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A Novel Therapeutic Approach Targeting Articular Inflammation Using the Filarial Nematode-Derived Phosphorylcholine-Containing Glycoprotein ES-62

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Understanding modulation of the host immune system by pathogens offers rich therapeutic potential. Parasitic filarial nematodes are often tolerated in human hosts for decades with little evidence of pathology and this appears to reflect parasite-induced suppression of host proinflammatory immune responses. Consistent with this, we have previously described a filarial nematode-derived, secreted phosphorylcholine-containing glycoprotein, ES-62, with immunomodulatory activities that are broadly anti-inflammatory in nature. We sought to evaluate the therapeutic potential of ES-62 in vitro and in vivo in an autoimmune disease model, namely, collagen-induced arthritis in DBA/1 mice. ES-62 given during collagen priming significantly reduced initiation of inflammatory arthritis. Crucially, ES-62 was also found to suppress collagen-induced arthritis severity and progression when administration was delayed until after clinically evident disease onset. Ex vivo analyses revealed that in both cases, the effects were associated with inhibition of collagen-specific pro-inflammatory/Th1 cytokine (TNF-α, IL-6, and IFN-γ) release. In parallel in vitro human tissue studies, ES-62 was found to significantly suppress macrophage activation via cognate interaction with activated T cells. Finally, ES-62 suppressed LPS-induced rheumatoid arthritis synovial TNF-α and IL-6 production. Evolutionary pressure has promoted the generation by pathogens of diverse mechanisms enabling host immune system evasion and induction of “tolerance.” ES-62 represents one such mechanism. We now provide proof of concept that parasite-derived immunomodulatory strategies offer a novel therapeutic opportunity in inflammatory arthritis. The Journal of Immunology, 2003, 171: 2127–2133.

Rheumatoid arthritis (RA) is a common chronic inflammatory disease for which current treatment strategies remain suboptimal. Recently, immune-based, anti-inflammatory therapies (e.g., TNF-α-targeting agents) have shown promise (1, 2), clearly demonstrating that the discovery of safe, novel immunomodulators could be of considerable clinical benefit. We have explored the hypothesis that the evolutionary pressure on parasite-host interactions to move toward harmony provides an opportunity to find new immunomodulators. Filarial nematodes, arthropod-transmitted parasites of vertebrates including humans, achieve long-term infection via suppression or modulation of the host immune system. In infected humans, reduced production of IFN-γ and increased production of IL-4/IL-10 together with elevated IgG4 levels suggest a predominant Th2/anti-inflammatory phenotype conducive to both parasite survival and host health (3, 4). Consistent with this, the majority of humans who harbor viable filarial nematodes exhibit little parasite-induced inflammatory response or local tissue destruction/disruption.

We have identified and characterized a glycoprotein, ES-62, secreted by the rodent filarial nematode Acanthocheilonema viteae, which has homologues in related human parasites (reviewed in Ref. 5). Mediated in part through posttranslational addition of phosphorylcholine (PC) attached to an N-type glycan, ES-62 exhibits broad immunomodulatory activities in vitro that are in general anti-inflammatory. Thus, ES-62 reduces Ag-driven B and T lymphocyte proliferation, inhibits the ability of macrophages to produce Th1/proinflammatory cytokines such as IL-12, TNF-α, and IL-6, modulates dendritic cell (DC) maturation to preferentially elicit Th2-polarized responses, and induces spleen cells and B1 B cells to produce IL-10 (reviewed in Refs. 6 and 7). Moreover, and importantly, our recent studies using osmotic pumps implanted in mice to release ES-62 at “physiological” concentrations indicate that the molecule is active in vivo (Refs. 8 and 9 and our unpublished observations). Filarial nematode infection has also been shown in some cases to modulate the human immune response to heterologous Ags, including vaccines. Of particular interest, the Th2-polarizing effect of infection can convert a response that would normally be Th1 (e.g., to mycobacterial Ag) to Th2 dominance (10).

The recently defined “Hygiene Hypothesis” proposes that increased incidence of disease associated with aberrant immune responses in the West reflects an absence of appropriate priming of the immune response by infectious agents such as parasitic helminths during childhood (11). Consistent with this, it is long recognized that several autoimmune disorders exhibit reduced incidence and severity in geographic regions with high parasite load (reviewed in Ref. 11). RA, for example, is reduced in filaria endemic areas, leading to suggestions that environmental factors may subtly alter disease progression (12).
Although the etiology of RA is unknown, dysregulated immune responses clearly play a significant role. In particular, within inflamed RA synovial membrane, the levels of proinflammatory cytokines (particularly TNF-α and IL-1β) exceed those of anti-inflammatory agents (IL-1Ra antagonist, IL-10). Such cytokine imbalance contributes directly to cartilage/bone erosion through matrix metalloproteinase production and dysregulated chondrocyte/osteoclast function (13, 14). The synovial cytokine response arises in part through the action of activated T lymphocytes. Synovial T cell populations contain oligoclonal subsets and several autoantigens have been identified to which synovial T cells exhibit enhanced responses (15, 16). T cell effector function may be via cytokine secretion, e.g., IFN-γ or IL-17, or through cell contact-dependent cognate interactions with macrophages via ligand pairs such as LFA-1/ICAM-1 and CD40/CD154 (17–27). Most data therefore suggest that RA is mediated via a type 1 T cell (Th1) response associated with excess monokine production (23–27). Given that it is anti-inflammatory/Th2 inducing, we hypothesized that ES-62 should possess powerful immune modulatory, antiarthritic properties. We now report studies in vivo using the collagengenerated-induced arthritis (CIA) model and in vitro using murine CIA and human RA-derived tissues that clearly demonstrate that ES-62 can potently suppress articular inflammation.

Materials and Methods

Animals

Male DBA/1 mice obtained from Harlan Olac (Bicester, U.K.) were used at 8–10 wk old and maintained at the Joint Animal Facilities, University of Glasgow (Glasgow, U.K.). All animals in experiments conducted in this study were cared for in accordance to the Home Office, U.K. animal guidelines.

Preparation of ES-62

ES-62 is a major secreted glycoprotein of the rodent filarial nematode Acanthocheilonema viteae and homologue of molecules found in filarial nematodes that parasitize humans. The molecule consists of a tetramer of identical monomers of 62 kDa which contain PC attached to N-type glycosylation sites (reviewed in Ref. 5). ES-62 was purified using an Endosafe kit (Charles River Laboratories, Kent, U.K.). ES-62 is used at a working concentration that has an effect on expression of MHC class II, CD40, CD80, and OX40 ligand expression on murine bone marrow-derived DC (29).

Induction and assessment of CIA

Male DBA/1 mice received 200 μg of bovine type II collagen (CII; Sigma-Aldrich) in CFA (Difco, Detroit, MI) by intradermal injection (day 0). Collagen (200 μg in PBS) was given again on day 21 by i.p. 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. Paw thickness was measured with a dial caliper (Kroeplin, Munich, Germany). For histological assessment, mice were sacrificed and the hind limbs were removed and fixed in 10% neutral-buffered Formalin, then decalcified in 5% formic acid and embedded in paraffin. Sections (5 μm) were stained with H&E or toluidine blue (Sigma-Aldrich). Quantification of arthritis was performed by two treatment-blinded observers as described elsewhere (30).

Briefly, H&E sections were scored on an Olympus BX51 light microscope (Olympus, Melville, NY) for the presence of synovial lining hyperplasia, inflammatory infiltrate, and cartilage/bone erosion (confirmed on toluidine blue parallel section). Each parameter was analyzed separately against a set of predefined, “standard” sections graded 0 (normal), 1 (mild), 2 (moderate), or 3 (severe).

Treatment protocols

For the prophylactic protocol, male DBA/1 mice were treated with 2 μg ES-62 s.c. on day 2, day 0 (day of immunization with CII in CFA), and day 21 (i.p. collagen challenge). The multitreatment group received 2 μg ES-62 s.c. as above followed by administration daily from days 22 to 24 and every 3 days thereafter until day 48. For the therapeutic studies, DBA/1 mice were treated daily with 2 μg ES-62 s.c. for a total of 14 days commencing 1 day after CIA was clinically detectable. Control mice received PBS alone at the same time points.

Collagen-specific in vitro culture

Draining lymph node cells were cultured at 2 × 10^6/ml for up to 96 h in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all Life Technologies). Cells were stimulated with graded concentrations of CII (50 μg/ml proved optimal and therefore data for this are shown) or Con A (5 μg/ml). Proliferation assays were performed in triplicate in U-bottom 96-well plates (Nunc, Roskilde, Denmark) at 2 × 10^5/ml for 96 h. [3H]Thymidine (Amersham, Arlington Heights, IL) was added for the last 18 h of culture. Supernatants from parallel triplicate cultures were stored at −70°C until estimation of the cytokine content by ELISA.

Human studies

Samples were derived with approval from Glasgow Royal Infirmary Ethical Committee (Glasgow U.K.). RA patients fulfilled the American College of Rheumatology diagnostic criteria. Peripheral blood (PB) T cell and monocyte subpopulations were prepared as described previously (17). Briefly, peripheral blood donor or RA patient-derived PB T cells were stimulated for 72 h with PHA (5 μg/ml)/PMA (10 nM) in RPMI 1640 with 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (all Sigma-Aldrich) and then fixed in 4% paraformaldehyde. Control non-activated T cells were also fixed for comparative purposes. Fixed PB T cells were then cocultured with myelomonocytic THP-1 cells that were pretreated with ES-62 for 18 h. ES-62 was used at 2 μg/ml, a concentration that we have previously reported to be within the active range with respect to effects on a number of cells of the immune system (macrophages, DC, B lymphocytes (8, 28, 29)). Supernatants were harvested after 48 h of coculture for TNF-α estimation by ELISA. Primary synovial membrane cultures were obtained as previously described by collagenase (Worthington Biochemical, Lakewood, NJ)/DNase (Sigma-Aldrich) digestion of synovial membrane samples obtained at knee arthroplasty (17). Synovial fluid was obtained from RA patients and mononuclear cells were prepared in Lymphoprep (Axis- Shield, Norway) by density gradient centrifugation. Synovial membrane or synovial fluid cultures were at 2 × 10^6/ml in the presence or absence of LPS (100 ng/ml) for 48 h.

ELISA

All cytokines and anti-collagen Ab levels were detected by ELISA. TNF-α, IFN-γ, IL-5, IL-6, and IL-10 (all BD PharMingen, San Diego, CA) assays were performed 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 and IFN-γ both at 20 pg/ml. Similarly, human TNF-α and IL-6 (BioSource International, Nivelles, Belgium) were assayed by ELISA. Lower limits of detection were 30 pg/ml. Anti-collagen II Ab titers of individual sera were detected with bovine-conjugated anti-mouse IgG1 or IgG2a (BD Pharmingen), followed by conjugated avidin peroxidase (Sigma-Aldrich) and developed with tetramethylbenzidine substrate (Kirkegaard & Perry, Gaithersburg, MD).
Clinical and histological scores were analyzed with the nonparametric Mann-Whitney U test. Cytokine- and collagen-specific IgG levels were compared using Student’s t test.

**Results**

**ES-62 suppressed development of CIA**

We first investigated the effect of ES-62 on the development of CIA in DBA/1 mice. ES-62 significantly suppressed the severity of developing CIA when administered during CII priming and i.p. challenge (days −2, 0, and 21; Fig. 1). In a second protocol, continued further administration of ES-62 (on days 22–24 and then every 3 days until day 50) reinforced this suppression of articular disease. To explore the mechanisms whereby such effects were achieved, CIA-specific immune responses were examined in vitro in draining lymph nodes obtained at days 33 and 50. CII-induced IFN-γ, TNF-α, and IL-6 release was significantly suppressed in mice receiving ES-62 in both protocols at day 33 (Fig. 2 and results not shown) and was associated with significant reduction in CII-induced cell proliferation (Fig. 2). In contrast, CII-induced production of IL-10 was significantly increased in ES-62 recipients. Neither IL-4 nor IL-5 release was detected. Immune modulation by ES-62 in vivo was not generalized since Con A-induced proliferation and production of IFN-γ, TNF-α, IL-5, and IL-10 in parallel cultures was not affected (data not shown). By day 50, T cell proliferation and IFN-γ release to CII in lymph node cultures remained significantly suppressed in both treatment arms. However, TNF-α and IL-6 release to CII was suppressed only in those mice receiving ongoing ES-62 injections (Fig. 2 and results not shown). Finally, serum collagen-specific IgG2α, but not IgG1, levels were significantly reduced in recipients of multiple ES-62 doses (Fig. 2). Together, these data clearly indicate that ES-62 significantly reduced the severity of developing CIA and that these effects were mediated at least in part through suppression of the CII-specific Th1 response.

**ES-62 suppresses established CIA**

It was important to determine whether similar effects could be obtained if ES-62 treatment was commenced after disease onset as this has previously been predictive in developing cytokine-targeting therapies in RA (31). ES-62 was therefore administered daily to DBA/1 mice commencing 1 day after CIA became clinically detectable. Significant reduction of arthritis progression compared with vehicle-treated controls was apparent within 3 days. This was evident in the mean articular index and in the number of subsequently recruited arthritic joints, but also in reduced progression of articular swelling in the initially inflamed joint (Fig. 3). To determine whether ES-62 administration prevented articular inflammation and destruction, we evaluated synovial inflammation and cartilage and bone integrity histologically. Toluidine blue staining was used to evaluate peptidoglycan loss. Progression of synovial hyperplasia and cartilage and bone erosion was significantly suppressed by ES-62 (Fig. 4). Together these data indicate that ES-62 potently suppressed inflammatory CIA even when commenced after the onset of clinically detectable disease. Importantly, such activity could prevent progression of articular damage.

We next determined whether ES-62 could modify the ongoing collagen-specific Th1 response when administered after the onset of established clinical disease. Draining lymph nodes were obtained 1 day after completion of ES-62 administration. ES-62 significantly reduced spontaneous and CIA-induced IFN-γ production and CII-induced cellular proliferation (Fig. 5). Neither IL-4 nor IL-5 production was detected. CII-induced TNF-α and IL-6 release...
activated PB T cells (surrogate for bystander activation of T cells) with respect of local Ag recognition (17, 18, 32, 33). PHA/PMA-activated macrophages drive RA synovial proinflammation primarily through cell contact with macrophages, irrespective of local Ag recognition. PHA/PMA-activated PB T cells (surrogate for bystander activation of T cells) from normal human subjects were paraformaldehyde-fixed and then cocultured with myelomonocytic THP-1 cells that had been cultured in the presence or absence of ES-62. Coculture-induced macrophage TNF-α release was significantly suppressed by ES-62 (Fig. 6A). Similar data were obtained when identical experiments were performed using PB T cells derived from RA patients (Fig. 6B). IL-10 was not detected in these cultures under either control or ES-62-treated conditions (data not shown). Finally, to confirm that cytokine release from RA synovium-derived tissues could be reduced by ES-62, we prepared primary cultures from RA synovial fluid and membrane and compared the capacity for monokine release in the presence or absence of ES-62. Significant suppression of LPS-induced TNF-α and IL-6 was observed in the presence of ES-62, clearly indicating that the latter can modify critical proinflammatory pathways ex vivo (Fig. 6, C and D, and results not shown) in disease-relevant tissues. In contrast, no effect was observed on IL-10 release in either resting or LPS-stimulated RA synovial membrane cultures.

**Discussion**

The present report provides powerful proof of the concept that molecular characterization of the host-parasite relationship offers therapeutic potential in the context of autoimmune disease. We have clearly shown for the first time that a defined filarial nematode-derived product can suppress inflammatory arthritis. ES-62, given either during Ag priming or after the onset of clinically evident disease, was effective in reducing synovial inflammation and articular structural damage. This was associated with Ag-specific immune suppression whereas mitogen-induced responses remained intact. However, ES-62 was active in reducing macrophage cytokine release induced by T cells previously activated in a non-Ag-driven manner, analogous to Ag-independent pathways that operate in RA synovitis (18, 33). Currently available therapeutics in RA are characterized often by unfavorable toxicity: benefit ratios. Recent cytokine-targeting therapies have proven encouraging but as yet provide a significant magnitude of response.
FIGURE 5. Suppression of established disease by ES-62 is associated with reduction in proinflammatory cytokine production in vitro and anti-collagen IgG generation in vivo. A, Draining lymph node cells from arthritic mice (n = 3) treated with either ES-62 or PBS were cultured with medium (□) or CII (■) and T cell proliferation and cytokine production were assessed as previously described. Data are means ± SEM. B, Serum anti-CII IgG2a and IgG1 concentrations were determined by ELISA and expressed as means ± SEM (n = 5/group). *, p < 0.05 ES-62 vs PBS (Student’s t test).

(American College of Rheumatology 50% response or better) in only 40–50% of patients (1, 2). Therefore, there remains unmet clinical need for novel therapies. The therapeutic strategy explored herein capitalizes on evolutionary pressure operating over millennia to safely and specifically modulate host immune responses to facilitate long-lasting nematode tolerance in vivo. Human hosts exhibit high levels of circulating PC-containing molecules such as ES-62 commensurate with worm retention via Th2 polarization (reviewed in Ref. 34), but apparently remain capable of effectively clearing routine infections. Thus, chronic inflammatory diseases may be amenable to similar ES-62-mediated immune modulation while allowing the capacity for novel immune response generation to remain intact.

Several mechanisms can be put forward to explain the data we have obtained. ES-62 administration clearly reduced collagen-specific Th1 responses associated with significantly reduced cytokine expression, in particular, TNF-α and IFN-γ, even when such responses were established in vivo before administration of the parasite product. Similarly, when used in vitro ES-62 effectively suppressed TNF-α release induced in primary synovial cultures and that arising from cognate interactions between T cells and macrophages derived from RA patients. Critically therefore ES-62 effectively reduces TNF-α expression that in turn is unequivocally linked to RA pathogenesis by clinical intervention studies (1, 2). The mechanism by which ES-62 inhibits TNF-α production has yet to be fully defined. However, we have found that it can directly suppress TNF-α production from LPS-stimulated macrophages and this is associated with inhibition of p38 mitogen-activated protein kinase activation (our unpublished observations) that is required for such cytokine induction (35). Also of interest, although we have found that ES-62 does not appear to polarize T cell cytokine profiles directly (our unpublished results), the parasite product may operate to modulate T cell-derived cytokines through altered DC function. Thus, using an OVA-transgenic model, we have previously shown that murine DC maturation in the presence of ES-62 is polarized to preferentially induce subsequent Th2 responses (29). Since DC in various maturation states are detected in RA synovial membrane (36, 37), this provides an intriguing potential antiarthritic action for ES-62 in altering local synovial DC maturation, autoantigen presentation, and Th1 functional polarization along with downstream effects on cytokine-releasing cells, including macrophages. Future studies will be required to address this possibility.

Consistent with its ability to suppress collagen-specific Th1 cytokine responses, ES-62 significantly suppressed collagen-specific IgG2a expression in vivo and was associated with increased IL-10 release. The effect on Ab production may play a key role in ES-62-mediated amelioration of CIA as it is increasingly recognized that B cell-mediated pathology is of critical importance in RA. For example, B cell depletion using anti-CD20 Ab induces RA clinical improvement (38). Moreover, synovial B cells exhibit somatic hypermutation and several autoantibody specificities have recently been associated with RA clinical subsets and adverse prognosis (39–41). The murine KRN model in which anti-GPI autoantibodies promote the effector phase of articular destruction via complement, Fc receptor, and mast cell-dependent pathways potently exemplifies the potential for Ig-mediated articular pathology (42–44).

FIGURE 6. ES-62 inhibits TNF-α production from human T cells and macrophages in vitro. A and B, Paraformaldehyde-fixed PMA/PHA-activated (■) or medium control (□) PB T cells from normal donors (n = 3) or RA patients (n = 5) were cultured with THP-1 cells either pre-treated for 18 h with ES-62 (2 μg/ml) or medium alone. Activated PB T cells from both RA and normal donors induced TNF-α production in a cell contact-dependent fashion and ES-62 pretreatment of THP-1 cells significantly inhibited TNF-α synthesis compared with medium control. *, p < 0.05 (Student’s t test, data are means ± SEM). C and D, Single-cell suspensions prepared from RA synovial membranes were pretreated for 2 h with ES-62 (2 μg/ml; ○) or medium alone (□) and subsequently stimulated with LPS (1000 ng/ml or 100 ng/ml) for 24 h. Release of TNF-α and IL-6 into culture supernatants was measured by ELISA. Data are means ± SEM of triplicate cultures and are representative of three similar experiments.
44). We have previously shown that exposure to ES-62 suppresses subsequent Ag-mediated proliferation of B cells by uncoupling the Ag receptors (B cell receptor) from extracellular signal-regulated kinase/mitogen-activated protein kinase-dependent proliferative signaling (45–47). Such desensitization of B cell clonal expansion can be mimicked by the PC component of ES-62 (48). Similarly, the PC moiety of ES-62 polarizes the murine humoral response to the protein backbone of ES-62 from mixed IgG1/IgG2a exclusively to an IgG1 phenotype in an IL-10-dependent manner and thus appears to be responsible for preventing generation of a Th1 Ab response (49).

The Hygiene Hypothesis links the increase in autoimmune diseases in developed communities to lack of exposure to childhood infections resulting in improper priming of the immune system. Helminths represent a prominent candidate infection since worms remain prevalent throughout the tropics/subtropics but have largely been eliminated in the developed world. Our present strategy therefore relies on a logical extension of this idea with the goal of reversing the progression of autoimmune diseases. By using a molecule that is released by worms and that is known to polarize the immune system, we offer a convincing starting point for the development of new and potent immunomodulators for therapeutic use. In practice, we envisage using small molecule derivatives of ES-62 for use as therapeutic entities. This is not attributable to perceived problems with loss of function due to immunogenicity. Although ES-62 is a large “foreign” molecule, the persistence of PC-containing molecules in the bloodstream of filarial patients (34) indicates that immunogenicity would be unlikely to be a major problem. Consistent with this, we have found that ES-62 is active in mice even in the presence of an Ab response against the parasite molecule (9). The problem rather is that ES-62 is chemically complex, its synthesis being dependent on a biosynthetic pathway that is unique to nematodes. Defining the component of ES-62 that is functionally active in the present study is thus paramount. Our data obtained to date on the basic immunomodulatory activities of ES-62 suggest that this activity will be at least partly dependent on PC. Nonimmunogenic peptide mimetics of PC (50) or synthetic PC-containing structures will shortly be available to explore this possibility. However, we cannot rule out at this stage a role for the other components of ES-62—two distinct N-glycans that lack PC and the protein background that shows homology to amino/carboxyl peptides (5). A complete dissection of the molecule is thus warranted.

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