IL-17-dependent [50,58,59]. Importantly,

however, they also indicate that while ES-62 can dampen the pathological responses associated with inflammatory autoimmune and allergic disorders, unlike current biologics such as TNF blockers, it is unlikely to compromise the host in terms of vaccine responses or mounting responses to other pathogens [54–57].

Future perspectives

The finding that ES-62 can suppress the pathology associated with autoimmune and allergic inflammatory disorders without compromising the host's ability to fight disease suggests that therapies based on the mode of action of ES-62 may provide the starting point in the development of safe effective drugs for treatment of such human diseases. Thus, to address identifying a 'blueprint' of the anti-inflammatory action of ES-62, we conducted gene microarray studies on synovial membrane cultures from rheumatoid arthritis patients. Analysis of the genes modulated by LPS and ES-62 in synovial membrane cultures from rheumatoid arthritis, but not osteoarthritis, patients (Table 1 and unpublished results) showed that many classes of immunoregulatory genes, including chemokines, cytokines, inflammatory mediators and their receptors, were targeted differentially. Similarly, genes involved in antigen processing and trafficking as well as intracellular signalling elements such as kinases and phosphatases, components of the ubiquitin pathways such as E3 ligases, de-ubiquitin enzymes (DUBs) and proteosomal subunits as well as transcription factors which are likely to be involved in directing the phenotype of the immune response were regulated differentially. Moreover, in some cases, co-culture with ES-62 could reverse the LPS phenotype. Thus, while many of the observed changes were small, LPS, for example, up-regulated expression of pro-inflammatory mediators (e.g. CXCL6, CCL11, HMGB1), pattern recognition receptors (e.g. TLR-4 and various scavenger receptors), co-stimulatory and adhesion molecules [e.g. CD80, lymphocyte function-associated antigen 1 (LFA-1) and LFA-3] as well as folistatin, a novel autoantigen/biomarker that has been shown recently to play a critical role in arthritis [61]. By contrast, ES-62 promoted the up-regulation of negative immunoregulatory receptors such as the immunoreceptor tyrosine-based inhibition motifs (ITIM)-containing LILRB members of the leucocyte immunoglobulin-like receptor family. Interestingly, both membrane and soluble forms of LILRB2 have been shown to exhibit potent immunosuppressive activity, and therefore have been proposed as exploitable in the treatment of transplant rejection, cancer, asthma and autoimmunity [62–64]. With relevance to arthritis, for example, LILRB2 receptors have been implicated in the suppression of osteoclasts responsible for bone resorption and hence articular erosion [63]. Interestingly, polymorphisms in the LILRB class of receptors have been associated with human leucocyte antigen (HLA)-DRB1 shared epitope-negative rheumatoid arthritis and, indeed, LILRB1 is encoded within the leucocyte receptor complex on chromosome19q13.4, implicated previously as a susceptibility region for systemic lupus erythematosus [65]. Closer analysis revealed that while many genes were up-regulated by both LPS and ES-62 (Table 2), a number such as complement receptor of the immunoglobulin family (CRIg), macrophage receptor with collagenous structure (MARCO), LILRB2, neuromedin U, fucosyltransferase 4 (FUT4) and cytochrome P450 2J2 (CYP2J2) were quite differentially regulated by these TLR-4 ligands, being very strongly up-regulated by ES-62 but not LPS. Interestingly, reduced CRIg [67,68] and MARCO [69–71] signalling has been implicated in the development of autoimmune inflammatory disorders while FUT4 [85,92] and neuromedin [81–84] appear to act to polarize towards Th2 responses and CYP2J2 [86,87] acts to produce anti-inflammatory arachidonic acid metabolites. While this type of analysis indicates that the mechanisms underlying ES-62-mediated suppression of inflammatory pathology are complex and manifold, the challenge is to assess experimentally and dissect the relative contribution of such signals to the anti-inflammatory action of ES-62 *in vivo*. Neverthless, this approach indicates that such studies involving a range of parasite-derived immunomodulators that exploit distinct immunoregulatory mechanisms are likely to be of value in the development of 'blueprints' predictive of anti-inflammatory pathways in clinically relevant tissues that may identify safe, novel therapeutic targets.

The great challenge, however, is to develop small molecule derivatives (SMDs) or peptide mimetics of ES-62 (and other nematode-derived immunomodulatory molecules) for use as anti-inflammatory drugs to target such pathways. One possible avenue of exploration is provided by our findings that the PAMP-PC moiety appears to be responsible for many of the immunomodulatory and therapeutic effects of ES-62 [38,40,44,45]. Thus, as we have also shown that glycosphingolipids of *Ascaris suum* have PC immunomodulatory moieties [93], as a first step in generating SMDs we tested the anti-inflammatory potential of synthetic PC-containing glycolipids in *in vitro* assays of macrophage and DC cytokine production which indicated that although PC is an immunomodulatory component, other structural features are necessary to allow it to act [94]. We found a similar picture in preliminary studies with PC-containing peptides and this led us to develop a range of compounds based upon these structures which have shown promise both in *in vitro* assays of cytokine and autoantibody production from blood and PBMC cultures from rheumatoid arthritis patients, and also prophylactically and therapeutically in the CIA model (GB Patent 0707724·1 and unpublished results). Collectively, these data suggest that synthetic PC-based molecules have the potential to be successful anti-inflammatory drugs.

A potential second approach builds on the ability of ES-62 to modulate signal transduction pathways selectively in

Table 1. Differential regulation of genes in synovial membranes cultured with lipopolysaccharide (LPS) or ES-62 from rheumatoid arthritis patients.

LPS	ES-62	ES-62/LPS	Name	Description
1·75	−1·11	1·49	CXCL6	Chemokine ligand 6 (granulocyte chemotactic protein 2)
3·46			CCL11	Chemokine ligand 11 (eotaxin)
−1·04	1·27	1·11	CSF2RB	Colony stimulating factor 2 receptor, beta chain
	−2·30	−1·88	LIFR	Leukaemia inhibitory factor receptor
2·50	−1·37	2·39	IL-2RG	Interleukin-2 receptor, gamma chain
1·25	−2·44	−1·54	IL-8	Interleukin-8
2·13	−1·66	1·29	IL-8	Interleukin-8 C-terminal variant (IL-8)
	2·00		IL-11RA	Interleukin-11 receptor, alpha chain
−2·45			IL-13RA2	Interleukin-13 receptor, alpha 2 chain
	−1·77	2·11	TGF-BR3	Transforming growth factor, beta receptor III
3·03		4·35	TNF-SF15	Tumour necrosis factor (ligand) superfamily, member 15
	−1·85		TNF-RSF11B	Tumour necrosis factor receptor superfamily, member 11b
2·68		1·73	TNF-RSF21	Tumour necrosis factor receptor superfamily, member 21
	2·30		RIPK1	Receptor (TNFRSF)-interacting serine-threonine kinase 1
1·40		1·35	HMGB1	High-mobility group box 1
−2·13			C1QBP	Complement component 1q- binding protein
	−1·84	−2·41	MASP2	Mannan-binding lectin serine protease 2
−1·20	2·99	2·06	DEFB1	Defensin, beta 1
−4·50			HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
	2·38		LILRB1	Leucocyte immunoglobulin-like receptor, subfamily B1 (ITIM)
	33·36	23·10	LILRB2	Leucocyte immunoglobulin-like receptor, subfamily B2 (ITIM)
	7·57		LILRB3	Leucocyte immunoglobulin-like receptor, subfamily B3 (ITIM)
	11·71	12·38	Z39IG	Immunoglobulin superfamily protein
	1·87	2·25	LAIR1	Leucocyte-associated Ig-like receptor 1
3·41		5·39	IGSF6	Immunoglobulin superfamily, member 6
2·03		1·97	CD80	CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)
1·16	−1·06	1·02	CLECSF2	C-type (calcium dependent, carbohydrate-recognition domain) lectin
−1·48	1·00	−1·10	CLECSF6	C-type (calcium dependent, carbohydrate-recognition domain) lectin
2·57		2·97	COLEC12	Collectin subfamily member 12
2·50		2·19	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)
5·28		3·89	LMAN1	Lectin, mannose-binding, 1
3·36			ITGAL	Integrin, alpha L (CD11A (p180), LFA-1)
1·84		2·11	CD58	CD58 antigen, (LFA- 3)
	2·16		LGALS9	Lectin, galactoside-binding, soluble, 9 (galectin 9)
3·78	−1·64	1·68	MSR1	Macrophage scavenger receptor 1
−1·38	1·65	1·31	MRC2	Mannose receptor, C type 2
	1·53	1·36	SCARB2	Scavenger receptor class B, member 2
3·84			TLR-4	Toll-like receptor 4
	−1·67		IRAK3	IL-1 receptor-associated kinase 3
−1·24	1·44	1·62	PELI2	Pellino homologue 2 (Drosophila)
2·97		1·93	CASP1	Caspase 1, apoptosis-related cysteine protease (IL-1β, convertase)
1·05	−13·18	−1·09	HSPCA	Heat shock 90 kDa protein
2·77		2·27	HSPA8	Heat shock 70 kDa protein 8
3·03		2·19	FSTL1	Follistatin-like 1
	−2·31	−1·09	ACVR1	Activin A receptor, type I
1·45	−1·19	1·78	ARIH1	Ariadne homologue, ubiquitin-conjugating enzyme E2 binding protein-1
1·18	−1·00	1·07	FBXW2	F-box and WD-40 domain protein 2
1·11	−2·25	1·83	UBE3A	Ubiquitin protein ligase E3A
3·14	−1·53	2·66	RNF13	Ring finger protein 13
	−1·31		PSMC3	Proteasome (prosome, macropain) 26S subunit, ATPase, 3
−2·71		−1·04	LRAP	Leucocyte-derived arginine aminopeptidase
1·25	−1·04	−1·14	ADAM10	A disintegrin and metalloproteinase domain 10
1·16	−1·08	1·64	LOC64167	Aminopeptidase
	4·06		CPVL	Carboxypeptidase, vitellogenic-like

Table 1. *Continued*

LPS	ES-62	ES-62/LPS	Name	Description
	2·50		KEL	Kell blood group
	2·75		TLL1	Tolloid-like 1
	−1·35	1·46	TIMP3	Tissue inhibitor of metalloproteinase 3 (pseudoinflammatory)
	−2·81	−4·66	CST6	Cystatin E/M
	−2·07		CAPN7	Calpain 7
	1·97	1·56	CTSF	Cathepsin F
	1·51		SERPINB6	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin) 6
	−2·35		SIAT1	Sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)
1·49	−1·37	1·53	CANX	Calnexin
1·45	−1·05	1·41	SNX5	Sorting nexin 5
1·56	−1·01	1·37	FLJ11116	Similar to rab11-binding protein
2·25	−1·66	2·16	GDI2	GDP dissociation inhibitor 2
2·79		2·00	RNP24	Coated vesicle membrane protein
	2·13	2·22	NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha
2·25	−1·66	2·16	GDI2	GDP dissociation inhibitor 2
	−1·46	1·34	SH3BP5	SH3-domain binding protein 5 (BTK-associated)
1·32	−1·28	1·35	AWP1	Protein associated with PRK1
−1·09	1·29	1·34	PRKRIR	Protein-kinase, interferon-inducible dsRNA dependent P58 repressor
−1·19	1·16	−1·01	SNK	Serum-inducible kinase
−2·03			MARK3	MAP/microtubule affinity-regulating kinase 3
1·75		1·93	CSNK1A1	Casein kinase 1, alpha 1
1·46		1·48	NCK1	NCK adaptor protein 1
	−1·83	2·27	LIM	LIM protein (similar to rat protein kinase C-binding enigma)
−1·83		−2·45	DYRK2	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
	−1·92	−1·19	STK17B	Serine/threonine kinase 17b (apoptosis-inducing)
	2·53		MAP2K5	Mitogen-activated protein kinase kinase 5
	−1·55	−1·82	MAP3K4	Mitogen-activated protein kinase kinase kinase 4
	−2·01		MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3
	−2·14		MAP4K5	Mitogen-activated protein kinase kinase kinase kinase 5
2·50	−1·38	1·84	CALM1	Calmodulin 1 (phosphorylase kinase, delta)
1·78	−2·14	1·78	CAMK2G	Calcium/calmodulin-dependent protein kinase II gamma
	−1·96		PTPRB	Protein tyrosine phosphatase, receptor type, B
3·01			PTPRO	Protein tyrosine phosphatase, receptor type O
−2·19			PTPN21	Protein tyrosine phosphatase, non-receptor type 21
−1·51	2·08	2·07	DUSP22	Dual specificity phosphatase 22
1·22	−1·09	1·33	GNAI1	G protein-alpha inhibiting activity polypeptide 1
	2·77	2·57	RGS1	Regulator of G-protein signalling 1
	2·08		GNA15	G protein-alpha 15 (Gq class)
1·62		1·66	GNB1	G protein-beta polypeptide 1
	−1·78	−2·20	RACGAP1	Rac GTPase activating protein 1
−2·50		−1·77	ARF6	ADP-ribosylation factor 6
1·75	−1·23	2·68	ARHGAP5	Rho GTPase activating protein 5
−2·38			HUMAUANTIG	Nucleolar GTPase
2·35		1·57	GTPBP2	GTP binding protein 2
	2·27	−1·67	DAPP1	Dual adaptor of phosphotyrosine and 3-phosphoinositides
	10·85		KCND3	Shal-type potassium voltage-gated channel-3
	2·11	2·35	KCNMA1	M-type potassium large conductance calcium-activated channel-1
	−1·62		PDE4B	cAMP phosphodiesterase 4B
−2·53		−2·04	PLD1	Phosphatidylcholine-specific phospholipase D1
2·58		1·79	PPAP2B	Phosphatidic acid phosphatase type 2B
3·12		2·14	SGPP1	Sphingosine-1-phosphate phosphatase 1
	4·72		PLCL1	Phospholipase C-like 1
2·46		1·05	DGKA	Diacylglycerol kinase, alpha 80 kDa
	−2·95		SPRY2	Sprouty homologue 2 (Drosophila)
	−2·53	−4·06	WIF1	WNT inhibitory factor 1

Table 1. *Continued*

LPS	ES-62	ES-62/LPS	Name	Description
1·38	−1·01	1·48	BCAA	RBP1-like protein
1·19	−1·03	1·20	SP110	SP110 nuclear body protein
1·12	−1·15	1·12	IFI16	Interferon-γ-inducible protein 16
1·01	−1·06	1·11	NAP1L4	Nucleosome assembly protein 1-like 4
	−2·08		RB1	Retinoblastoma 1
	−2·60	−2·57	RARB	Retinoic acid receptor, beta
	2·66	1·60	NFYB	Nuclear transcription factor Y, beta
	1·92	2·06	CCND2	Cyclin D2
−2·03			CDK8	Cyclin-dependent kinase 8
	−3·27		PPM1D	Protein phosphatase 1D magnesium-dependent, delta isoform
1·12	−1·01	1·11	PDCD6	Programmed cell death 6
1·10	−1·21	−1·00	DAPK3	Death-associated protein kinase 3

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 (5 ¥ 106 cells) from RA and osteoarthritis (OA) patients were obtained by digestion using collagenase as described previously [44] and were then exposed to media or ES-62 (2 mg/ml) for 2 h before being stimulated with media or LPS (100 ng/ml) for a further 4 h. After extraction of mRNA, microarray analysis was carried out using the Affymetrix Human Genome Focus array (HG Focus). The Affymetrix GeneChip version 5·0 software was used for image acquisition for the HG Focus chips. Data normalization, log transformation, statistical analysis and pattern study were performed with GeneSpring version 7·0 software (Silicon Genetics, Redwood City, CA, USA) setting the cut-off signal ratio at 1·5-fold change from the control OA samples, with the resultant data being expressed as fold change relative to the control RA sample and differential gene expression identified by a two-way analysis of variance approach, as described previously [60].

Table 2. Genes up-regulated strongly by ES-62 in rheumatoid arthritis synovial membrane cultures.

Gene	Biological process	LPS	ES-62	ES-62/LPS
Inhibitory leucocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 2 (LILRB2)	Inhibitory receptors of which both membrane and soluble forms have potent immunosuppressive activity which have potential for treating transplant rejection, autoimmunity, asthma and cancer. LILRB suppress development of osteoclasts responsible for bone resorption, transduction, cellular defence response [62–64]		33·36	23·1
Oxidized low-density lipoprotein (lectin-like) receptor 1 (OLR1)	Binds, internalizes and degrades oxidized low-density lipoprotein and may play a role as a scavenger receptor associated with inflammatory disorders such atherosclerosis and arthritis [66]	12·13	2·04	12·73
Complement receptor of the immunoglobulin superfamily (CRIg; Z39IG)	Expressed on tissue resident macrophages of synovium: clearance receptor and inhibitor of alternative complement pathway [67,68]		11·71	12·38
Macrophage receptor with collagenous structure (MARCO)	Scavenger receptor involved in clearance of pathogens and apoptotic cells; reduced expression contributes to development of systemic lupus erythematosus-like disease in mice [69–71]	4·20	33·82	23·75
CD163 antigen (CD163)	Macrophage scavenger receptor: after shedding, the soluble form may play an anti-inflammatory role, and may be diagnostic of macrophage activation in inflammatory conditions. May be a marker of alternatively activated macrophages [72,73]	10·56	13·55	13·64
Signalling lymphocytic activation molecule (SLAMF1)	Self-ligand involved in inhibiting CD40-mediated proinflammatory responses in DCs and their ability to prime Th1 responses and also IL-4/IL-10-secreting NKT2 differentiation in NOD mice [74,75]	7·94	9	8·51
DAP12 (TYRO protein tyrosine kinase binding protein)	ITAM-containing adaptor: transduces TREM-1 and 2 signals to activate myeloid cells. Although TREM signalling can synergize with that of LPS, TREM-1 activation also attenuates the induction of some LPS target genes, including those that encode IL-12 cytokine family subunits. This is because TREM-2 via DAP12 negatively regulates TLR and FcR signals. In addition, acts, via APC, to control the level of anti-microbial type 1 T cell activation and immunopathology [76,77]	4·59	5·78	3·92
Major histocompatibility complex, class II (HLA-DRA)	Antigen presentation	10·93	6·32	8·11

Table 2. *Continued*

Gene	Biological process	LPS	ES-62	ES-62/LPS
GPR109A (nicotinic acid receptor: PUMA-G, HM74)	Up-regulated by IFN-γ in macrophages	6·87	8·57	7·41
Chemokine (C-C motif) ligand 4 (CCL4; MIP-1β)	Proinflammatory chemokine involved in the host response to infection and inflammatory disorders including asthma and arthritis [78]	4·86	9·58	10·34
Chemokine (C-X-C motif) ligand 13	Contributes to inflammation and ectopic G C formation in synovial tissue and is a potential biomarker for rheumatoid arthritis. Also associated with asthma-like inflammation [79,80].	6·15	6·15	6·19
Neuromedin U (NMU)	Neuropeptide that promotes Th2 cell, mast cell, eosinophil and monocyte inflammation in asthma, sepsis [81–84]		29·86	
Complement 1qα (C1QA)	Complement activation, classical pathway	19·43	20·11	16·22
Fucosyltransferase 4 (alpha (1,3) fucosyltransferase, myeloid-specific) (FUT4)	Cell adhesion based on E-selectin ligand activity, particularly eosinophils [85]		12·04	8·63
Epoxygenase [cytochrome P450, family 2, subfamily J, polypeptide 2 (CYP2J2)]	Produces the anti-inflammatory arachidonic acid metabolites, epoxyeicosatrienoic acids (EETs), which regulate vascular tone, cellular proliferation, migration, inflammation and cardiac function. While down-regulation of epoxygenases leads to renal damage, overexpression of CYP2J2 decreases cytokine-induced endothelial cell adhesion molecule expression. Moreover EETs prevent leucocyte adhesion to the vascular wall by inhibition of IκB kinase and NF-κB [86,87]		13·09	
Aquaporin 3 (AQP3)	Water/glycerol transport channels implicated in hydration and repair of epithelial/endothelial barriers; down-regulated by TNF in skin. Up-regulated in IL-13-associated asthma and altered localization may be a contributing factor to diarrhoea during bacterial infection [88]	4·99	7·52	6·11
Potassium voltage-gated channel, Shal-related subfamily, member 3 (KCND3)	Potassium ion transport		10·85	
Haemopoietic cell kinase (HCK)	Regulation of phagocytosis, adhesion and migration and hence inflammation [89]	5·1	5·17	4·03
UBP43 (ubiquitin-specific protease 18)	De-ubiquitination enzyme (DUB) that deconjugates ISG15 which is an IFN-inducible ubiquitin-like protein whose expression and conjugation to target proteins are induced dramatically upon viral or bacterial infection. UBP43-deficient mice are hypersensitive to LPS-induced lethality	6·59	5·78	7·26
Myeloid cell nuclear differentation antigen (MNDA)	Regulation of transcription and cell death. Highly expressed in arthritis patients and member of family implicated in SLE susceptibility [90,91]	95·67	247·28	183·55

APC: antigen-presenting cells; IL: interleukin; LPS: lipopolysaccharide; NOD: non-obese diabetic; NF-κB: nuclear factor kappa B; Th1: T helper type 1; TNF: tumour necrosis factor; TREM: triggering receptor expressed on myeloid cells; TLR: Toll-like receptor.

immune system cells, including those dependent upon phosphoinositide-3 (PI-3) kinase, sphingosine kinase/phospholipase D (SphK/PLD), mitogen-activated protein (MAP) kinases and nuclear factor kappa B (NF-κB) [42,48,49], suggesting that targeting of such signals may ameliorate inflammatory disease. Inhibitors of many of these pathways are currently undergoing clinical trials in a variety of inflammatory diseases, including asthma and arthritis, but due to their widespread expression, multiple isoforms and essential roles there are generally debilitating associated side effects, although the recent development of isoform-specific inhibitors may go some way to addressing these problems [51,95,96]. Another approach is to target particular isoforms specifically by *in vivo* siRNA therapy [97], and in this way we have recently used siRNA specific for SphK1 to ameliorate pathology successfully in mouse models of arthritis, airway hyper-reactivity and mast cell-dependent anaphylaxis [98–100]. Alternatively, as many of these signalling elements exert their effects via protein–protein (or protein–DNA, protein–lipid) interactions using well-defined modular (e.g. SH2, SH3, PH) domains rather than/in addition to their intrinsic enzyme activities, cell-permeable peptides that disrupt these interactions have been used successfully to block such signals both *in vitro* and *in vivo* [39,101,102]. Thus, building upon the data generated by microarray (Tables 1 and 2) and consequent proteomics studies to identify the precise 'blueprint' of key signalling molecules targeted by ES-62 in a particular inflammatory

cell or target organ could allow the development of such target-based therapeutics, rather than PC-based SMDs, to mimic ES-62-mediated suppression of disease-specific inflammatory signalling pathways.

For example, peptides targeting interactions of inhibitor of κB (IκB) and NF-κB essential modulator (NEMO) prevent activation of NF-κB and consequent release of inflammatory mediators without affecting basal or protective NF-κB activity [101,102]. Indeed, employing a variety of cell-permeable protein transduction domain (PTD) peptides to provide systemic delivery of one such peptide (NBD) demonstrated it to be therapeutic in mouse models of arthritis, inflammatory bowel disease, diabetes and muscular dystrophy [101,102]. Moreover, as many signalling elements often have more than one adaptor domain, it should be possible to select peptides that target particular individual interactions [103,104], allowing subtle modulation of cellular responses. This approach has been used successfully for immune cell surface receptors (e.g. the chemokine receptors, CCR2, CCR3, CCCR5 and CXCR4 [104,105]) and intracellular signalling molecules involved in cytokine signalling, such as signal transducer and activator of transcription 3 (STAT3) [103], and could be applied to mimicking of ES-62-mediated disruption of TLR signalling that requires TLR dimerization and/or interaction with TIR-containing adaptors such as myeloid differentiation primary response gene 88 (MyD88) for initiating proinflammatory cytokine secretion.

Perhaps of particular relevance to ES-62, given its selective action towards protein kinase C isoforms [48,49], are the peptide inhibitors specific for enzyme-substrate/pseudosubstrate interaction sites [106] which might mimic ES-62 by blocking, for example, isoform-specific PKCs. Alternatively, peptidomimetic compounds (e.g. phosphonate-based pTyr mimetics) that disrupt SH2-phosphotyrosine interactions [106,107] might reproduce ES-62-mediated targeting of certain Src-related protein tyrosine kinases and consequent uncoupling of the B cell antigen receptor (BCR) from the RasMAPkinase pathway driven by recruitment of SHP-1 phosphatase [48,49]. Importantly, the structural modifications (e.g. mono- or difluoro-derivatives) of the phosphonates used to promote stability and bioavailability [106,107] have also allowed differential targeting of distinct SH2 domains. For example, while a difluoro-derivative of a phosphonate peptide displays equivalent affinity to the PI-3K SH2 domain, it was more potent towards Src SH2 domains and less effective against Grb2 SH2 domains than the parent pTyr-peptide [106,108,109]. Other key targets of ES-62 action, the extracellular-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) MAP kinases, can be regulated via interaction with scaffold proteins containing kinase interaction motifs (KIM) such as kinase interacting protein (KIP) and JNK-interacting protein (JIP) [107]. Inhibitory peptides that resemble the KIM of JIP have been developed which when fused to the TAT cell-permeable PTD sequence have been found to block Jnk activity [110]. As ES-62 selectively targets these kinases to differentially modulate IL-12 and TNF-α secretion from macrophages [48,49,111], combination therapy using such peptides may be able to modulate specifically both the precise phenotype and the extent of the inflammatory response. Indeed, given the therapeutic potential of ES-62 in a variety of inflammatory pathologies, the recent development of a peptide phage display library screen for tissue specific delivery that has produced efficient PTDs that transport proteins selectively into tumours, airway epithelia, synovial fibroblasts and cardiac tissue render this approach clinically useful *in vivo* [112]. Moreover, by testing whether such pharmacological modulation of helminth targets results in a similar 'blueprint' to the anti-inflammatory action of parasite-derived immunomodulators in clinically relevant tissues, this parasite-based approach may identify safe, novel therapeutic targets collectively, in that they have been validated over millennia by the host–parasite relationship.

Disclosure

MM Harnett, AJ Melendez and W Harnett report no conflicts of interest.

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