The Parasitic Helminth Product ES-62 Suppresses Pathogenesis in Collagen-Induced Arthritis by Targeting the Interleukin-17–Producing Cellular Network at Multiple Sites

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Objective. **Among many survival strategies, parasitic worms secrete molecules that modulate host immune responses. One such product, ES-62, is protective against collagen-induced arthritis (CIA), a model of rheumatoid arthritis (RA). Since interleukin-17 (IL-17) has been reported to play a pathogenic role in the development of RA, this study was undertaken to investigate whether targeting of IL-17 may explain the protection against CIA afforded by ES-62.**

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 cell populations, and in situ analysis of joint inflammation in mice with CIA.**

Results. **ES-62 was found to down-regulate IL-17 responses in mice with CIA. First, it acted to inhibit priming and polarization of IL-17 responses by targeting a complex IL-17–producing network, involving signaling between dendritic cells and γ/δ or CD4+ T cells. In addition, ES-62 directly targeted Th17 cells by downregulating myeloid differentiation factor 88 expression to suppress responses mediated by IL-1 and Toll-like receptor ligands. Moreover, ES-62 modulated the migration of γ/δ T cells and this was reflected by direct suppression of CD44 up-regulation and, as evidenced by in situ analysis, dramatically reduced levels of IL-17–producing cells, including lymphocytes, infiltrating the joint. Finally, there was strong suppression of IL-17 production by cells resident in the joint, such as osteoclasts within the bone areas.**

Conclusion. **Our findings indicate that ES-62 treatment of mice with CIA leads to unique multisite manipulation of the initiation and effector phases of the IL-17 inflammatory network. ES-62 could be exploited in the development of novel therapeutics for RA.**

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 where helminth infections are endemic, 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-containing glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae*, has broad immunomodulatory activities and can exert powerful antiinflammatory 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 the suppression of tumor necrosis factor α (TNFα) 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 against disease in mice, while overexpression of IL-17 exacer-

bates 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, previous studies have shown that IL-17 levels are increased in serum and synovial fluid samples from patients with RA compared to those from patients with osteoarthritis or healthy control subjects (9). In contrast, it has been proposed that interferon-γ (IFNγ) may play a protective role, since IFNγR$^-$/$^-$ mice are more susceptible to the development of CIA (10), perhaps reflecting abrogation of counterregulation 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 proinflammatory IL-17 production, and thus to readdress its protective role, but in the perspective of IL-17–associated pathology.

MATERIALS AND METHODS

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

Ex vivo analysis. Draining lymph node (LN) cells (10^6/ml) were incubated with or without 50 ng/ml phorbol myristate acetate (PMA) plus 500 ng/ml ionomycin for 1 hour, followed by addition of 10 μg/ml brefeldin A (Sigma-Aldrich) for 5 hours at 37°C with 5% CO_2. Phenotypic markers were labeled using allophycocyanin (APC)–conjugated anti–Toll-like receptor 4 (anti–TLR-4; R&D Systems), biotinylated anti-CD44 (BioLegend; detected with phycoerythrin (PE)–conjugated streptavidin [BD PharMingen]), PerCP-conjugated anti-CD4 or biotinylated anti-CD4 (detected with Alexa Fluor 450–conjugated streptavidin [BD PharMingen]), or fluorescein isothiocyanate (FITC)–conjugated anti-$\gamma\delta$ (BioLegend) antibodies before the cells were fixed and permeabilized according to BioLegend protocols. Cells were then labeled using APC-conjugated anti–IL-17A or PerCP-Cy5.5–conjugated anti–IL-

17A (BioLegend), anti–retinoic acid receptor–related orphan nuclear receptor γt (RORγt) (eBioscience; detected with APC-conjugated anti-rat IgG), and anti–myeloid differentiation factor 88 (anti-MyD88) (Abcam; detected with PE-conjugated anti-rabbit IgG) antibodies for 30 minutes prior to flow cytometry, with gating according to appropriate isotype controls.

Cytokine analysis. Enzyme-linked immunosorbent assays (ELISAs) for IL-17A, IL-10 (BioLegend), TNFα, IL-6, IL-23, and IL-27 (eBioscience) were performed according to the recommendations of the manufacturer. 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 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum) supplemented with 10% conditioned medium from the granulocyte–macrophage colony-stimulating factor–transfected X63 myeloma cell line and 50 μM 2-mercaptoethanol at 37°C in 5% CO_2 for 6 days. Naive CD4+CD62L+ T cells and γ/δ T cells were isolated using Miltenyi magnetic bead technology. For BMDC–T cell cocultures, BMDCs were incubated with ES-62 (2 μg/ml), matured with lipopolysaccharide (LPS) (*Salmonella minnesota*; Sigma), and then pulsed with ovalbumin (OVA) peptide (0–300 nM) before incubation with naive T cells derived from OVA-specific DO.11.10/BALB/c or OT-II/C57BL/6 mice for 4 days. For in vitro polarization of Th17 cells, naive LN T cells from BALB/c mice were incubated, in plates precoated with anti-CD3 (4 μg/ml), with anti-CD28 (1.5 μg/ml), anti-IFNγ (5 μg/ml), and anti–IL-4 (5 μg/ml) antibodies and recombinant IL-6 (rIL-6) (20 ng/ml), transforming growth factor β (rTGFβ; 4 ng/ml), and rIL-1 (10 ng/ml) with or without ES-62 (0–1 μg/ml) for 4 days. The γ/δ T cells from BALB/c mice were activated with rIL-1 plus rIL-23 (both at 10 ng/ml) overnight with or without ES-62 (2 μg/ml) before being incubated with BMDCs at different γ/δ:DC ratios (1:2, 1:5, and 1:20). Culture supernatants were collected after 24 hours.

Immunofluorescence analysis. Tissue sections (7 μm) were deparaffinized in xylene and dehydrated in ethanol, and antigen was retrieved by incubation at 60°C for 2 hours 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 a goat IgG isotype control and DAPI as a counterstain, at 4°C for 12 hours, followed by staining with a biotinylated rabbit anti-goat IgG antibody and streptavidin–Alexa Fluor 647. Images were obtained using an LSM 510 META confocal laser coupled to an Axiovert 200 microscope (Zeiss) and analyzed with Zeiss LSM Image Browser software.

Laser scanning cytometry. Draining LNs were fixed in 10% formalin at 4°C for 24 hours, transferred to 30% sucrose in PBS for 48 hours before being frozen in liquid nitrogen in OCT compound (Bayer), and stored at −70°C. Sections (7 μm) were stained with FITC-conjugated anti-B220 and PE-conjugated anti–γ/δ T cell receptor (anti–γ/δ TCR) or isotype controls (BD PharMingen) and mounted in Vectashield (Vector). Fluorescence was quantified by laser scanning cytometry (CompuCyte) to generate tissue maps of the draining LNs using WinCyte software version 3.6 (CompuCyte). Briefly, setting a gate for positive-staining

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B220+ B cells generated a tissue map of the localization of B220+ B cells that allowed generation of the indicated gates designating the paracortical (T cell) and follicular (B220+ B cell) regions that were subsequently copied onto the γ/δ TCR+ T cell tissue map. This allowed unbiased statistical quantitation of γ/δ TCR+ T cells within follicular regions by the WinCyte software following merging of the γ/δ TCR+ T cell and B220+ B cell tissue maps (15).

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Quantitative RT-PCR and reverse transcription of RNA were performed according to the recommendations of the manufacturer (Applied Biosystems). High-performance liquid chromatography–purified probes (VH Bio; Integrated DNA Technologies) contained the reporter 5′-6-carboxyfluorescein (FAM) and quencher TAMRA dyes, and the sequences were as follows: for RORγt, forward 5′-CCGCTGAGAGGGCTTCAC-3′, reverse 5′-TGCAGGA-GTAGGCCACATTACA-3′, and 5′-FAM-AAGGGCTTCT-TCCGCCGCCAGCAG-TAMRA-3′. Applied Biosystems assay kits for IL-17A, MyD88, and GAPDH (Mm00439618_m, NM_010851.2, and 4352339E1, respectively) were used. Data were analyzed using RQ Manager software (Applied Biosystems), and were normalized to the reference reporter GAPDH.

Statistical analysis. Parametric data were analyzed by Student's unpaired 2-tailed *t*-test or by one-way analysis of variance followed by the Newman-Keuls post-test. Normalized data were analyzed by Kruskal-Wallis test, and the Mann-Whitney test was used for the analysis of clinical CIA scores. *P* values less than 0.05 were considered significant.

RESULTS

Association of ES-62 protection against CIA with down-regulation of IL-17 responses. ES-62 exhibits antiinflammatory action, as evidenced by the significant reduction in articular score and hind paw swelling observed in ES-62–treated mice with CIA (Figure 1A). Disease incidence was also delayed and reduced in these ES-62–treated mice (Figure 1A). Consistent with the notion that IL-17 plays a pathogenic role in CIA, we observed a strong positive correlation of serum levels of IL-17 (IL-17A) (Figure 1B), but not IFNγ (data not shown), with disease scores in animals with CIA. Thus, to assess whether protection by ES-62 is associated with
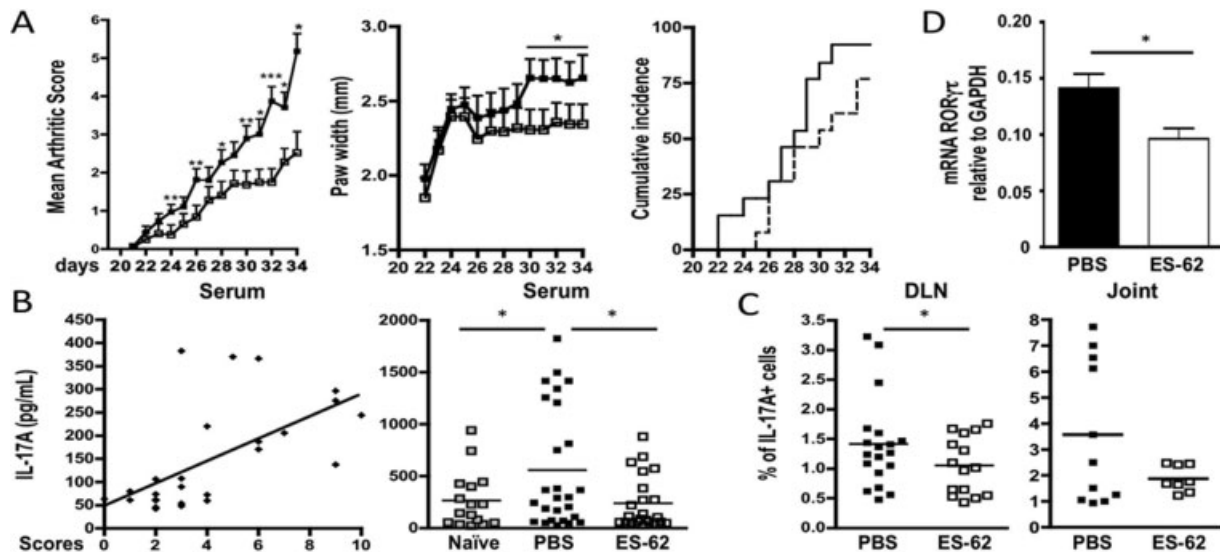

Figure 1. ES-62 protects against collagen-induced arthritis (CIA). **A,** Mean ± SEM clinical score (left) and paw width (middle) in mice with CIA treated with phosphate buffered saline (PBS) (solid squares; n = 43 for clinical score and n = 9 for paw width) or ES-62 (open squares; n = 32 for clinical score and n = 9 for paw width), and disease incidence in mice with CIA treated with PBS (solid line) or ES-62 (broken line) (right). Disease incidence was defined as the percentage of animals that developed a severity score of ≥1. **B,** Serum interleukin-17 (IL-17) levels in mice with CIA. The left panel shows a significant correlation between serum IL-17 level and clinical score in mice with CIA (number of XY pairs = 26; Pearson's r = 0.6050, *P* < 0.001). The right panel shows serum IL-17 levels in naive mice (n = 16), mice with CIA treated with PBS (n = 26), and mice with CIA treated with ES-62 (n = 23). Symbols represent the mean of triplicate analyses of individual mice; horizontal lines represent the mean value for the treatment group. **C,** Percentages of IL-17+ draining lymph node (DLN) cells and joint cells from mice with CIA treated with PBS or ES-62. The percentage of IL-17+ draining LN cells was determined after ex vivo stimulation with phorbol myristate acetate plus ionomycin. Squares represent individual mice; horizontal lines represent the mean (n = 19 PBS-treated mice and 15 ES-62–treated mice for analysis of draining LNs and n = 11 PBS-treated mice and 8 ES-62–treated mice for analysis of joints). **D,** Retinoic acid receptor–related orphan nuclear receptor γt (RORγt) mRNA levels, relative to GAPDH, in mice with CIA treated with PBS (n = 4) and mice with CIA treated with ES-62 (n = 3). Values are the mean ± SEM. * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.001.

suppression of IL-17–mediated pathology, the effect of administration of the helminth product ES-62 on serum cytokine levels was analyzed. Significantly higher levels of IL-17 (Figure 1B), but not IFNγ (data not shown), were detected in the serum of mice with CIA than in the serum of naive animals, and exposure to ES-62 in vivo reduced these to levels similar to those observed in naive mice.

Consistent with these findings, significant differences between the PBS-treated mice, but not the ES-62–treated mice, and naive mice were found in terms of total numbers of draining LN cells (results not shown), and significantly higher proportions of draining LN cells from animals with CIA treated with PBS than from animals with CIA treated with ES-62 produced IL-17 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 messenger RNA (mRNA) levels were sig-

nificantly lower in draining LN cells from the ES-62–treated mice than in draining LN cells from the PBS-treated 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).

Suppression of the levels of IL-17–producing CD4+ and γ/δ T cells by ES-62. CD4+ and γ/δ T cells were the 2 major IL-17–producing compartments (>90%) in the draining LNs of mice from all treatment groups (Figure 2A). Although the mice with CIA (treated with PBS) tended to have higher numbers of draining LN CD4+ T cells than those from both the naive and ES-62–treated groups (Figure 2B), there were no significant differences between any of these groups, in terms of either proportions or absolute numbers of CD4+ T cells spontaneously producing IL-17 (results not shown). In contrast, following ex vivo stimulation with PMA plus ionomycin, while 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 naive
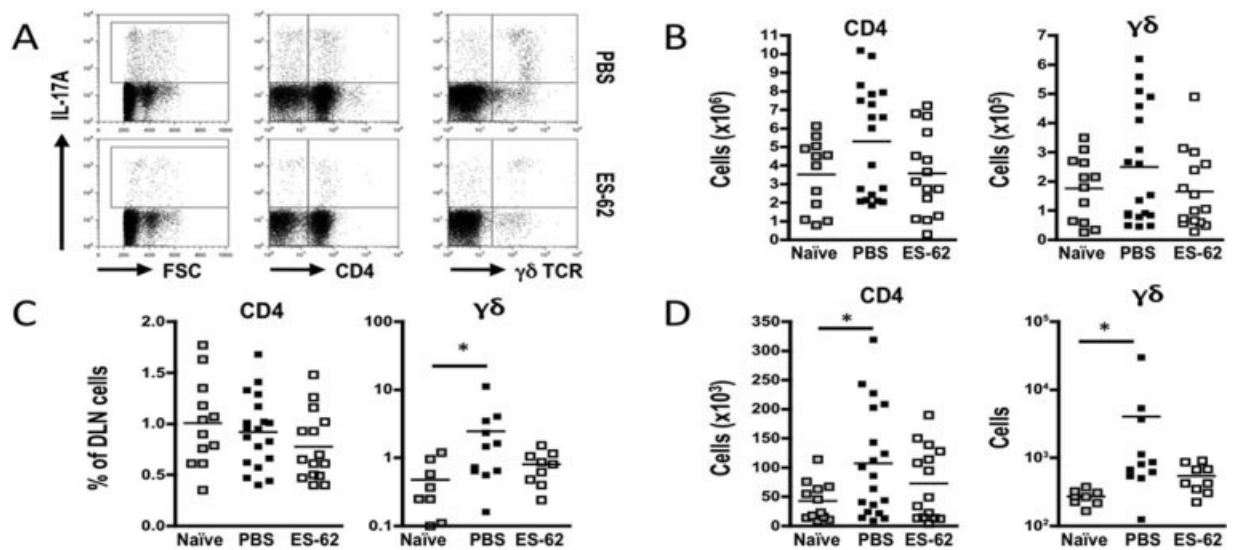

Figure 2. ES-62 targets IL-17–producing CD4+ and γ/δ T cells. **A,** Representative plots of the gating patterns of intracellular IL-17 expression by draining LN cells from mice with CIA treated with PBS or ES-62, showing forward scatter (FSC) on the x-axis versus IL-17 expression on the y-axis as well as the cellular expression of IL-17 by CD4+ cells and γ/δ T cell receptors (γ/δ TCR). **B,** Numbers of CD4+ T cells (left) and γ/δ T cells (right) present in the draining LNs of naive mice (n = 12), mice with CIA treated with PBS (n = 19), and mice with CIA treated with ES-62 (n = 15). **C and D,** Percentages (**C**) and absolute numbers (**D**) of IL-17+ CD4+ T cells in the draining LNs of naive mice (n = 12), mice with CIA treated with PBS (n = 19), and mice with CIA treated with ES-62 (n = 15) after stimulation with phorbol myristate acetate plus ionomycin (left panels), and of γ/δ T cells that spontaneously produced IL-17 in the draining LNs of naive mice (n = 8), mice with CIA treated with PBS (n = 11), and mice with CIA treated with ES-62 (n = 9) (right panels). In **B–D,** squares represent individual mice; horizontal lines represent the mean. ∗ = $P < 0.05$. See Figure 1 for other definitions.

group, and this was reduced by exposure to ES-62 (Figure 2D).

Analysis of γ/δ T cell responses showed that there were no significant differences between the groups in terms of the total numbers of such cells present in the draining LNs (Figure 2B). However, both the proportions and the absolute numbers of γ/β T cells that spontaneously produced IL-17 were higher in the mice with CIA, but not those exposed to ES-62 in vivo, when compared to those from the naive group (Figures 2C and D). No differences were detected among the groups, however, following ex vivo stimulation with PMA plus ionomycin (data not shown). Interestingly, while unlikely to be related to its protective effects (given the lack of correlation between serum IFNγ levels and disease score mentioned above and previously reported findings [10]), we found that ES-62 reduced the percentages of CD4+, γ/δ+, and CD8+ T cells spontaneously producing IFNγ (results not shown), which is consistent with the results of our previous studies showing that ES-62 suppressed IFNγ recall responses in CIA (2,3).

Attenuation of Th17 responses by both indirect and direct effects of ES-62. ES-62 modulates DC-mediated priming and polarization of Th cell responses in healthy mice (16–18). Thus, we next investigated whether ES-62 modulated the capacity of DCs to prime Th17 responses in mice with CIA, by preincubating BMDCs derived from naive DBA/1 mice with ES-62 before maturing them with LPS in vitro. Although the LPS-stimulated release of IL-10 was unaffected (data not shown), we observed that ES-62 significantly inhibited the LPS-induced secretion of the proinflammatory cytokine TNFα and 2 cytokines associated with the polarization and survival of Th17 cells, IL-6 and IL-23 (Figure 3A). Similarly, BMDCs derived from one group of DBA/1 mice with CIA (mean ± SEM articular score 7.1 ± 0.68) produced reduced levels of TNFα, IL-6, and IL-23 when treated with ES-62 prior to LPS maturation in vitro (Figure 3B). Moreover, while IL-23 could not be detected, BMDCs derived from a second group of DBA/1 mice with CIA (mean ± SEM articular score 5.4 ± 1.6) spontaneously produced significantly more
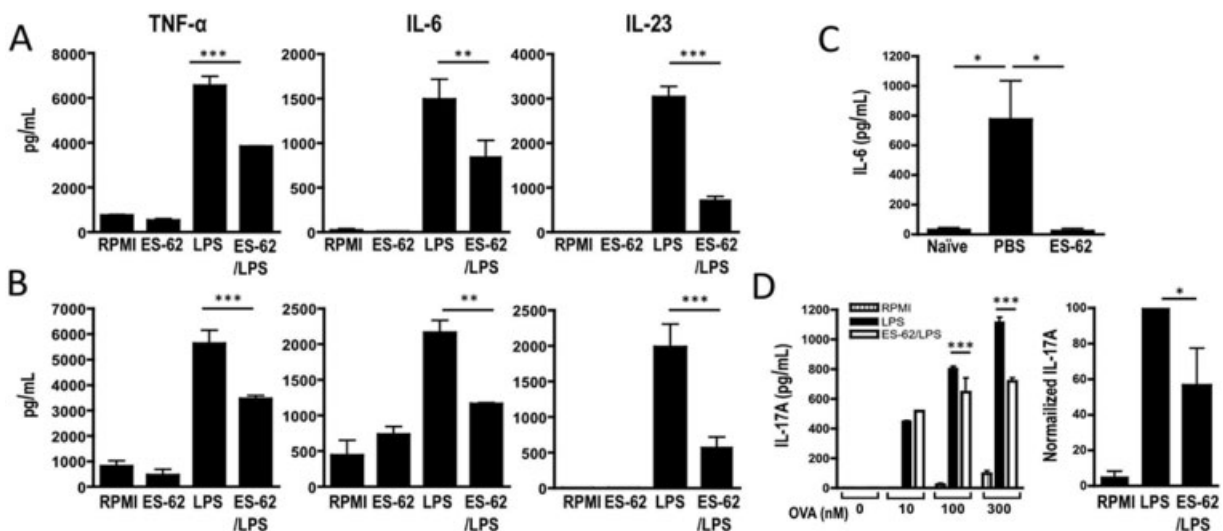

Figure 3. ES-62 down-regulates dendritic cell (DC)–driven Th17 cell priming in vitro. **A** and **B,** Levels of tumor necrosis factor α (TNFα), IL-6, and IL-23 in bone marrow–derived DCs (BMDCs) from naive DBA/1 mice (**A**) and DBA/1 mice with CIA (**B**). Mouse BMDCs were preincubated with ES-62 (n = 4 naive mice and n = 4 mice with CIA) or without ES-62 (RPMI; n = 5 naive mice and n = 7 mice with CIA) for 24 hours prior to stimulation with lipopolysaccharide (LPS) for 24 hours, and TNFα, IL-6, and IL-23 levels were then analyzed. Values are the mean ± SEM of triplicate samples from individual mice. **C,** Spontaneous production of IL-6 by BMDCs from naive DBA/1 mice, DBA/1 mice with CIA treated with PBS, and DBA/1 mice with CIA treated with ES-62. Values are the mean ± SEM of triplicate samples from individual mice (n = 4 mice per group). **D,** IL-17A levels, measured by enzyme-linked immunosorbent assay, in ovalbumin (OVA)–pulsed LPS-matured or immature (RPMI) BMDCs from C57BL/6 mice. BMDCs had been preincubated with or without ES-62 and cocultured with naive OT-II T cells for 4 days. Values in the left panel are the mean ± SD of triplicate samples from a single experiment. Values in the right panel are the mean ± SEM percent maximum (LPS) response of pooled results from 5 independent experiments where data were normalized to the LPS response at 300 nM OVA. ∗ = $P < 0.05$; ∗ ∗ = $P < 0.01$; ∗∗∗ = $P < 0.001$. See Figure 1 for other definitions.

IL-6, but not TNFα or IL-10, than those derived from either naive DBA/1 mice (articular score 0) or DBA/1 mice with CIA that had been exposed to ES-62 in vivo (mean ± SEM articular score 1.8 ± 0.5) (Figure 3C and results not shown). Taken together, these results suggest that ES-62 suppresses the generation of Th17-polarizing cytokines by DCs in mice with CIA. Consistent with these findings, ES-62–treated DCs showed a reduced ability to skew naive OVA-specific T cells toward a Th17 phenotype (Figure 3D).

We next investigated whether ES-62 also directly affects Th17 cells. Naive 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 cells were coincubated with the parasite product, ES-62 directly down-regulated 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 that the expression of TLR-4, which is required for ES-62 action (19), was 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, while ES-62 did

not appear to modulate either the surface or intracellular levels of TLR-4 (data not shown), it did induce down-regulation of the TLR signal transducer, MyD88 (Figure 4C), and this was reflected at the mRNA level (Figure 4D).

DCs are necessary for ES-62 targeting of IL-17 production by γ/δ T cells. To address whether ES-62 likewise directly modulated IL-17 production by γ/δ T cells, γ/δ T cells from naive mice were stimulated to produce IL-17 in vitro in a TCR-independent manner, using rIL-1 plus rIL-23 (20). Such "activated," γ/δ T cells produced large amounts of IL-17, whereas resting γ/δ T cells did not. However, ES-62 did not modulate this response (Figure 5A). Perhaps consistent with these findings, TLR-4 expression was not detected, and culture with LPS did not induce γ/δ T cell activation (results not shown). Nevertheless, we found that ES-62 inhibited γ/δ T cell activation, as indicated by its ability to prevent up-regulation of the cell surface marker CD44 in vitro (Figure 5A) and in vivo (Figure 5B).

Therefore, we next investigated whether DCs regulated the production of IL-17 by γ/δ T cells. LPS-matured DCs were cocultured with resting or IL-1/IL-23–stimulated γ/δ cells that had been exposed to ES-62 or left untreated. We found that IL-17 production was
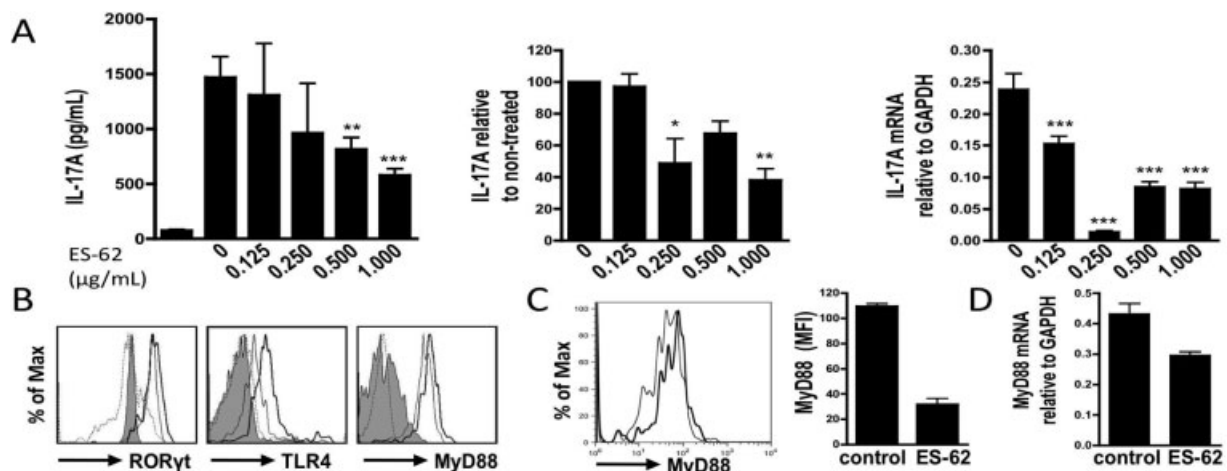

Figure 4. ES-62 directly inhibits Th17 polarization in vitro. **A,** Levels of IL-17, determined by enzyme-linked immunosorbent assay, in Th17 cells from BALB/c mice, differentiated in vitro and left untreated or treated with ES-62 (0–1 μg/ml). Values in the left panel are the mean ± SD of triplicate samples from a single representative experiment. Values in the middle panel are the mean ± SEM of samples pooled from 3 independent experiments, normalized to the values in control (untreated) Th17 cells. Values in the right panel are the mean ± SD mRNA expression in triplicate samples from a single experiment, relative to GAPDH. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, versus untreated cells. **B,** Expression of RORγt, surface Toll-like receptor 4 (TLR-4), and myeloid differentiation factor 88 (MyD88) during in vitro Th17 polarization, determined by flow cytometric analysis. Expression levels relative to isotype control (broken lines) are shown for day 0 (gray areas), day 2 (thin lines), and day 4 (thick lines). **C,** Reduction in the expression of MyD88 (black line) in Th17 cells treated with ES-62 (1 μg/ml; gray line), as determined by flow cytometric analysis (left) and geometric mean analysis (mean fluorescence intensity [MFI]; right). Values are the mean ± range from 2 independent experiments. **D,** MyD88 mRNA expression, relative to GAPDH, in control and ES-62–treated Th17 cells. Values are the mean ± range from 2 independent experiments. See Figure 1 for other definitions.

reduced in such ES-62–treated DC–γ/δ T cell cocultures (Figure 5C). Furthermore, IL-17 and RORγt mRNA levels were reduced when the activated γ/δ T cells had been exposed to ES-62 (Figure 5C). DC maturation is required for these conditioning effects on γ/δ T cells, as such immunomodulation did not occur with immature DCs. Also, while the results did not reach significance, the observed effects were associated with increased generation of IL-27, a cytokine that antagonizes IL-17 production (21,22), in the cocultures containing ES-62–treated γ/δ T cells (data not shown).

ES-62–mediated modulation of γ/δ T cell responses also appears to occur during CIA in vivo. Thus, such draining LN γ/δ T cells not only displayed reduced expression of CD44 when analyzed ex vivo (Figure 5B), but in situ analysis also demonstrated that γ/δ T cells in ES-62–treated mice exhibited altered localization within draining LNs, showing reduced distribution in the B cell follicles compared to those from PBS-treated animals with CIA (Figure 5D).

Reduction in the levels of IL-17–positive cells in the joints of mice with CIA treated with ES-62. Consistent with a pathogenic effector role of IL-17 in the joint, in situ analysis showed that, while little or no IL-17 expression was detected in joints from naive mice (Figure 6A), there was strong expression of this cytokine in joints from 2 representative PBS-treated mice with CIA (articular scores 7 and 8). In contrast, IL-17 expression was dramatically reduced in the joints of 2 representative ES-62–treated mice (articular scores 3 and 0). Furthermore, examination of the cells producing IL-17 indicated that these cells consisted of both cells infiltrating the joint (Figures 6B and C), including large numbers of lymphocytes as indicated by size and morphology (Figure 6C), and cells in the bone, such as multinucleated osteoclasts (Figure 6B). IL-17 levels appeared to be
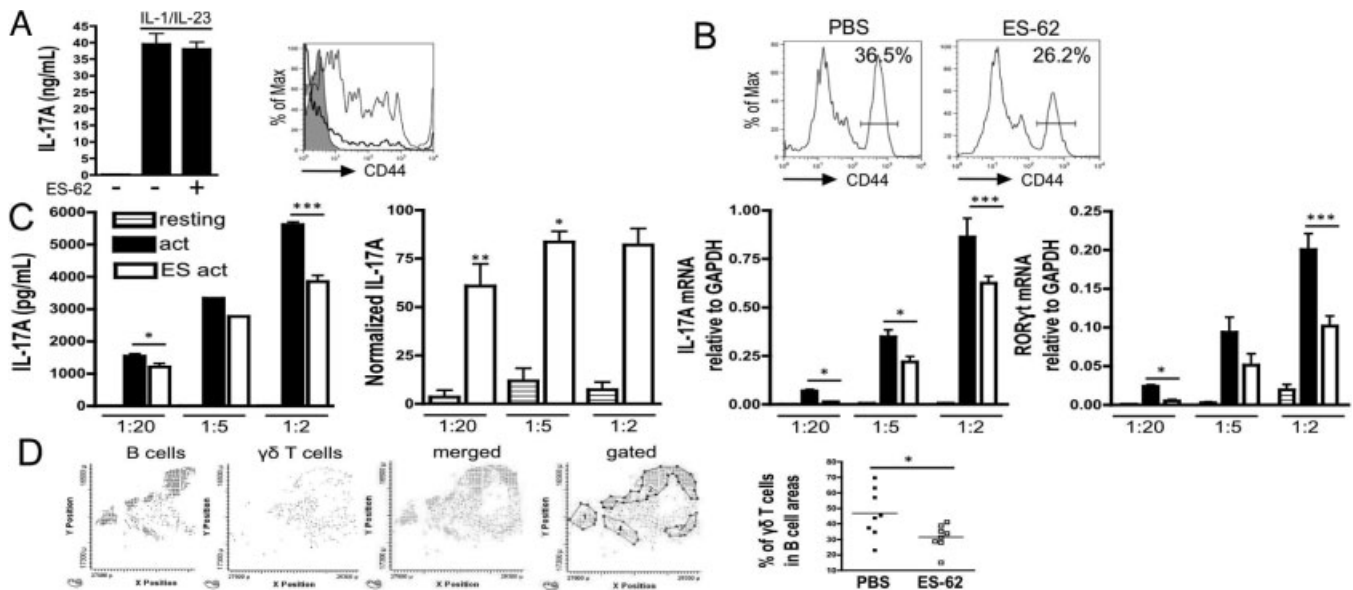

Figure 5. ES-62 modulates cross-talk between γ/δ T cells and dendritic cells (DCs) in vitro. **A,** IL-17 release (left) and CD44 expression (right) in γ/δ T cells from BALB/c mice. The γ/δ T cells were activated in vitro with recombinant IL-1 (rIL-1) plus rIL-23, with or without ES-62 (2 μg/ml). IL-17 release and CD44 expression were analyzed at 24 hours. CD44 expression is shown for resting cells (gray area), cells activated with rIL-1 plus rIL-23 (thin line), and cells activated with rIL-1, rIL-23, and ES-62 (thick line). Values in the left panel are the mean ± SD (n = 3 samples per group). **B,** Percentage of γ/δ T cells expressing CD44 in draining LNs from mice with CIA treated with PBS or ES-62. **C,** IL-17 levels, and IL-17 and RORγt mRNA levels relative to GAPDH, in resting, activated (act), or ES-62–exposed activated γ/δ T cells cocultured with lipopolysaccharide-activated DCs at the indicated ratios. IL-17 levels were determined at 24 hours. Values in the left panel are the mean ± SD of triplicate samples from a single experiment. Values in the middle panel are the mean ± SEM pooled results of 4 independent experiments, normalized to values in control activated γ/δ T cells. Values in the right panels (mRNA levels) are the mean ± SD (n = 3 from a single representative experiment). **D,** Laser scanning cytometry of B220+ cells (black) and percent of γ/δ TCR+ cells (gray) within B cell follicles from mice with CIA, gated as described in Materials and Methods. The first 4 panels show results for a representative PBS-treated mouse with CIA. In the last panel, squares represent the mean of 2 sections from each mouse; horizontal lines represent the mean value for the treatment group (n = 8 mice per group). ∗ = P < 0.05; ∗∗ = P < 0.01; ∗∗∗ = P < 0.001. See Figure 1 for other definitions.
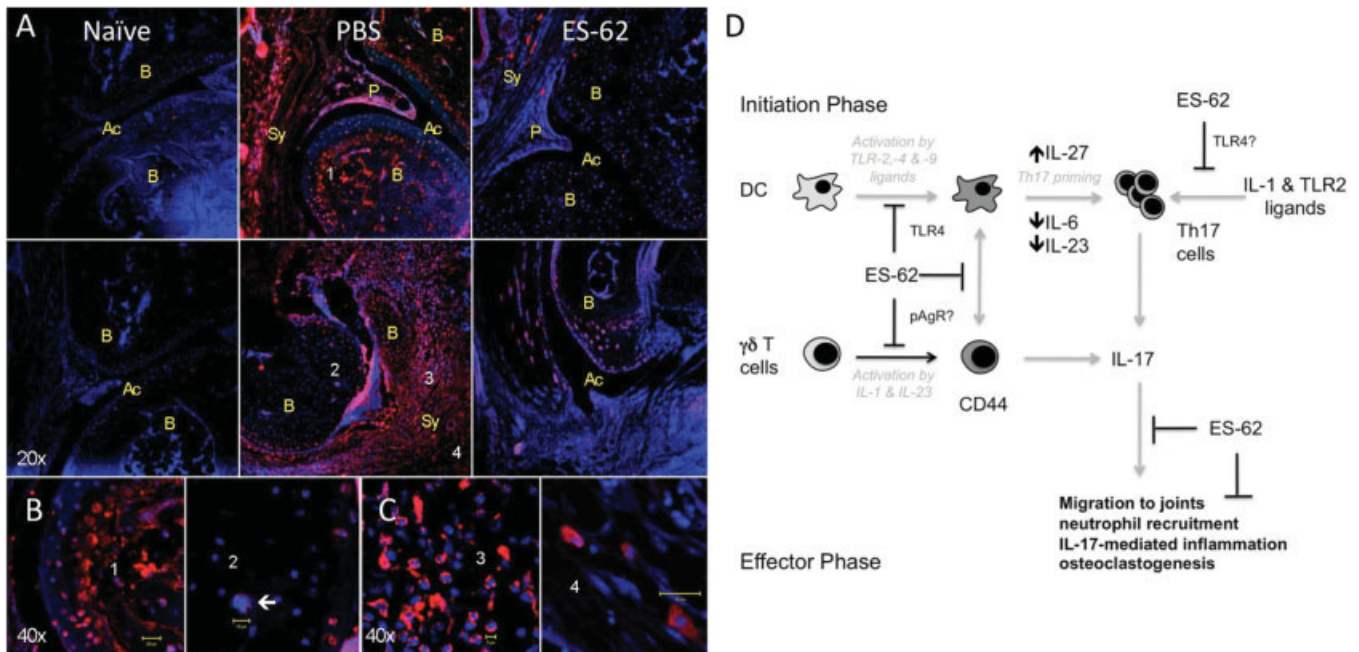
Figure 6. ES-62 suppresses the levels of IL-17–producing cells in the joints of mice with CIA. **A,** Joint sections from 2 representative naive mice, 2 representative mice with CIA treated with PBS (1 with an articular score of 7 and 1 with an articular score of 8), and 2 representative mice with CIA treated with ES-62 (1 with an articular score of 3 and 1 with an articular score of 0). Red indicates IL-17; blue indicates nuclei. Isotype control sections were IL-17 negative. **B** = bone; **Ac** = articular cavity; **Sy** = synovium; **P** = pannus. Original magnification × 20. **B** and **C,** IL-17+ cells in the bone (**B**) and synovium (**C**) in the numbered sections from the mice with CIA treated with PBS in **A** (1–4). Original magnification × 40. **Arrow** indicates a multinucleated cell. Bars indicate relative magnification (bar in left panel of **B** = 20 μm [scan zoom ×2.3]; bar in right panel of **B** = 10 μm [scan zoom ×2.1]; bar in left panel of **C** = 5 μm [scan zoom ×2.5]; bar in right panel of **C** = 20 μm [scan zoom ×2.6]). **D,** A model of the mechanism of action of ES-62 modulating a complex network of dendritic cell (DC), CD4+ T cell, and γ/δ T cell interactions to suppress pathogenic IL-17 responses in mice with CIA. TLR-2 = Toll-like receptor 2; pAgR = phosphoantigen receptor (see Figure 1 for other definitions).

reduced at both sites in mice treated with ES-62 (Figure 6A). These data suggest that, as well as suppressing the early IL-17–driven proinflammatory responses in the draining LNs that are associated with the initiation of pathogenesis, exposure to ES-62 in vivo reduces 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 cellular producers of this cytokine might provide a mechanism for suppression of disease severity by ES-62 (2,3). Consistent with this notion, the highly elevated levels of IL-17 observed in the serum of mice with CIA, compared to naive animals, were significantly reduced in mice with CIA treated with ES-62 in the present study. Furthermore, ES-62 reduced the percentage of IL-17+ draining LN cells, relative to the control cohorts with CIA, such that it was not significantly different from that

in naive DBA/1 mice. Although prophylactic treatment with ES-62 on days −2, 0, and 21 resulted in an ~50–60% reduction in the articular score, it is likely that more frequent and/or higher doses of ES-62 would have further reduced IL-17 responses and the resultant pathology. Alternatively, since ES-62 typically reduces IL-17 responses to levels near those observed in naive DBA/1 mice, the residual pathology observed in the presence of ES-62 could reflect IL-17–independent pathogenic effector mechanisms.

It is certainly the case that CD4+ and γ/δ T cell–driven pathogenesis in CIA relies on the ability of these cells to initiate IL-17–dependent responses (14,23–25), although it has recently been suggested that the induction of γ/δ T cells may be a result of CFA-associated inflammation (14,23). However, we found that the levels of IL-17+ γ/δ T cells were not upregulated in mice immunized with CFA alone (results not shown), indicating that IL-17 production by both

CD4+ and γ/δ T cells plays a role in the collagen response and, importantly, that ES-62 targets both of these major IL-17–producing compartments in the CIA model used in this study.

DCs are a major target of ES-62 action in modulating the priming and polarization of Th cell responses (16–18). Thus, we hypothesized that the reduction in the numbers of Th17 cells reflected suppression of Th17 cell priming by DCs. We subsequently found that in vitro conditioning of BMDCs with ES-62 significantly inhibited LPS-driven production of the cytokines TNFα, IL-6, and IL-23, the latter two of which are implicated in the development and maintenance of the Th17 phenotype, and a reduction in OVA-specific priming of IL-17 production by naive CD4+ Th cells.

However, we also observed that ES-62 modulated Th17 responses directly. Although naive CD4+ T cells do not express TLR-4, which is the receptor required for ES-62 to mediate its antiinflammatory effects in antigen-presenting cells (19,26), we observed up-regulation of TLR-4 and MyD88 in parallel with up-regulation of the signature transcription factor, RORγt, during in vitro polarization to the Th17 phenotype. ES-62–mediated suppression of the resultant IL-17 response therefore likely reflects not only the fact that TLRs can be expressed by most T cell subsets, but also the fact that TLR agonists (e.g., for TLR-3, -5, -7, and -9) can modulate Teff or Treg cell responses in the absence of antigen-presenting cells (for review, see ref. 27) and LPS/TLR-4 signaling can both induce and enhance IL-23–stimulated IL-17 release from Th17 cells differentiated in vitro (28).

We have found 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 (Pineda MA, et al: unpublished observations), a cytokine that has been shown to commit naive T cells to a Th17 phenotype via STAT-3 activation independently of MyD88 recruitment (29). Therefore, ES-62 subversion of signaling via TLR-4, with consequent down-regulation of MyD88, a key signal transducer of the TLR/IL-1 receptor (IL-1R) family (30), would provide a molecular rationale for the observed decrease in Th17 polarization, given that it has recently been reported (31,32) that IL-1R–associated kinase 4 (IRAK-4) and IRAK-1, the downstream effectors of IL-1R/MyD88 signaling, are required for such polarization.

In contrast, ES-62 did not directly down-regulate IL-17 production by γ/δ T cells in response to activation with IL-1/IL-23. Consistent with this finding, we were unable to detect TLR-4 expression by γ/δ T cells,

supporting the notion that modulation of γ/δ T cell responses by LPS requires cooperation with DCs (33). It was surprising, therefore, that we found that ES-62 suppressed the up-regulation of CD44 resulting from the activation of γ/δ T cells in response to IL-1/IL-23. These data suggested that ES-62 might directly modulate γ/δ T cell activation, but not cytokine production, through some undefined receptors, such as those involved in the recognition of small phosphorylated molecules present in mycobacteria that lead to DC activation by γ/δ T cells (34,35), in a TLR-independent manner (36–39). In turn, mature DCs can stimulate γ/δ 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 γ/δ T cells (41). Thus, since the active phosphorylcholine moiety of ES-62 (3) is structurally reminiscent of the phosphorylated mycobacterial molecules, this suggested that ES-62 was possibly targeting γ/δ T cells via such receptors to modulate bidirectional interaction with DCs, a hypothesis supported by the fact that ES-62 down-regulated the production of IL-17 and tended to up-regulate the production of IL-27, a cytokine that suppresses CIA (22,42,43), in DC–γ/δ cocultures.

The targeting of CD44 expression by γ/δ T cells that was observed both in vitro and in vivo in the present study may be physiologically relevant to ES-62–mediated protection against CIA, since such modulation would impact lymphocyte migration during CIA (44), particularly to the joint (45). Indeed, in situ laser scanning cytometry revealed that exposure to ES-62 in vivo modulates the localization of γ/δ T cells within the draining LNs of mice with CIA, and this may, in turn, modulate bidirectional signaling between γ/δ T cells and DCs to subvert initiation of the inflammatory phenotype driving autoimmunity. Moreover, and perhaps reflecting suppression of the CD44-mediated migration of IL-17–producing lymphocytes to the site of inflammation, we have also shown that ES-62 dramatically reduces the levels of IL-17+ infiltrating cells in the joints. This is likely to be of importance therapeutically, since IL-17 produced during the initiator phase induces the recruitment and accumulation of inflammatory cells, particularly neutrophils, to the joints and the release of proinflammatory chemokines, cytokines, and matrix metalloproteinases (7,8,46), which 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, since some of the IL-17+ cells in the bone appeared multinucleated (Fig-

ure 6B), suggesting that they could be osteoclasts. Moreover, the infiltrating cells in the joints of mice with CIA contained large numbers of small IL-17+ mononuclear cells that appeared to be lymphocytes, consistent with ES-62 blocking the migration of pathogenic effector Th17 and/or IL-17–producing γ/δ T cells to the site of inflammation. Importantly, levels of all classes of IL-17–producing cells in the joint appeared to be reduced in the ES-62–treated mice.

Taken together, 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 targets cells of the innate immune system (DCs and γ/δ T cells) to inhibit initiation of pathogenic responses and also, by acting directly on Th17 cells, to suppress ongoing adaptive responses. Mechanistically, given the increasing evidence of TLR signaling in the initiation (DC) and amplification of Th17 cell– and γ/δ T cell–mediated IL-17 responses and autoimmune inflammation (28,31,48), it is pertinent that ES-62 rewires TLR-2–, TLR-4–, and TLR-9–driven maturation of DCs to an antiinflammatory phenotype in a TLR-4–dependent manner (19). This was reflected in the present study by the inhibition of LPS-induced TNFα, IL-6, and IL-23 production, as well as by the release of increased levels of IL-27, resulting in the 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 TLR-4–mediated down-regulation of MyD88, leading to uncoupling of IL-1R from IRAK-1/4 signals that are essential for Th17 polarization (31,32). Since MyD88 is a key signal transducer for all TLR family members except for TLR-3 (interestingly, signaling of which is not modulated by ES-62 [19]), the recent finding that Th17 responses and consequent autoimmune pathogenesis are promoted by TLR-2 signaling 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 γ/δ T cells, possibly via phosphoantigen receptors, not only to modulate the bidirectional DC–γ/δ cell interactions required to drive subsequent adaptive Th17 responses, but also to down-regulate CD44 expression and suppress migration of such pathogenic cells to the joint. 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 Teff cell responses through subversion of TLR-4 signaling, without compromising the host immune response to infection (26), constitutes a highly appealing therapeutic strategy for RA.

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