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The parasitic helminth product ES-62 suppresses pathogenesis in CIA by targeting of the IL-17-producing cellular network at multiple sites

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Abbreviations: CIA: collagen-induced arthritis; PC: phosphorylcholine; RA: rheumatoid arthritis

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ABSTRACT

Objectives: Among many survival strategies, parasitic worms secrete molecules to modulate host immune responses. One such product, ES-62, is protective in the collagen-induced arthritis (CIA) model of rheumatoid arthritis. As IL-17 has been reported to play a pathological role in the development of rheumatoid arthritis, we investigated whether targeting of IL-17 may explain the protection afforded by ES-62 in the CIA model.

Methods: DBA/1 mice progressively display arthritis following immunization with type-II collagen. The protective effects of ES-62 were assessed by determination of cytokine levels, flow cytometric analysis of relevant cellular populations and *in situ* analysis of joint inflammation.

Results: ES-62 was found to downregulate IL-17 responses in the CIA model. Firstly, it acts to inhibit priming and polarisation of IL-17 responses by targeting a complex IL-17-producing network, involving signalling between dendritic cells and γδ or $CD4^+$ T cells. In addition, ES-62 directly targets Th17 cells by downregulating MyD88 expression to suppress responses mediated by IL-1 and TLR ligands. Moreover, ES-62 modulates migration of γδ T cells and this is reflected by direct suppression of CD44 upregulation and, as evidenced by *in situ* analysis, dramatically reduced levels of IL-17-producing cells, including lymphocytes, infiltrating the joint. Finally, there is strong suppression of IL-17 production by cells resident in the joint, such as osteoclasts within the bone areas.

Conclusion: Such unique multi-site manipulation of the initiation and effector phases of the IL-17 inflammatory network could be exploited in the development of novel therapeutics for rheumatoid arthritis.

Key words: collagen-induced arthritis, inflammation, ES-62, IL-17

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory condition, which, despite recent advances in cytokine therapy, continues to increase in incidence in the Western World. However, in areas of the world endemic for helminth infections, rates of autoimmune diseases such as RA remain low leading to the hypothesis that certain helminth infections may protect against the development of autoimmunity [1]. In support of this theory, we have previously shown that ES-62, a phosphorylcholine (PC)-containing glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae*, has broad immunomodulatory activities and can exert powerful anti-inflammatory action in the mouse collagen-induced arthritis (CIA) model of RA [2, 3].

Originally it was proposed that the ability of ES-62 to inhibit disease severity in the CIA model reflected suppression of TNF-α production and associated Th1-mediated inflammation [2, 3]. However, it has become increasingly clear that Th17, rather than Th1 cells, appear to be the pathogenic drivers of inflammation in many autoimmune conditions, including CIA and RA [4]. Consistent with this, neutralization of IL-17 protects mice from disease, whilst over-expression of IL-17 exacerbates pathology [5]. Moreover, Th17 cells may be vital in promoting the chronic destructive phase of arthritis, due to their ability to induce the expression of RANKL and activate osteoclasts, thereby leading to bone resorption [6] as well as stimulating matrix metalloproteinases resulting in cartilage breakdown [7, 8]. Indeed, studies from RA patients have shown that IL-17 levels are raised in serum and synovial fluid samples compared to those from osteoarthritis or healthy control subjects [9]. By contrast, it has been proposed that IFNγ may play a protective role as IFNγR$^{-/-}$ mice are more susceptible to the development of CIA [10], perhaps reflecting abrogation of counter-regulation of Th17 development by IFNγ-producing Th1 cells [11]. Moreover, IFNγ is a potent antagonist of

osteoclastogenesis in mice and humans [12, 13] and thus may also act to prevent joint erosion.

Therefore, given these new insights into CIA pathology, it was important to ascertain the effect, if any, that ES-62, a molecule being considered in the context of therapeutic intervention, has on pro-inflammatory IL-17 production, and thus to re-address its protective role, but in the perspective of IL-17-associated pathology.

MATERIALS AND METHODS

CIA

Animals were bred (BALB/c and C57BL/6 background) and/or maintained in the Biological Services Units in accordance with the Home Office UK Licences PPL60/3580, PPL60/3119 and PIL60/12183 and the Ethics Review Board of the University of Glasgow. CIA was induced in male DBA/1 mice (8-10 weeks old; Harlan Olac; Bicester, UK) by intradermal immunization with bovine type II collagen (CII, MD Biosciences) in complete Freund's adjuvant (FCA) and mice were treated with purified endotoxin-free ES-62 (2 μg/dose) or PBS subcutaneously on days -2, 0 and 21 [2, 3] and cells were recovered from joints [14] as previously described.

***Ex vivo* analysis**

Draining lymph node (DLN) cells (10^6/ml) were incubated ± 50 ng/ml PMA plus 500 ng/ml ionomycin for 1 h before addition of 10 μg/ml Brefeldin A (Sigma-Aldrich, UK) for 5 h at 37^{o}C with 5 % CO_2. Phenotypic markers were labelled using anti-TLR4-APC (R&D Systems), biotinylated anti-CD44 (BioLegend; detected by streptavidin-PE, BD Pharmingen), anti-CD4-PERCP or biotinylated anti-CD4 (detected by Alexa Fluor-450 streptavidin, BD Pharmingen), or anti-γδ-FITC (BioLegend) antibodies before the cells were fixed and permeabilised using BioLegend protocols. Cells were then labelled using anti-IL-17A-APC or anti-IL-17A-PerCP-Cy5.5 (BioLegend), anti-RORγt (eBioscience; detected by anti-Rat IgG-APC) and anti-MyD88 (Abcam; detected by anti-Rabbit IgG-PE) antibodies for 30 min prior to flow cytometry, gated according to appropriate isotype controls.

Cytokine analysis

ELISAs for IL-17A, IL-10 (BioLegend), TNF-α, IL-6, IL-23 and IL-27 (eBioscience) were performed according to manufacturer's instructions. Alternatively, IL-17A was detected by cytometric bead assay (FlowCytomix).

In vitro **cell culture**

Bone marrow-derived dendritic cells (bmDCs) from male DBA/1, C57BL/6 or BALB/c mice (6-8 weeks old) were derived by *in vitro* culture in complete RPMI 1640 medium (containing 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin and 10% FCS) supplemented with 10% conditioned medium from the GM-CSF-transfected X63 myeloma cell line and 50 µM 2-ME at 37^{o}C and in 5% CO_2 for 6d. Naïve $CD4^{+}CD62L^{+}$ T cells and γδ T cells were isolated using Miltenyi magnetic bead technology. For bmDC-T cells co-cultures, bmDCs were incubated with ES-62 (2 µg/mL), matured with LPS (*Salmonella minnesota*; Sigma) and then pulsed with ovalbumin (OVA) peptide (0-300 nM) before incubation with naïve T cells derived from OVA-specific DO.11.10/BALB/c or OT-II/C57BL/6 mice for 4d. For *in vitro* polarisation of Th17 cells, naïve LN T cells from BALB/c mice were incubated in plates pre-coated with anti-CD3 (4 µg/mL) with anti-CD28 (1.5 µg/mL), anti-IFN-γ (5 µg/mL) and anti-IL-4 (5 µg/mL) antibodies and rIL-6 (20 ng/mL), rTGF-β (4 ng/mL), and rIL-1 (10 ng/mL) ± ES-62 (0-1 µg/mL) for 4d. γδ T cells from BALB/c mice were activated with rIL-1+ rIL-23 (both at 10 ng/mL) overnight ± ES-62 (2 µg/mL) before being incubated with bmDCs at different γδ:DC ratios (1:2, 1:5 and 1:20) and culture supernatants collected after 24 h.

Immunofluorescence

Tissue sections (7 μm) were deparaffinized in xylene, dehydrated in ethanol and antigen retrieved by incubation at 60°C for 2 h in 10 mM Tris-1 mM EDTA-0.05% Tween 20 buffer, pH 9.0. Samples were stained with a goat anti-mouse IL-17 antibody (R&D Systems; or goat IgG isotype control) and DAPI as counterstain, at 4°C for 12 h followed by a biotinylated rabbit anti-goat IgG antibody and streptavidin-Alexa Fluor 647. Images were obtained using a LSM510 meta confocal laser coupled to an Axiovert 200 (Zeiss) microscope and analyzed with the software Zeiss LSM Image Browser.

Laser Scanner Cytometry (LSC)

DLNs were fixed in 10% formalin at 4°C for 24 h, transferred to 30% sucrose in PBS for 48 h before being frozen in liquid nitrogen in OCT (Bayer) and stored at -70°C. Sections (7 μm) were stained with anti-B220-FITC and anti-γδ TCR-PE (or isotype controls; BD Pharmingen) and mounted in Vectashield (Vector Laboratories). Quantitation of fluorescence was by LSC (CompuCyte) to generate tissue maps of the DLNs using WinCyte software version 3.6 (CompuCyte). Briefly, setting of a positive staining $B220^+$ B cell gate generated a tissue map of the localisation of $B220^+$ B cells that allowed generation of the indicated gates designating the paracortical (T cell area) and follicular ($B220^+$ B cell area) regions that were subsequently copied onto the γδ TCR^+ T cell tissue map. This allowed unbiased statistical quantitation of γδ TCR^+ T cells within follicular regions by the WinCyte software following merging of γδ TCR^+ T cell and $B220^+$ B cell tissue maps [15].

qRT-PCR

qRT-PCR and reverse transcription of RNA were according to the manufacturer's instructions (Applied Biosystems). HPLC purified probes (VH Bio; Integrated DNA technologies) contained the reporter 5'-6-carboxy-fluorescein (FAM) and quencher 3'-6-carboxy-tetramethyl rhodamine (TAMRA) dyes and sequences were: RORγt: F- 5'CCG CTGAGAGGGCTTCAC3', R- 5'TGCAGGAGTAGGCCACATTACA3' and 5'-FAM-AAGGGCTTCTTCCGCCGCCAGCAG-TAMRA-3'. Applied Biosystems assay kits for IL-17A, MyD88 and GAPDH (Mm00439618_m; NM_010851.2 and 4352339E1) were used. Data were analyzed by RQ Manager software (Applied Biosystems), normalized to the reference reporter GAPDH.

Statistics

Parametric data were analysed by the unpaired two-tailed Student's t test or by 1-way ANOVA followed by the Newman-Keuls post-test. Normalised data were analysed by the Kruskal-Wallis test whilst the Mann-Whitney test was used for analysis of clinical CIA scores where *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

RESULTS

ES-62 protection against CIA is associated with down-regulation of IL-17 responses

ES-62 exhibits anti-inflammatory action in terms of significant reduction of articular score and hind paw swelling in mice undergoing CIA (Figure 1A). Disease incidence was also delayed and reduced in such ES-62-treated mice (Figure 1A). Consistent with IL-17 playing a pathogenic role in CIA, we observed a strong positive correlation of IL-17 (IL-17A), but not IFNγ, levels in serum with disease scores in animals with CIA (Figure 1B and results not shown). Thus to assess whether protection by ES-62 is associated with suppression of IL-17-mediated pathology, the effect of administration of the helminth product on serum cytokine levels was analyzed. Significantly higher levels of IL-17, but not IFNγ, were detected in the serum of mice with CIA, compared to naïve animals, and exposure to ES-62 *in vivo* reduced these towards the levels observed in naïve mice (Figure 1B and results not shown).

In accordance with this, significant differences between the PBS-, but not ES-62-, treated, and naïve groups mice were found in terms of total numbers of DLN cells (results not shown) and significantly higher proportions of DLN cells from CIA-PBS animals produced IL-17 relative to the ES-62-treated CIA mice following *ex vivo* stimulation with PMA plus ionomycin (Figure 1C). Moreover, although the differences did not reach statistical significance, analysis of spontaneous IL-17 production by cells recovered from the site of inflammation also showed a reduction in the proportion of IL-17$^+$ cells infiltrating the joint in the ES-62 treated animals (Figure 1C). Corroboration that ES-62 suppressed Th17 responses was provided by data showing that RORγt mRNA levels were significantly lower in DLN cells from the ES-62-treated compared to PBS-treated CIA mice (Figure 1D). Targeting of RORγt and IL-17-associated responses by ES-62 was specific, since expression of the Th1-

associated transcription factor, t-Bet was not affected by exposure to the parasite product (data not shown).

ES-62 suppresses the levels of IL-17-producing CD4 and γδ T cells

CD4 and γδ T cells were the two major IL-17 producing compartments (>90%) in the DLN of all treatment groups (Figure 2A). Although the mice with CIA (PBS) tended to have higher numbers of DLN $CD4^+$ T cells than those from both the naïve and ES-62-treated groups (Figure 2B), there were no significant differences amongst any of these groups, either in terms of proportions or absolute numbers of $CD4^+$ T cells spontaneously producing IL-17 (results not shown). By contrast, following *ex vivo* stimulation with PMA plus ionomycin, whilst there were no differences in the proportions of such IL-17^+ T cells (Figure 2C), significantly higher numbers of $CD4^+$ T cells from the mice with CIA expressed IL-17 relative to the naïve group and this was reduced by exposure to ES-62 (Figure 2D). Analysis of γδ T cell responses showed that there were no significant differences amongst the groups in terms of the total numbers of such cells present in the DLN (Figure 2B). However, both higher proportions and absolute numbers of γδ T cells from the mice with CIA, but not those exposed to ES-62 *in vivo*, spontaneously produced IL-17 when compared to those from the naïve group (Figure 2C&D). No differences were detected amongst the groups, however, following *ex vivo* stimulation with PMA plus ionomycin (results not shown). Interestingly, whilst unlikely to be related to its protective effects (lack of serum IFNγ-disease correlation as mentioned earlier and [10]) we found that ES-62 suppressed the % of $CD4^+$, $\gamma\delta^+$ and $CD8^+$ T cells spontaneously producing IFNγ (results not shown), data consistent with our previous reports that ES-62 suppressed IFNγ recall responses in CIA [2, 3].

ES-62 can attenuate Th17 responses by both indirect and direct effects

ES-62 modulates DC-mediated priming and polarisation of Th cell responses in healthy mice [16-18]. Thus, we next investigated whether ES-62 modulated the capacity of DC to prime Th17 responses in CIA by pre-incubating bmDC first derived from naïve DBA/1 mice with ES-62 before "maturing" them with LPS *in vitro*. Although LPS-stimulated release of IL-10 was unaffected (results not shown), we observed that ES-62 significantly inhibited the LPS-induced secretion of the pro-inflammatory cytokine, TNF-α and two cytokines associated with polarisation and survival of Th17 cells, IL-6 and IL-23 (Figure 3A). Similarly, bmDC derived from DBA/1 mice undergoing CIA (mean articular score of 7.1 ± 0.68) produced reduced levels of TNF-α, IL-6 and IL-23 when treated with ES-62 prior to LPS maturation, *in vitro* (Figure 3B). Moreover, whilst IL-23 could not be detected, bmDC derived from DBA/1 mice undergoing CIA (mean articular score 5.4 ± 1.6) spontaneously produced significantly more IL-6, but not TNF-α or IL-10, than those derived from either naïve DBA/1 (articular score 0) mice or DBA/1 mice undergoing CIA that had been exposed to ES-62 (mean articular score 1.8 ± 0.5) *in vivo* (Figure 3C and results not shown). Collectively, these findings suggested that ES-62 could suppress the generation of Th17-polarising cytokines by DC in CIA, and consistent with this ES-62-treated DCs show a reduced ability to skew naïve OVA-specific T cells towards a Th17 phenotype (Figure 3D).

We next investigated whether ES-62 could also directly affect Th17 cells. Naïve T cells were primed using anti-CD3 plus anti-CD28 antibodies in the presence of the cytokines, IL-6, TGFβ and IL-1β and neutralizing antibodies specific for IFNγ and IL-4 to induce *in vitro* differentiation of Th17 cells. When co-incubated with the parasite product, we observed that ES-62 was able to directly down-regulate IL-17 production in a significant and dose-

dependent manner and this reduction in IL-17 release was reflected by reduced IL-17 mRNA levels (Figure 4A). We found expression of TLR4, which is required for ES-62 action [19], to be up-regulated during *in vitro* priming and differentiation of Th17 cells, in parallel with that of MyD88 and RORγt (Figure 4B). From a mechanistic point of view, whilst ES-62 did not appear to modulate either the surface or intracellular levels of TLR4 (data not shown), it did induce downregulation of the TLR signal transducer, MyD88 (Figure 4C) and this was also reflected at the mRNA level (Figure 4D).

DCs are necessary for ES-62-targeting of IL-17 production by γδ T cells

To address whether ES-62 could likewise directly modulate IL-17 production by γδ T cells, γδ T cells from naïve mice were stimulated to produce IL-17 *in vitro* in a TCR-independent manner using rIL-1+rIL-23 [20]. Such "activated", but not resting, γδ T cells produced large amounts of IL-17. However, ES-62 did not modulate this response (Figure 5A) and perhaps consistent with this, we could not detect TLR4 expression and culture with LPS did not induce γδ T cell activation (results not shown). Nevertheless, we found that ES-62 could inhibit γδ T cell activation as indicated by its ability to prevent upregulation of the cell surface marker CD44 *in vitro* (Figure 5A) and *in vivo* (Figure 5B).

We therefore next investigated whether DCs were regulating production of IL-17 by γδ T cells by co-culturing LPS-matured DCs with resting or IL-1/IL-23-stimulated γδ cells that had been exposed or not to ES-62 and found that IL-17 production was reduced in such ES-62-treated DC-γδ T cell co-cultures at all ratios tested (Figure 5C). Furthermore, IL-17 and RORγt mRNA levels were also reduced when the activated γδ T cells had been exposed to ES-62 (Figure 5C). DC maturation is required for these conditioning effects on γδ T cells as such immunomodulation did not occur with immature DCs. Also, whilst the results did not reach significance, the observed effects were associated with increased generation of IL-27, a

cytokine that antagonises IL-17 production [21, 22], in the co-cultures containing ES-62-treated γδ T cells (results not shown).

ES-62-mediated modulation of γδ T cell responses also appears to occur during CIA *in vivo*. Thus, such DLN γδ T cells not only displayed reduced expression of CD44 when analysed *ex vivo* (Figure 5B) but also *in situ* analysis demonstrated that γδ T cells in such ES-62-treated mice exhibited altered localisation within DLN, showing reduced distribution in the B cell follicles compared to those from PBS-treated CIA animals (Figure 5D).

ES-62 reduces the levels of IL-17 positive cells in the joints of CIA animals

Consistent with a pathogenic effector role for IL-17 in the joint, *in situ* analysis showed that whilst little or no IL-17 expression could be detected in the joints of naïve mice (Figure 6A), there was strong expression of this cytokine in the joints from PBS-treated CIA mice, (articular scores 7&8). By contrast, expression of IL-17 was dramatically reduced in the joints of ES-62-treated mice (articular scores 3&0). Furthermore, examination of the cells producing IL-17 indicated that this reflected cells both infiltrating the joint (Figure 6B&C), including large numbers of lymphocytes as indicated by size and morphology (Figure 6C), as well as those in the bone such as multinucleated osteoclasts (Figures 6B). IL-17 produced at both sites appeared to be reduced in mice treated with ES-62 (Figure 6A). These data suggest that as well as suppressing early IL-17-driven pro-inflammatory responses in the DLN associated with the initiation of pathogenesis, exposure to ES-62 *in vivo* is able to reduce effector IL-17 responses in the affected joints.

DISCUSSION

The recent proposal that IL-17 is a master regulator of CIA pathogenesis suggested that targeting of cellular producers of this cytokine might provide a mechanism for suppression of disease severity by ES-62 [2, 3]. Consistent with this, the highly elevated levels of IL-17 in the serum of mice with CIA, compared to naïve animals, were significantly reduced in the ES-62-treated CIA cohorts. Furthermore, ES-62 reduced the percentage of IL-17$^+$ DLN cells, relative to their control CIA cohorts, such that these were not significantly different to those of naïve DBA/1 mice. Although, prophylactic treatment with ES-62 at d-2, -0 and -21 results in some 50-60 % suppression of articular score, it is likely that more frequent and/or higher doses of ES-62 would have further reduced IL-17 responses and resultant pathology. Alternatively, since ES-62 typically reduces IL-17 responses towards the levels observed in naïve DBA/1 mice, the residual pathology observed in the presence of ES-62 could reflect IL-17-independent pathogenic effector mechanisms. However, it is certainly the case that CD4$^+$ and γδ T cell-driven pathogenesis in CIA relies on the ability of these cells to initiate IL-17 dependent responses [14, 23-25], albeit, it has recently been suggested that the induction of γδ T cells may be as a result of CFA-associated inflammation [14, 23]. However, we found that the levels of IL-17$^+$ γδ T cells were not up-regulated in mice immunized with CFA alone (results not shown), indicating that IL-17 production by both CD4$^+$ and γδ T cells plays a role in the collagen response and importantly, ES-62 targets both of these major IL-17 producing compartments in our CIA model.

DCs are a major target of ES-62 action in modulating priming and polarisation of Th cell responses [16-18]. Thus, we hypothesised that the reduction in Th17 cells reflected suppression of Th17 cell priming by DCs. We subsequently found that *in vitro* conditioning of bmDC with ES-62 significantly inhibited LPS-driven production of TNF-α, IL-6 and IL-

23, the latter two cytokines implicated in the development and maintenance of the Th17-phenotype, and a reduction of OVA-specific priming of IL-17 production by naïve $CD4^+$ Th cells. However we also observed that ES-62 could modulate Th17 responses, directly. Although naïve $CD4^+$ T cells do not express TLR4, the receptor required for ES-62 to mediate its anti-inflammatory effects in APC [19, 26], we found upregulation of TLR4 and MyD88 in parallel with the signature transcription factor, RORγt during *in vitro* polarisation to the Th17 phenotype. ES-62-mediated suppression of the resultant IL-17 response therefore likely reflects not only that TLRs can be expressed by most T cell subsets, but also that TLR agonists (e.g. for TLR3, 5, 7 and 9) can modulate effector or regulatory T cell responses in the absence of APC (reviewed in [27]) and LPS/TLR4 signalling can both induce and enhance IL-23-stimulated IL-17 release from *in vitro* differentiated Th17 cells [28]. We find that ES-62 suppresses IL-17 release from Th17 cells differentiated *in vitro* in response to IL-1 but not in response to IL-23 (manuscript in preparation), a cytokine that has been shown to commit naïve T cells to a Th17 phenotype via STAT-3 activation independently of MyD88 recruitment [29]. Therefore, ES-62 subversion of signalling via TLR4 with consequent downregulation of MyD88, a key signal transducer of the TLR/IL-1R family [30], would provide a molecular rationale for the observed decrease in Th17 polarisation given that it has recently been reported [31, 32] that IRAK4 and IRAK1, the downstream effectors of IL-1R/MyD88 signalling are required for such polarisation.

By contrast, ES-62 did not directly down-regulate IL-17 production by γδ T cells in response to activation with IL-1/IL-23 and consistent with this, we were unable to detect TLR4 expression by γδ T cells, supporting the proposal that modulation of γδ T cell responses by LPS requires cooperation with DCs [33]. It was surprising therefore that we found that ES-62 suppressed the upregulation of CD44 resulting from activation of γδ T cells in response to IL-

1/IL-23. These data suggested that ES-62 might directly modulate γδ T cell activation, but not cytokine production, through some undefined receptor like those involved in recognition of small phosphorylated molecules present in Mycobacteria that lead to DC activation by γδ T cells [34, 35] in a TLR-independent manner [36-39]. In turn, mature DCs can stimulate γδ T cells to promote sustained immune responses [37, 40] and perhaps of relevance to this study, DCs have been shown to modulate IL-17 production by γδ T cells [41]. Thus as the active phosphorylcholine moiety of ES-62 [3] is structurally reminiscent of the phosphorylated mycobacterial molecules, this suggested that ES-62 was possibly targeting γδ T cells via such receptors to modulate bi-directional interaction with DC, a hypothesis supported by ES-62 downregulating IL-17 and tending to upregulate IL-27, a cytokine that suppresses CIA [22, 42, 43], production in DC:γδ co-cultures.

The targeting of CD44 expression by γδ T cells observed *in vitro* and *in vivo* may be physiologically relevant to ES-62-mediated protection from CIA as such modulation would impact on lymphocyte migration during CIA [44], particularly to the joint [45]. Indeed, *in situ* LSC revealed that exposure to ES-62 *in vivo* modulates the localisation of γδ T cells within the DLN of mice with CIA and this may in turn modulate bidirectional signalling between γδ T cells and DC, to subvert initiation of the inflammatory phenotype driving autoimmunity. Moreover, and perhaps reflecting suppression of CD44-mediated migration of IL-17-producing lymphocytes to the site of inflammation, we have also shown that ES-62 can dramatically reduce the levels of IL-17$^+$ infiltrating cells in the joints. This is likely to be of importance therapeutically as IL-17 produced during the initiator phase, induces the recruitment and accumulation of inflammatory cells, particularly neutrophils to the joints and the release of pro-inflammatory chemokines, cytokines and matrix metalloproteinases [7, 8, 46], that ultimately results in osteoclastogenesis and bone destruction *in situ* [47].

Interestingly, our data suggest that during the effector phase, infiltrating cells and bone cells could both be producing IL-17 *in situ*, as some of the IL-17$^+$ cells in the bone appear multinucleated (Figure 6B), suggesting that they could be osteoclasts. Moreover, the infiltrating cells in the joints of mice with CIA contain large numbers of small IL-17$^+$ mononuclear cells that appear to be lymphocytes, consistent with ES-62 blocking the migration of pathogenic effector Th17 and/or IL-17-producing γδ T cells to the site of inflammation. Importantly, levels of all classes of IL-17 producing cells in the joint appear to be reduced in the ES-62-treated mice.

Collectively, these data suggest that ES-62 targets the IL-17 inflammatory axis at several regulatory points in order to optimize safe modulation of pathogenic IL-17 responses (Figure 6D). Thus, it both targets cells of the innate immune system (DCs and γδ T cells) to inhibit initiation of pathogenic responses and also, by acting directly on Th17 cells, suppress ongoing adaptive responses. Mechanistically, given the increasing evidence for TLR signalling in the initiation (DC) and amplification of Th17 and γδ T cell-mediated IL-17 responses and autoimmune inflammation [28, 31, 48], it is pertinent that ES-62 rewires TLR-2, -4 and -9 driven maturation of DC to an anti-inflammatory phenotype in a TLR4-dependent manner [19]. This is reflected here by the inhibition of LPS-induced TNF-α, IL-6 and IL-23 production as well as increased IL-27 release, resulting in suppression of differentiation and/or maintenance of the Th17 phenotype. ES-62 can also act directly on CD4$^+$ T cells to suppress IL-1-dependent Th17 differentiation and this likely involves TLR4-mediated downregulation of MyD88, leading to un-coupling of IL-1R from IRAK1/4 signals that are essential for Th17 polarisation [31, 32]. As MyD88 is a key signal transducer for all TLR family members except for TLR3 (interestingly, signalling of which is not modulated by ES-62 [19]), the recent finding that Th17 responses and consequent autoimmune

pathogenesis are promoted by TLR2 signalling *in vivo* [28] suggests that ES-62 may downregulate MyD88 expression as a general mechanism of targeting aberrant Th17 responses and inflammatory disease. Interestingly, ES-62 also acts directly on γδ T cells, possibly via phosphoantigen receptors (pAgR), not only to modulate the bidirectional DC-γδ interactions required to drive subsequent adaptive Th17 responses, but also to downregulate CD44 expression and suppress migration of such pathogenic cells to the joints. Certainly, it dramatically suppresses pathogenic IL-17 production by effector cells within the joint.

Hence, the use of ES-62 to modulate this highly inflammatory mediator by targeting both DC maturation and effector T cell responses through subversion of TLR4 signalling, without compromising the host immune response to infection [26], constitutes a highly appealing therapeutic strategy for RA.

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FIGURE LEGENDS

Figure 1. ES-62 protects against CIA.

(A) Clinical scores (PBS, n=43; ES-62, n=32; left-hand panel) and paw width (n=9; middle panel), expressed as mean scores ± SEM for PBS (filled squares) or ES-62 (open squares)-treatment groups of CIA mice and disease incidence (right-hand panel), indicated by the % of animals in the PBS- (solid line) and ES-62- (broken line) groups developing a severity score ≥ 1. (B) Serum IL-17 levels are plotted, as the means of triplicate analyses from individual mice showing a significant correlation with clinical score (number of XY pairs = 26, Pearson r = 0.6050; $p<0.001$; left-hand panel) and as mean values of triplicate IL-17 analyses of serum from individual mice (naïve, n = 16; PBS, n = 26; ES-62, n = 23; right-hand panel). (C) The % IL-17$^+$ DLN cells after *ex vivo* stimulation with PMA plus ionomycin; (PBS, n=19, ES-62, n=15; left-hand panel) and the % IL-17$^+$ joint cells (PBS, n=11, ES-62, n=8; right-hand panel). (D) Mean values ± SEM of RORγt/GAPDH mRNA (PBS, n = 4, ES-62, n = 3) as plotted for individual mice.

Figure 2. ES-62 targets IL-17-producing CD4$^+$ and γδ T cells.

(A) Exemplar plots of gating strategy of intracellular IL-17 expression by DLN cells from PBS- and ES-62-treated mice with CIA show forward scatter (FSC) on the x-axis versus IL-17 expression on the y-axis as well as the cellular expression of IL-17 by CD4 and γδ T cells. (B) The numbers of CD4$^+$ T cells (left-hand panel) and γδ T cells (right hand panel) present in the DLN of individual mice (naïve, n=12; PBS, n= 19 and ES-62, n= 15) are shown. (C) Percentages of IL-17$^+$ CD4$^+$ T cells (naïve, n=12; PBS, n=19; ES-62, n=15; left hand panel;) after PMA plus ionomycin and IL-17$^+$ γδ T cells (naïve, n=8; PBS, n=11; ES-62, n=9; right hand panel) spontaneously producing IL-17 cells in the DLN of individual mice. (D) Absolute numbers of IL-17$^+$ CD4$^+$ T cells (naïve, n=12; PBS, n=19; ES-62, n=15; left hand

panel;) after PMA plus ionomycin exposure and IL-17$^+$ γδ T cells (naïve, n=8; PBS, n=11; ES-62, n=9; right hand panel) spontaneously producing IL-17 cells in the DLN of individual mice.

Figure 3: ES-62 down-regulates DC driven Th17 cell priming *in vitro*.

bmDCs from naïve (A) or CIA (B) DBA/1 mice were preincubated ± ES-62 for 24h prior to stimulation ± LPS (24h) before analysis of TNF-α, IL-6 and IL-23. Data are means (of mean values of triplicate samples) ± SEM from individual mice (A; RPMI, n=5; ES-62, n=4; B; RPMI, n=7; ES-62, n=4). (C) Spontaneous production of IL-6 from bmDCs from naïve, CIA (PBS) or ES-62-treated CIA (ES-62) DBA/1 mice is presented as means (of mean values of triplicate samples) ± SEM, n=4 individual mice. (D) OVA-pulsed LPS-matured or immature (RPMI) C57BL/6 bmDCs, preincubated ± ES-62, were co-cultured with naïve OTII T cells for 4 d before measuring IL-17 levels by ELISA. Data are mean values ± SD of triplicate samples from a single experiment (left-hand panel) or pooled results from 5 independent experiments where data were normalized to the LPS response at 300 nM OVA and presented as mean % maximum (LPS) response ± SEM (right-hand panel).

Figure 4: ES-62 directly inhibits Th17 polarisation *in vitro*.

(A) Th17 cells were differentiated *in vitro* ± ES-62 (0-1 μg/mL) and IL-17 measured. ELISA data are presented as mean values ± SD of triplicate samples from a single representative experiment (left-hand panel) or pooled from three independent experiments where the levels of IL-17 were normalized relative to the control Th17 cells (100%=no ES-62) and presented as the mean value ± SEM (middle panel). IL-17 mRNA expression levels relative to GAPDH are presented where the data represent the mean values ± SD of triplicate samples from a single experiment (right-hand panel). (B) Expression of RORγt, surface TLR4 and MyD88

during *in vitro* Th17 polarisation was evaluated by flow cytometric analysis. Expression levels are shown at d-0 (tinted grey trace), d-2 (thin black line) and d-4 (thick black line) relative to isotype controls (broken line). (C) Expression of MyD88 (black line) was reduced by ES-62 (1 μg/ml; grey line) as indicated by flow cytometric analysis (left-hand panel) and geometric mean analysis (MFI; right-hand panel) and (D) MyD88 mRNA expression relative to GAPDH is presented where data represent the mean values ± range from two independent experiments.

Figure 5: ES-62 modulates crosstalk between γδ T cells and DCs *in vitro*.
(A) γδ T cells from BALB/c mice were activated *in vitro* with rIL-1+rIL-23 ± ES-62 (2 μg/mL) and IL-17 release (means ± SD n=3; left-hand panel) and CD44 expression (resting, grey; rIL-1+rIL-23, line; rIL-1+rIL-23+ES-62, bold line; right-hand panel) analysed at 24 h. (B) The % of γδ T cells expressing CD44 in DLN from PBS- and ES-62-treated CIA mice. (C) Resting, activated (act) or ES-62-exposed activated (ES act) γδ T cells and LPS-activated DCs were co-cultured at the indicated cell ratios and IL-17 detected at 24 h. Data are mean values ± SD, n=3 from a single experiment or pooled normalized (% activated γδ T cell control response) presented for each ratio as mean (of mean values) ± SEM, n=4 independent experiments. IL-17 and RORγt mRNA levels relative to GAPDH were measured; data represent mean ± SD, n=3 from a single representative experiment. (D) LSC analysis of $B220^+$ (black) cells and % of γδ TCR^+ cells (grey) within B cell follicles, gated as described in Methods. Plotted data are mean % (of two sections) ± SEM n=8 individual mice for both PBS and ES-62-treated CIA.

Figure 6: ES-62 suppresses the levels of IL-17-producing cells in the joints of CIA mice.
(A) Joint sections of naïve, PBS (articular scores 7& 8) and ES-62 treated (articular scores 3& 0) were imaged (magnification x20) for IL-17 (red) and nuclei (blue). Isotype control

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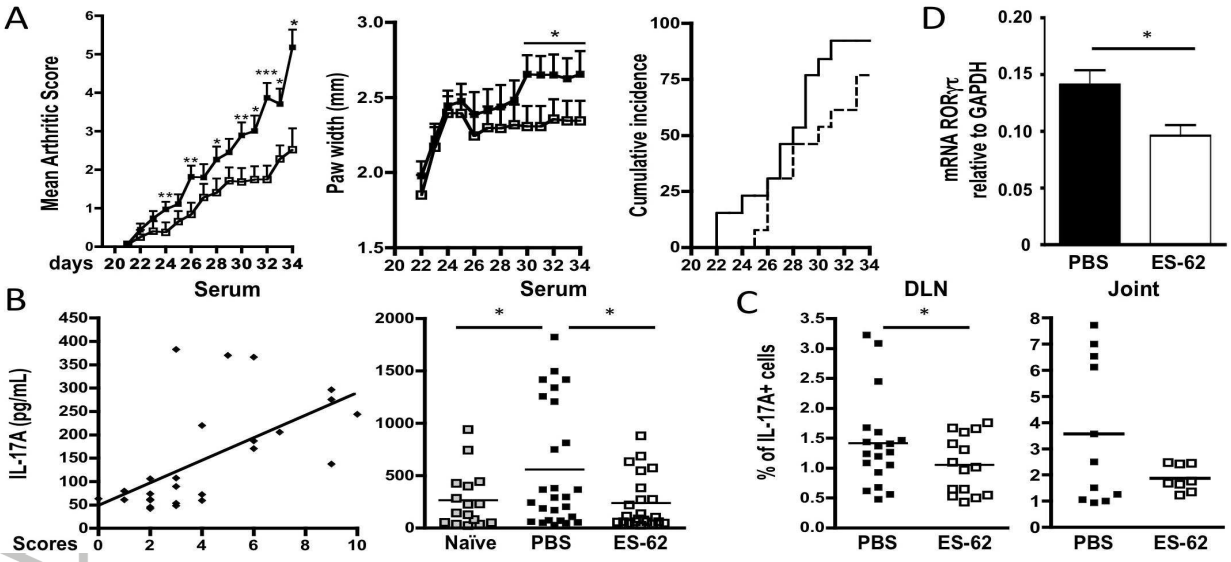

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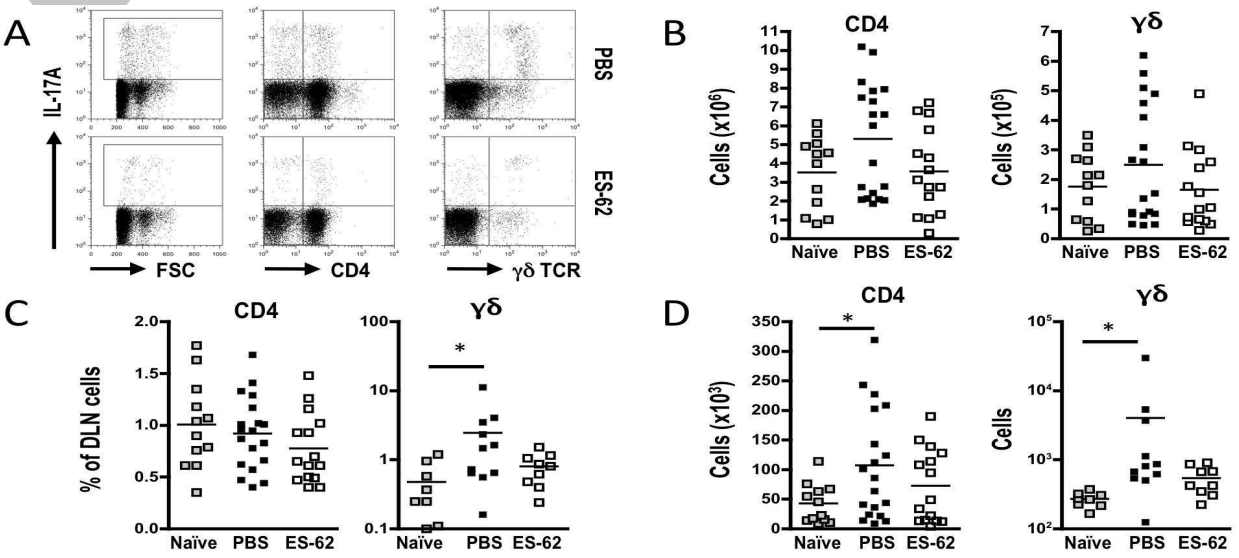

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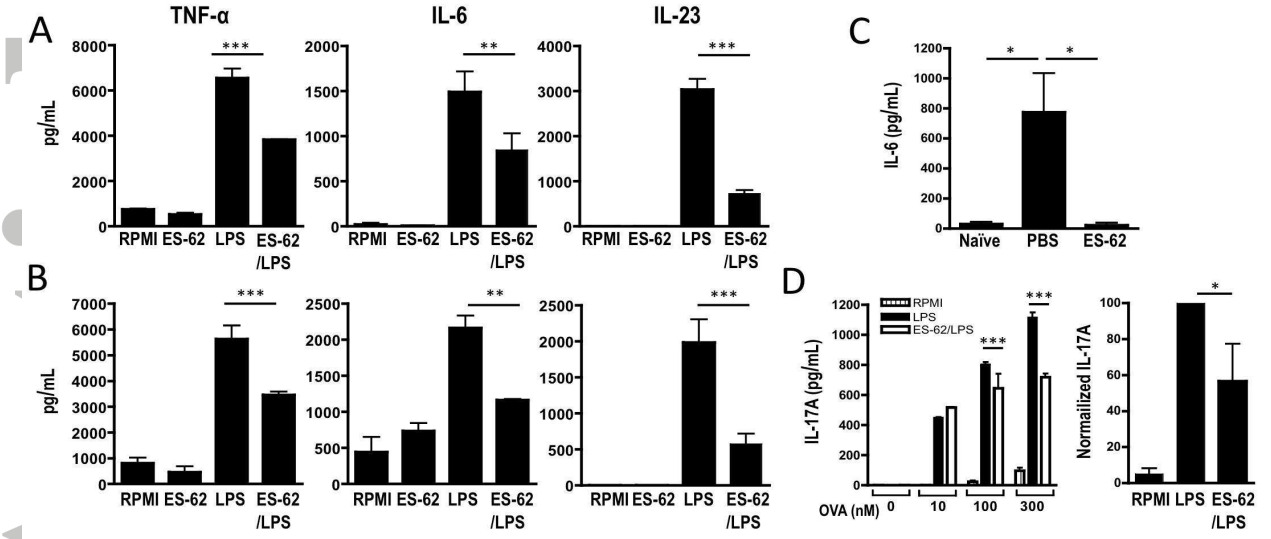

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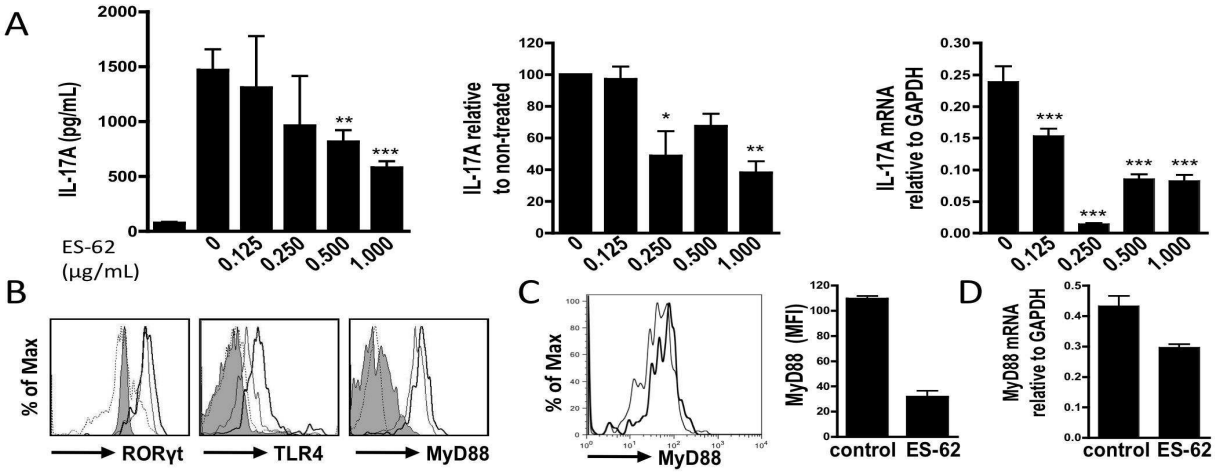

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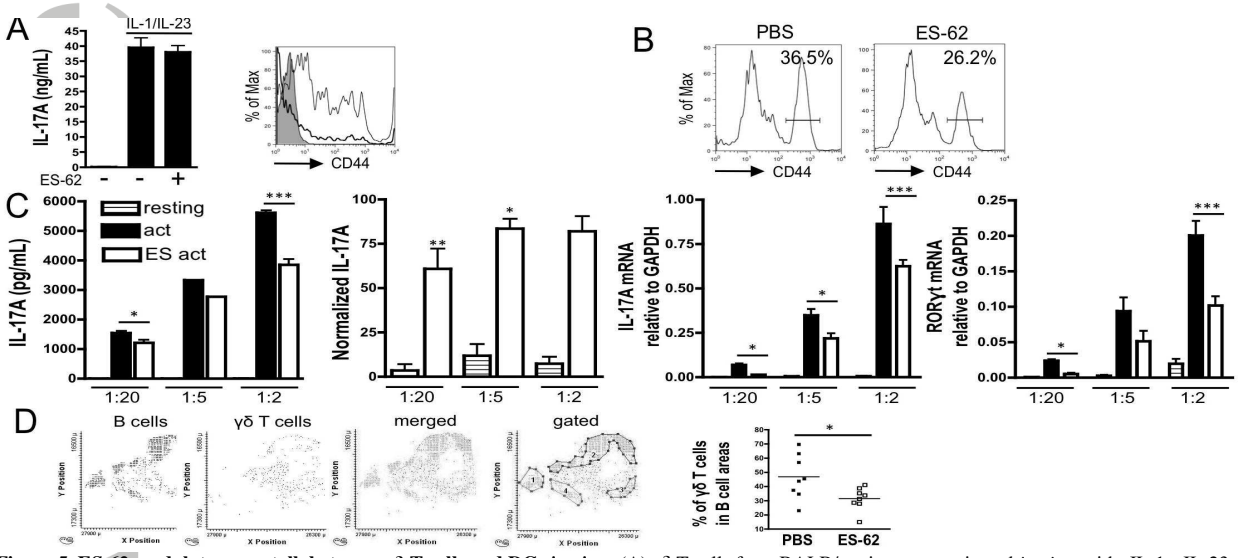

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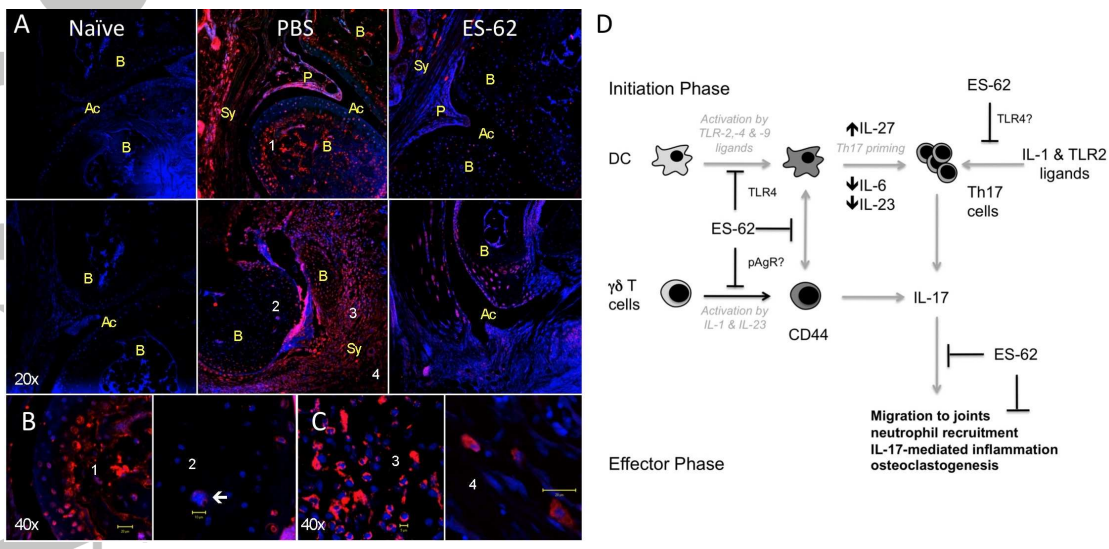

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