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RESEARCH PAPER

A formyl peptide receptor agonist suppresses inflammation and bone damage in arthritis

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BACKGROUND AND PURPOSE

Annexin A1 (AnxA1) is an endogenous anti-inflammatory protein and agonist of the formyl peptide receptor 2 (FPR2). However, the potential for therapeutic FPR ligands to modify immune-mediated disease has been little explored. We investigated the effects of a synthetic FPR agonist on joint disease in the K/BxN model of rheumatoid arthritis (RA) and RA fibroblast-like synoviocytes (FLS).

EXPERIMENTAL APPROACH

Arthritis was induced by injection of K/BxN serum at day 0 and 2 in wild-type (WT) or AnxA1$^{-/-}$ mice and clinical and histopathological manifestations measured 8–11 days later. WT mice were given the FPR agonist compound 43 (Cpd43) (6 or 30 mg·kg^{-1} i.p.) for 4 days. Effects of AnxA1 and Cpd43 on RANKL-induced osteoclastogenesis were assessed in RAW 264.7 cells and human RA FLS and macrophages.

KEY RESULTS

Treatment with Cpd43 before or after the onset of arthritis reduced clinical disease severity and attenuated synovial TNF-α and osteoclast-associated gene expression. Deletion of AnxA1 in mice exacerbated arthritis severity in the K/BxN model. *In vitro*, Cpd43 suppressed osteoclastogenesis and NFAT activity elicited by RANKL, and inhibited IL-6 secretion by mouse macrophages. In human RA joint-derived FLS and monocyte-derived macrophages, Cpd43 treatment inhibited IL-6 release, while blocking FPR2 or silencing AnxA1 increased this release.

CONCLUSIONS AND IMPLICATIONS

The FPR agonist Cpd43 reduced osteoclastogenesis and inflammation in a mouse model of RA and exhibited anti-inflammatory effects in relevant human cells. These data suggest that FPR ligands may represent novel therapeutic agents capable of ameliorating inflammation and bone damage in RA.

Abbreviations

AnxA1, annexin A1; CsH, cyclosporin H; Cpd43, compound 43; FLS, fibroblast-like synoviocytes; FPR2, formyl peptide receptor 2; ALX, lipoxin A4 receptor; MAPK, mitogen-activated protein kinase; RA, rheumatoid arthritis; TRAP, tartrate-resistant acid phosphatase

Table of Links

TARGETS	LIGANDS
FPR2/ALX	Annexin A1 Cyclosporin H WRWWWW (WRW4) Compound 43

This Table lists the protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, characterized by the production of autoantibodies against citrullinated peptides, cytokine imbalance, joint inflammation, synovial hyperplasia and invasion of the synovium into adjacent bone and cartilage, leading to progressive joint destruction. The pathogenesis of RA involves the interaction of multiple cell types, including macrophages, lymphocytes and fibroblast-like synoviocytes (FLS) (Bartok and Firestein, 2010). Although the mechanisms of activation of leukocytes and FLS are becoming increasingly better understood, the effects of endogenous anti-inflammatory pathways which temper the activation of these cells in RA remain incompletely understood.

Annexin A1 (AnxA1) was originally identified as a calcium- and phospholipid-binding protein induced by glucocorticoids but is now recognized as an endogenous anti-inflammatory mediator. The anti-inflammatory effects of AnxA1 have been documented *in vivo* and *in vitro*, and include inhibition of leukocyte recruitment, inhibition of pro-inflammatory cytokine expression, inhibition of phospholipase A_2 activity and induction of apoptosis (see Parente and Solito, 2004; Lim and Pervaiz, 2007; Perretti and D'Acquisto, 2009). A role for AnxA1 in the regulation of inflammatory arthritis has been supported by several studies. AnxA1 is expressed in human RA synovial tissue and cells (Goulding *et al.*, 1995; Sampey *et al.*, 2000; Morand *et al.*, 2006) and has been identified as an important endogenous anti-inflammatory mediator in several animal models of RA. For example, our previous studies demonstrated that deficiency of AnxA1 significantly exacerbated antigen-induced arthritis in mice, and reduced the inhibitory effect of glucocorticoids in this model (Yang *et al.*, 2004). This finding is compatible with the exacerbation of experimental arthritis in rats in response to neutralization of AnxA1 using a specific antibody (Yang *et al.*, 1999). Recent studies have also revealed that mediation of glucocorticoid effects by AnxA1 is associated with effects on the expression of glucocorticoid-induced anti-inflammatory genes, such as MAPK phosphatase-1 and glucocorticoid-induced leucine zipper (Yang *et al.*, 2006; 2009). Together, these data suggest AnxA1 is a significant factor in the inhibitory regulation of joint inflammation.

Formyl peptide receptor (FPR) 2, also known as the lipoxin A4 receptor (ALX), is a cell surface receptor for AnxA1 (Walther *et al.*, 2000; Perretti *et al.*, 2002). FPR2 belongs to the human FPR family of GPCRs, which also includes FPR1 and FPR3 (Ye *et al.*, 2009). These receptors share significant sequence homology, but have different functional properties (Chiang *et al.*, 2006). FPR2 is activated by lipoxin A4, AnxA1 or AnxA1-derived peptides, as well as by the acute phase reactant serum amyloid A. Mice deficient in FPR2/3, which is homologous to human FPR2, exhibit exacerbated disease in carrageenan-induced paw oedema and K/BxN serum transfer-induced arthritis (Dufton *et al.*, 2010), indicating that FPR2 mediates anti-inflammatory effects. Thus, the anti-inflammatory effects of AnxA1 in arthritis may be receptor-dependent, raising the possibility that synthetic ligands of FPR2 could have therapeutic potential. This hypothesis has yet to be extensively explored.

Compound 43 (Cpd43) is a low MW compound synthesized as an agonist for FPR2 (Burli *et al.*, 2006) and has been shown to have this function in human leukocytes (Sogawa *et al.*, 2009; 2011). *In vivo*, Cpd43 exerts anti-inflammatory effects in murine ear inflammation and air-pouch models, which require FPR2 (Burli *et al.*, 2006; Dufton *et al.*, 2010; Sogawa *et al.*, 2011). Mutation studies have identified specific domains in FPR2, which are required for the action of Cpd43 (Bena *et al.*, 2012), but in human neutrophils, Cpd43 has also been reported to interact with FPR1 (Forsman *et al.*, 2011). Its effects have not been studied in the context of arthritis.

Here, we investigated the effects of FPR ligation using Cpd43 on K/BxN serum transfer-induced arthritis *in vivo* and, *in vitro,* on the activation of macrophages, osteoclasts and FLS from RA patients, Our results indicated that Cpd43 treatment resulted in significant anti-inflammatory and anti-osteolytic effects in these experimental conditions, effects in line with the anti-inflammatory effects of endogenous AnxA1. These findings highlight the potential of members of the FPR family as therapeutic targets in RA and demonstrated for the first time that an FPR agonist could ameliorate bone damage.

Methods

Animals

All animal care and experimental procedures were approved by the Monash University Animal Ethics Committee. A total of 44 animals were used in the experiments described here. C57BL/6 mice were purchased from the Walter and Eliza Hall Institute (Melbourne, Australia). AnxA1$^{-/-}$ mice were generated as described (Hannon *et al.*, 2003), and backcrossed to the C57BL/6 background for at least nine generations.

K/BxN serum transfer arthritis

Mice were injected i.p. with K/BxN sera at days 0 and 2 (38 μL per mouse). To assess preventive effects, Cpd43, provided by Amgen (Thousand Oaks, CA, USA), was given i.p. daily at 6 or 30 mg·kg^{-1} from day 0 to 3 and disease monitored over 8 days. To evaluate therapeutic effects, mice with clinically evident K/BxN arthritis (day 2) were treated daily with Cpd43 (30 mg·kg^{-1}) or vehicle for 4 days and disease monitored over 10 days. K/BxN serum transfer-induced arthritis was also induced in AnxA1$^{-/-}$ and wild-type (WT) mice as described above, and mice were monitored over 11 days. Clinical disease and histological features were assessed as described previously (Monach *et al.*, 2008). In brief, each limb was scored daily on a scale of 0 (no observable redness or swelling) to 3 (severe redness and swelling). The scores of four limbs were added together to obtain the clinical index (maximum score = 12). Paw thickness was measured daily with callipers. For histological assessment, sagittal ankle sections were stained with Safranin-O and counter-stained with fast green/iron haematoxylin. Histological sections were scored 0-3 for each of five parameters: synovitis, inflammation, joint space exudate, cartilage degradation and bone damage, and total histological score calculated from the sum of these data as described elsewhere (Santos *et al.*, 2011).

Osteoclastogenesis assay

C57BL/6 mouse bone marrow cells (5 × 10^4 cells per well) were seeded in tissue culture wells (6 mm diameter) , cultured in minimal essential medium α containing 10% FBS (MEM/FBS) with M-CSF (30 ng·mL^{-1}) and RANKL (50 ng·mL^{-1}) for 7 days to produce osteoclasts. Cpd43 (1, 10 and 30 μM) was added to the culture at day 3. RAW 264.7 cells (5 × 10^4 per well in 96-well plates) were stimulated with RANKL (100 ng·mL^{-1}) for 7 days in MEM/FBS, and Cpd43 (1, 10 and 30 μM) was added to cultures at day 3. Cells were histochemically stained for tartrate-resistant acid phosphatase (TRAP) at the end of experiments, and TRAP$^+$ multinucleated cells (with more than three nuclei) were identified as osteoclasts.

NF-κB and NFATc1 luciferase reporter assays

RAW 264.7 cell lines were stably transfected with NF-κB and NFATc1 luciferase reporter constructs as previously described (Wang *et al.*, 2003; Singh *et al.*, 2012). The effect of Cpd43 (1, 10 and 30 μM) on transcriptional activity of NFATc1 and NF-κB was examined in the presence or absence of LPS (10 ng·mL^{-1}) or RANKL 100 ng·mL^{-1}) for 6 (NF-κB) or 24 h (NFAT). Luciferase activity was measured as previously described (Yang *et al.*, 2009).

Cell culture and materials

Tissue samples were obtained from RA patients with full informed consent and with the approval of the Human Research Ethics Committee, Monash Medical Centre. The patients fulfilled the ACR criteria for the classification of RA (Arnett *et al.*, 1988). FLS were obtained, as previously described (Leech *et al.*, 1999), from joint replacement surgery specimens in RA patients. Human FPR2 was blocked by the specific antagonist, WRWWWW-NH$_2$ (WRW4; 5 μM; Calbiochem, Merck Millipore, Kilsyth, VIC, Australia). siRNA directed at human AnxA1 was transfected into FLS as described previously (Jia *et al.*, 2013).

RAW 264.7 cells (2 × 10^5 per well in 96-well plates; from ATCC, Manassas, VA) were stimulated with LPS from *Escherichia coli* O111.B4 (10 ng·mL^{-1}; Sigma-Aldrich, Castle Hill, NSW, Australia) for 4 h in RPMI/FCS and Cpd43 (10 or 30 μM). Supernatants were collected and analysed by ELISA for IL-6.

Human THP-1 macrophages (ATCC) were generated by treating 1 × 10^6 THP-1 cells suspended in RPMI 1640 with 10% FCS with PMA (0.5 ng·mL^{-1}) in 96-well plates for 24 h. After washing, macrophages were cultured for 2 days before treatment with LPS (100 ng·mL^{-1}) and/or Cpd43 (30 μM), the FPR1 antagonist cyclosporin H (CsH, 1 μM; Chemieliva Pharmaceutical Co., Ltd., Chongqing, China) or the FPR2 antagonist WRW4 (5 μM) overnight. Supernatants were analysed by ELISA for IL-6.

RNA interference

RA FLS were transfected with 10 nM siRNA against human AnxA1 (Invitrogen, Mulgrave, Victoria, Australia) using lipofectamine RNAiMAX (Invitrogen) as described previously (Jia *et al.*, 2013). Culture was continued for 48 h. Silencing efficiency was monitored using RT-PCR for mRNA and Western blot for protein levels. For control experiments, non-targeting siRNA pool (Invitrogen) was used under the same conditions.

ELISA

IL-6 (human and mouse) in culture supernatants was measured using commercially available ELISAs (Quantikine M; R&D Systems, Minneapolis, MN, USA, and BioLegend, San Diego, CA, USA).

Confocal microscopy

Cells (2 × 10^3 cells per well) were cultured in 8-well Lab-Tek chamber slides (Thermo Fisher Scientific, Scoresby, VIC, Australia) and stained with rabbit anti-FPR2 mAb (Abcam, Cambridge, MA, USA) or rabbit IgG antibodies. Samples were analysed on a Nikon C1 confocal microscope (Nikon, Tokyo, Japan).

Quantitative PCR (qPCR)

Total RNA from peritoneal cells was extracted as described previously (Santos *et al.*, 2011). RNA was isolated from cells using the RNeasy mini kit (Qiagen, Melbourne, VIC, Australia). RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase and Oligo dT^{20} (Invitrogen, Mount Waverley, Victoria, Australia). qPCR was performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, New South

Wales, Australia) using power SYBR Green PCR master mix (Applied Biosystems, Scoresby, Victoria, Australia) according to the manufacturer's protocol. Primers used for the determination of murine TNF, IL-1, MIF and β-actin mRNA were as previously described (Yang *et al.*, 2004). Analysis of relative change in gene expression was calculated according to the 2^-ΔΔCt method using the housekeeping gene, β-actin, as the control.

Western blotting

Western blotting was performed as previously described (Toh *et al.*, 2004; Yang *et al.*, 2013b). Briefly, cell lysates were collected using cell lysis buffer supplemented with complete mini protease inhibitor cocktail. Protein (20 μg per well) was separated on 10% SDS-PAGE and transferred to Hybond-C extra nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were probed with antibodies against NFATc1 (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Sigma-Aldrich). Anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 700 (Rockland, Stepney, South Australia, Australia) and IRDye 800 (Rockland), respectively, were used to probe primary antibodies. Protein bands were detected and quantified by Western blotting with the Odyssey system (Li-Cor, Surry Hills, Victoria, Australia). Densitometry ratios were normalized to β-actin content and expressed as arbitrary units.

Data analysis

Results are expressed as means ± SEM. One-way ANOVA with Newman–Keuls multiple comparison *post hoc* test was used when more than one treatment was compared with a control. Student's *t*-test was used when only two variables were compared. *P*-values less than 0.05 were considered statistically significant.

Results

FPR agonist suppresses development of K/BxN serum-induced inflammatory arthritis

K/BxN serum was transferred to C57BL/6 mice to induce arthritis. Mice were treated with vehicle or Cpd43 (6 or 30 mg·kg^{-1}) daily for the first 4 days, then monitored over 8 days. At 6 mg·kg^{-1}, Cpd43 failed to inhibit development of arthritis (Figure 1A,B). However, treatment with Cpd43 at 30 mg·kg^{-1} significantly reduced clinical scores and paw swelling (Figure 1A,B). Consistent with the effects on clinical features, 30 mg·kg^{-1} Cpd43 significantly reduced histopathological changes in ankle joints in arthritic mice (Figure 1C), as measured by each of synovitis, soft-tissue inflammation and cartilage and bone damage, when compared with mice treated with vehicle alone. Total scores and individual parameters of histological severity are summarized in Figure 1D,E.

We next looked at the effects of Cpd43 treatment on the expression of pro-inflammatory cytokines in ankle joints of mice with K/BxN serum-induced arthritis. Treatment with Cpd43 significantly reduced the joint expression of TNF mRNA (Figure 1F). A non-significant inhibitory effect of Cpd43 treatment on joint IL-1β mRNA was also observed (data not shown). Expression of osteoclast-associated genes, including RANK, cathepsin K and OC-STAMP, was also decreased in Cpd43-treated mice (Figure 1G–I). These data demonstrate that Cpd43 exerts powerful anti-inflammatory effects during the development of arthritis in the K/BxN serum transfer model, and may have a novel protective effect to reduce arthritic bone damage.

The therapeutic effect of Cpd43 in established arthritis was also assessed. Mice developed clinical signs of arthritis at day 1 after serum injection. Mice with established arthritis (day 2) were treated with Cpd43 (30 mg·kg^{-1}) or vehicle daily until day 5 and clinical disease and paw thickness monitored. Cpd43 treatment significantly attenuated development and severity of the arthritis, as measured by both clinical scores and paw thickness (Figure 1J,K).

Exacerbation of K/BxN serum-induced inflammatory arthritis in AnxA1$^{-/-}$ mice

We next studied the effects of deficiency of endogenous AnxA1 in this model, by comparing disease severity in AnxA1$^{-/-}$ and WT mice. Compared with WT mice, AnxA1$^{-/-}$ mice developed more severe clinical manifestations of disease in the K/BxN model, as shown by higher clinical scores and increased ankle thickness (Figure 2A,B). Analysis of histopathological features of arthritis showed severe inflammation and cartilage damage in arthritic WT mice. Total histological severity, as well as scores for inflammation, synovitis, and cartilage damage, were increased in AnxA1$^{-/-}$ mice (Figure 2C–E). These data indicate that endogenous AnxA1 also had important inhibitory effects on joint inflammation in this model.

FPR agonist suppresses RANKL-mediated osteoclastogenesis in mouse macrophages

Osteoclasts are responsible for resorbing bone, both during bone development and remodelling, and in the pathogenesis of RA bone erosions. They develop from immature macrophages in a manner dependent on RANKL, a TNF-related protein produced locally in bone by osteoblasts and lymphocytes. As Cpd43 reduced the expression of osteoclast-associated markers in inflamed joints, we next examined its effects in RANKL-induced osteoclast formation *in vitro* using RAW 264.7 cells and primary bone marrow-derived macrophages (BMM). Osteoclast formation was identified by the presence of TRAP^{+} multinucleated cells. Osteoclast formation in both BMM and RAW264.7 cultures was significantly and dose-dependently inhibited by Cpd43 treatment (Figure 3A–C).

To examine the mechanism of action of Cpd43, we investigated its effects on the activity of NF-κB and NFATc1, two RANKL-induced transcription factors that are essential for osteoclastogenesis. Using RAW 264.7 cells stably transfected with luciferase reporter constructs, we found that Cpd43 significantly inhibited RANKL-induced NFATc1 activity (this construct responds to both NFATc1 and NFATc1-induced NFATc2), but did not affect RANKL-induced NF-κB activation (Figure 3D,E). Moreover, RANKL-induced NFATc1 protein expression was inhibited by treatment with Cpd43 (Figure 3F), consistent with its effect on NFATc1 activity.

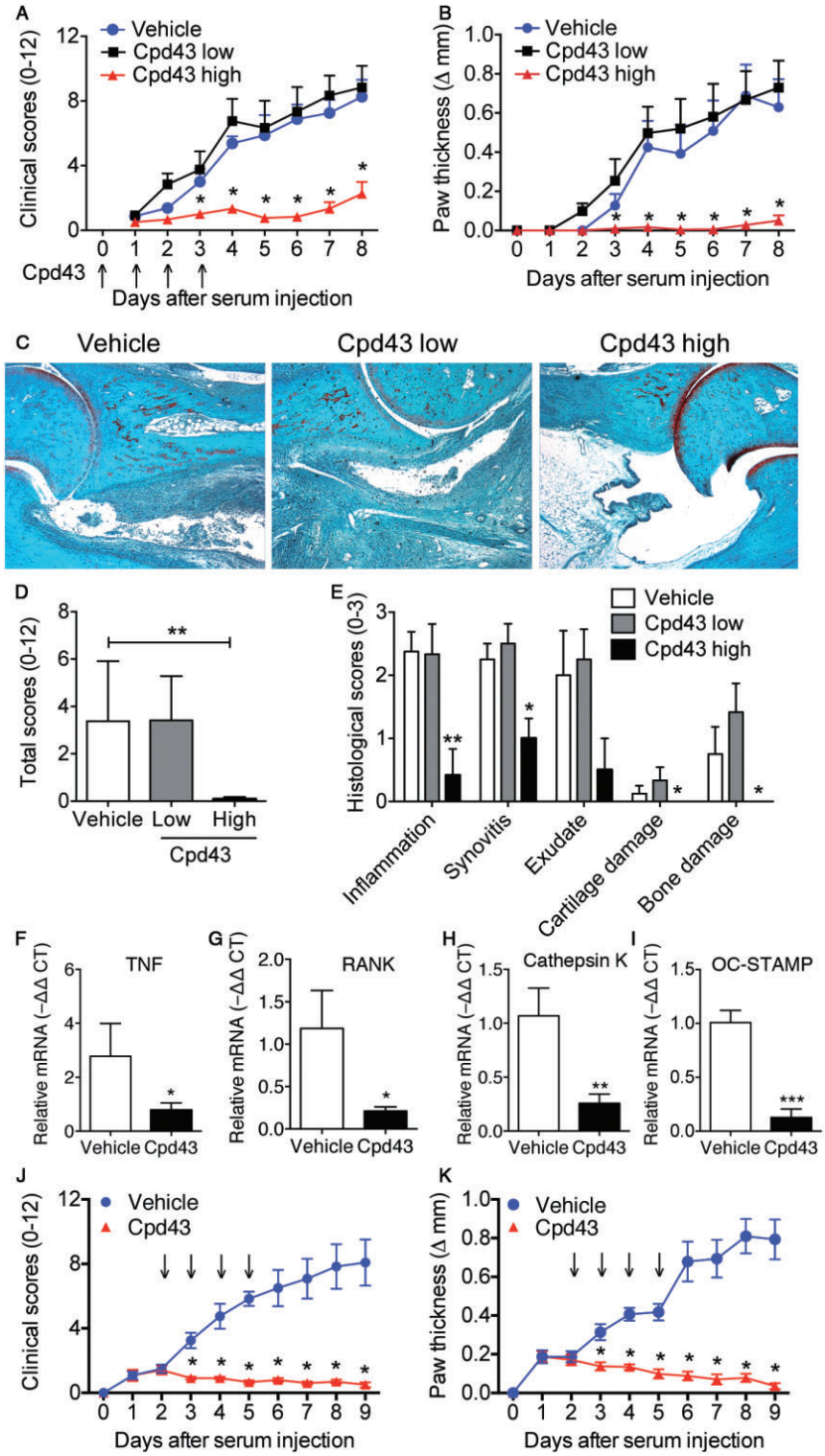

Figure 1

Cpd43 inhibits arthritis development and cytokine and osteoclast-relevant gene expression in a K/BxN serum transfer model. C57BL/6 mice were injected with K/BxN serum at days 0 and 2 i.p. Vehicle or Cpd43 at doses of 6 mg·kg^{-1} (low) or 30 mg·kg^{-1} (high) were injected i.p. daily from day 0 to day 3. Clinical scores (A) and paw thickness (B) were assessed daily. n = 6 for each group; *P < 0.01 versus vehicle treatment. (C) Representative Safranin-O sections of ankles from mice treated with vehicle, low or high doses of Cpd43, prepared for histological scoring on day 8. Original magnification ×50. (D) Total histological scores for joint inflammation. (E) Individual histological scores, including joint inflammation, synovitis, exudate, cartilage and bone damage. (F) TNF-α mRNA expression in ankle joints measured by qPCR. *P < 0.05, **P < 0.01, ***P < 0.001. (G–I) Quantitative analysis of osteoclast markers in inflamed joints, (G) RANK mRNA, (H) cathepsin K mRNA and (I) OC-STAMP measured by qPCR. (J,K) K/BxN arthritis mice were treated daily with Cpd43 (30 mg·kg^{-1}) or vehicle from day 2 to 5. Clinical scores (J) and paw thickness (K) were assessed daily. Data are presented as mean ± SEM; n = 6 per group. *P < 0.001, versus vehicle treatment or as indicated.

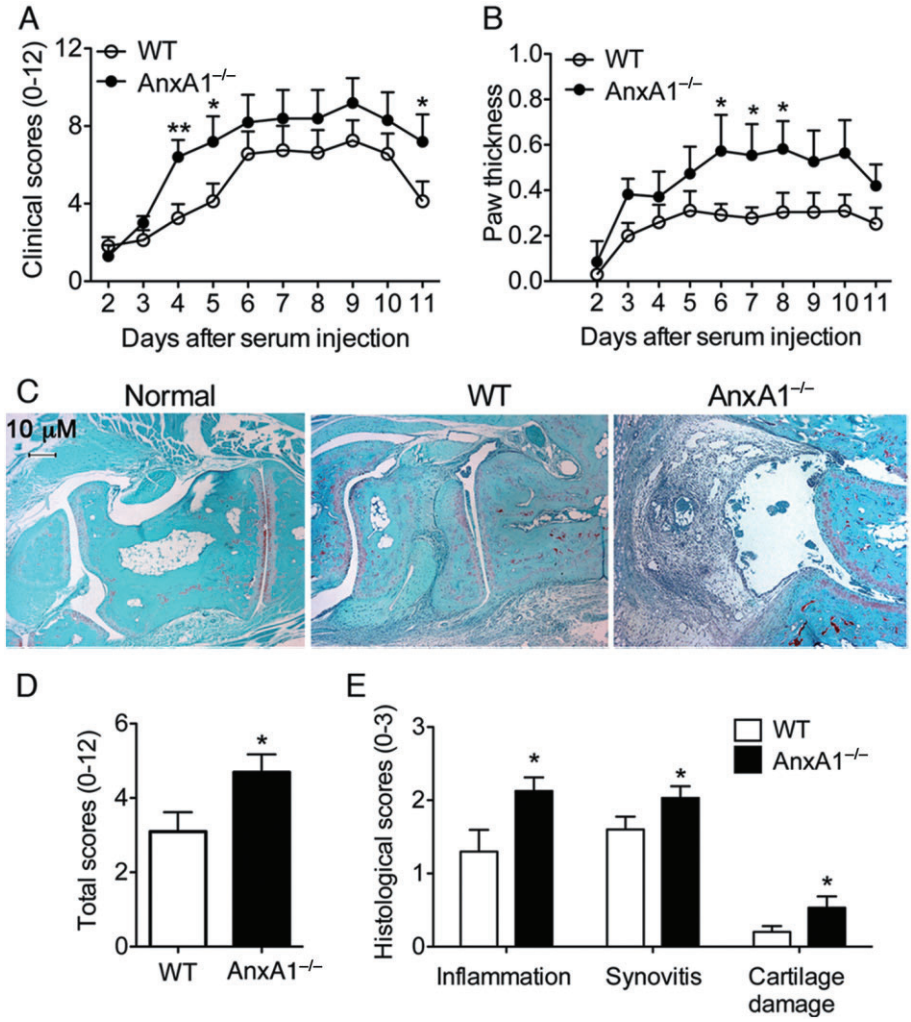

Figure 2

Increased severity of K/BxN serum-induced arthritis in AnxA1$^{-/-}$ mice. (A,B) K/BxN serum was injected i.p. into WT or AnxA1$^{-/-}$ mice at days 0 and 2. Clinical arthritis and paw thickness was measured. Values are the mean ± SEM (n = 8 mice per group). *P < 0.05; **P < 0.01 versus WT mice. (C) Representative Safranin-O sections of ankles from normal or mice with arthritis prepared for histological scoring on day 11. (D,E) Histological scores for joint inflammation, total (D) and individual scores, including joint inflammation, synovitis, exudate, cartilage and bone damage (E). Data are presented as mean ± SEM. *P < 0.05, versus WT.

FPR agonist suppresses LPS-mediated IL-6 release, NFATc1 and NF-κB activity in mouse macrophages

Because LPS-induced responses in macrophages involve similar pathways to those activated by RANKL, we also examined the actions of Cpd43 on LPS-mediated cytokine secretion and signalling pathways. Cpd43 significantly suppressed LPS-induced IL-6 release from RAW 264.7 cells (Figure 4A). Cpd43 also significantly inhibited both LPS-induced NFATc1 and NF-κB luciferase reporter activity in RAW 264.7 cells (Figure 4B,C). These data suggest that although Cpd43 inhibits bone damage by osteoclasts through the regulation of NFATc1, the inhibition of pro-inflammatory cytokine responses and clinical joint inflammation may be dependent on the regulation of NFATc1, NF-κB or both. To evaluate the receptors utilized by Cpd43, and test the effects of Cpd43 in human cells, we used the THP-1 human monocyte-derived macrophage cell line. Cpd43 significantly inhibited IL-6 release induced by LPS, from THP-1 cells (Figure 4D). Blocking FPR1 with CsH failed to prevent the inhibitory effect of Cpd43 on IL-6 release induced by LPS (Figure 4D). In contrast, when FPR2 was blocked using WRW4, Cpd43 inhibition of LPS-induced IL-6 release was clearly decreased (Figure 4D). These findings suggest that, at least in these cells, the action of Cpd43 was mediated via FPR2.

FPR agonist inhibits IL-6 release in RA tissue-derived FLS

To investigate effects of Cpd43 on inflammatory responses relevant to human RA beyond its effects on macrophages, we looked at its effects on human RA-derived FLS. Using confocal microscopy, we detected the expression of FPR2, but not that

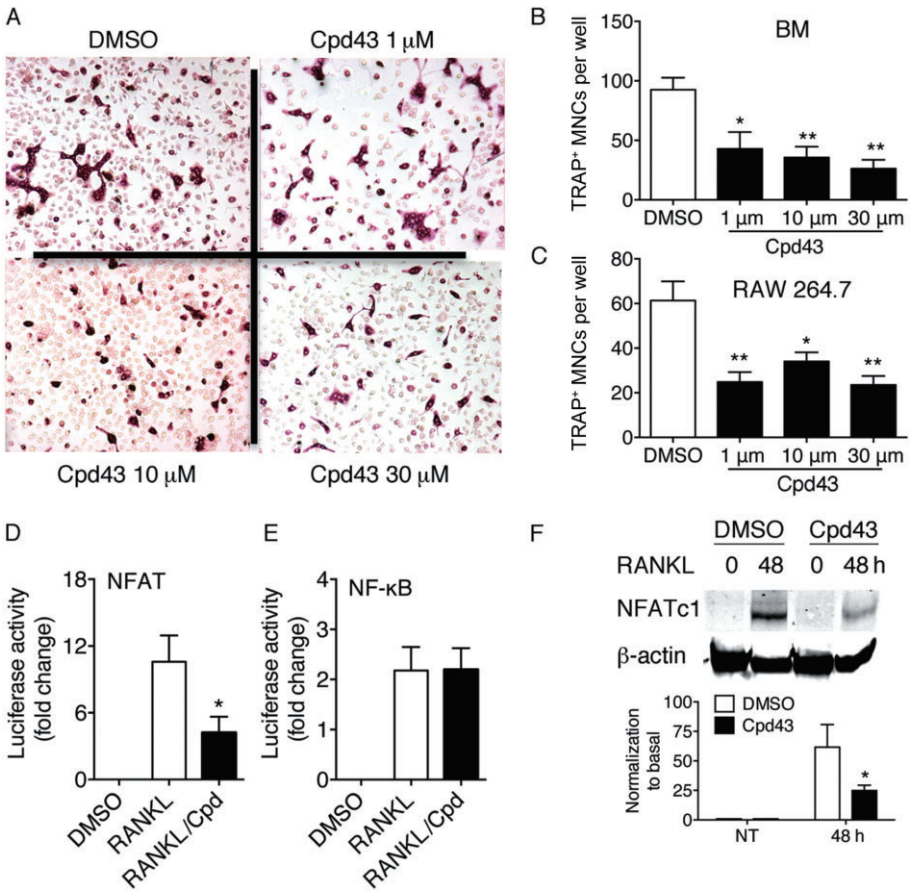

Figure 3

Cpd43 inhibits osteoclastogenesis as well as RANKL-induced signals *in vitro*. (A,B) Murine bone marrow (BM) macrophages were stimulated with RANKL (50 ng·mL^{-1}) and M-CSF (30 ng·mL^{-1}) for 7 days with Cpd43 at indicated concentrations added on day 3. Cells were histochemically stained for TRAP (red colour) and osteoclasts identified as TRAP$^+$ cells with three or more nuclei (TRAP$^+$ MNCs) (A). Original magnification × 200 (representative images of at least three separate experiments). Quantitative analysis of TRAP$^+$ MNCs (B). (C) RAW 264.7 cells were stimulated with RANKL (100 ng·mL^{-1}) and Cpd43 as above. TRAP$^+$ MNCs were quantitated in (B). (D, E) NFAT and NF-κB reporter RAW cells were treated with RANKL (100 ng·mL^{-1}) and Cpd43 (30 μM) for 6 h (NF-κB) or 24 h (NFAT). Luciferase activity was measured. $n = 3$ independent experiments. (F) RAW 264.7 cells were treated with Cpd43 (30 μm) and RANKL (100 ng·mL^{-1}) for 48 h. The Western blot is representative of three individual experiments. Data expressed as mean ± SEM; $n = 3$; *$P < 0.05$, **$P < 0.01$, versus control.

of FPR1, on FLS (Figure 5A), suggesting that these cells would respond to Cpd43. As was observed in mouse macrophages, Cpd43 dose-dependently inhibited IL-6 secretion by FLS (Figure 5B). Correspondingly, blocking FPR2 with the selective antagonist WRW4 increased IL-6 secretion (Figure 5C). Consistent with this, siRNA silencing of AnxA1 also increased IL-6 secretion by FLS (Figure 5D). These data indicated that endogenous AnxA1 and exogenous Cpd43 exerted significant inhibitory effects on FLS activation.

Discussion

Arthritis caused by K/BxN serum transfer is induced by passive transfer of autoantibodies to glucose-6-phosphate isomerase. Disease expression requires the participation of macrophages and neutrophils, and is also dependent on cytokines such as IL-1 and TNF-α (Monach *et al.*, 2008). AnxA1 has numerous anti-inflammatory effects that overlap with the mechanisms operative in K/BxN serum transfer arthritis, including inhibition of leukocyte recruitment and pro-inflammatory cytokine expression (Perretti and D'Acquisto, 2009; Yang *et al.*, 2013a). AnxA1 has been shown to ameliorate disease in several animal models of inflammation, including arthritis (Yang *et al.*, 1999; 2004; 2013b; Patel *et al.*, 2012).

AnxA1 is most likely to exert its anti-inflammatory effects through interaction with FPR2 on the cell surface (Ye *et al.*, 2009). Cpd43 was developed as a ligand for FPR2, and has been shown to inhibit neutrophil chemotaxis and markedly reduce mouse ear inflammation (Burli *et al.*, 2006; Sogawa *et al.*, 2011). Alterations in the structure of FPR2 prevent the action of Cpd43, suggesting the presence of specific sites on FPR2 with which Cpd43 interacts (Bena *et al.*, 2012), although this has not been demonstrated physically, for example, using crystallography.Notably, in human

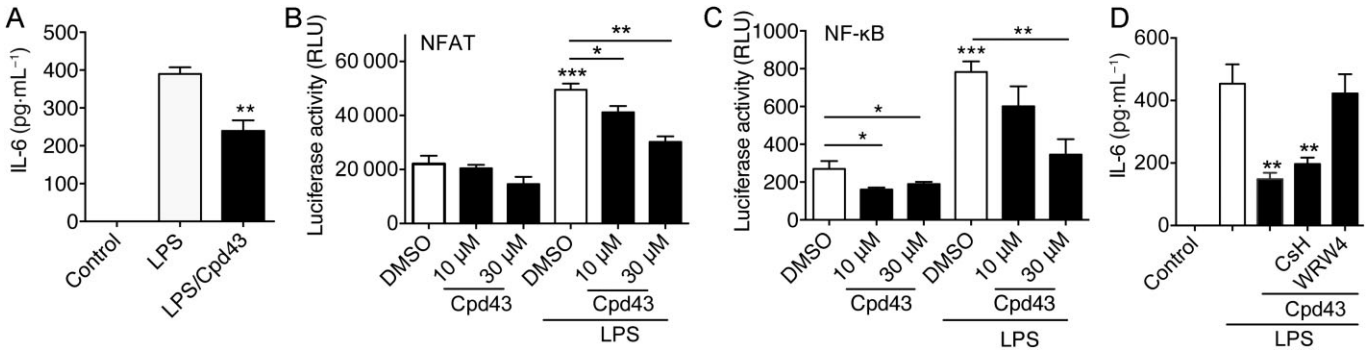

Figure 4

Cpd43 inhibits LPS-induced signals *in vitro*. (A) RAW 264.7 cells were treated with LPS (10 ng·mL^{-1}) and Cpd43 (30 μM) for 4 h. IL-6 concentration in the supernatants was measured by ELISA. (B) NFAT reporter RAW cells were treated with Cpd43 and LPS (10 ng·mL^{-1}) for 24 h and luciferase activity measured. (C) NF-κB reporter RAW cells treated and Cpd43 and LPS (1 ng·mL^{-1}) for 6 h and luciferase activity measured. (D) Human THP-1 cell-derived macrophages were treated with LPS and Cpd43 (30 μM) and/or antagonists of FPR1 (CsH, 1 μM) or FPR2 agonist (WRW4, 5 μM) overnight. Supernatant IL-6 was measured by ELISA. Data expressed as mean ± SEM; $n = 3$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, versus control or as indicated.

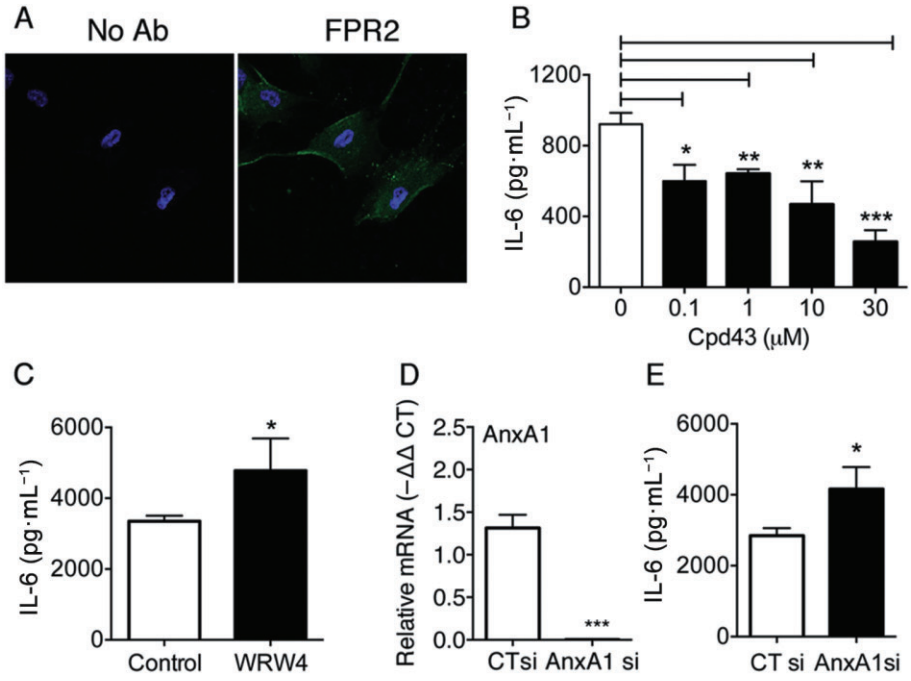

Figure 5

Cpd43 suppresses activation of FLS from RA patients. (A) FLS were labelled with anti-FPR2 mAb or rabbit IgG. Nuclei were stained with DAPI and cells analysed by confocal microscopy. (B) FLS were treated with vehicle (control) or Cpd43 at indicated concentrations for 4 h. IL-6 in the supernatants was measured by ELISA. (C) FLS were treated with vehicle (control) or WRW4 for 8 h. IL-6 in the supernatants was measured by ELISA. (D) FLS were transiently transfected with control (CT) or AnxA1-targeting siRNA using RNAiMAX lipofectamine for 48 h, and AnxA1 mRNA expression measured by qPCR. (E) IL-6 concentrations in the supernatants of siRNA-transfected FLS were measured by ELISA. Data are means ± SEM; $n = 4$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$ versus controls.

neutrophils, Cpd43 has been reported to interact with both FPR1 and FPR2 (Forsman *et al.*, 2011), suggesting that, in at least some conditions, it is a FPR1/2 dual agonist.

The discovery of compounds like Cpd43 permits the examination of putative FPR ligands as potential therapies for diseases like RA. Based on the known anti-inflammatory effects of AnxA1, and exacerbation of K/BxN serum transfer-induced arthritis in FPR2$^{-/-}$ mice (Dufton *et al.*, 2010), we hypothesized that Cpd43 might act as a potent inhibitor of inflammatory arthritis. We have shown here that treatment of mice with Cpd43 at a dose of 30 mg·kg^{-1}, either before or after the onset of disease, almost completely inhibited arthri-

tis in the K/BxN serum transfer model. These anti-arthritic effects were observed at both clinical and histopathological levels, and were associated with suppression of TNF expression in the joint. There was no effect of Cpd43 at the lower dose tested, 6 mg·kg^{-1}. The lowest effective dose has not been established by these experiments and more extensive dose–response experiments would be required to establish this.

Our data also show that Cpd43 inhibits osteoclast differentiation *in vitro*, consistent with its effects on histological evidence of bone damage in mice with K/BxN arthritis. Cpd43 also inhibited the expression of the osteoclast markers, cathepsin K and OC-STAMP, suggesting a reduction in the very high levels of osteoclast maturation that occurs in inflammatory arthritis. Osteoclasts are multinucleated cells that originate from the myeloid cell lineage and are responsible for bone resorption. Although bone loss is secondary to arthritis, reduction of osteoclastic bone resorption is an attractive second aspect to target in the treatment of RA, as periarticular bone erosion is a major source of deformity, pain and disability in this disease. The inhibitory effects of Cpd43 appear to be mediated by suppression of NFATc1, a major mediator of RANKL actions and a critical transcription factor in osteoclast differentiation (Ishida *et al.*, 2002).

We also observed inhibitory effects of Cpd43 on human FLS and macrophage cytokine production, suggesting that FPR ligation with Cpd43 inhibits the activities of many cells that drive joint damage in arthritis, and thus that FPR ligand drugs may exert many therapeutically desirable actions. Both blocking FPR2 using the antagonist compound WRW4 and silencing endogenous AnxA1 increased IL-6 release by human FLS. Blocking FPR2, but not FPR1, also abolished the inhibitory effect of Cpd43 on IL-6 release by human macrophages, supporting the conclusion that Cpd43 interacts with FPR2 in order to exert its inhibitory role in this context. These data on human cells further support an anti-inflammatory role for FPR2 ligands in arthritis, and suggest that the effects of AnxA1 and Cpd43 are most likely dependent on FPR2. We were unable to detect FPR1 in FLS or THP-1 human macrophages (data not shown), but it remains possible that interactions of Cpd43 with FPR1 in other cells of relevance to RA may be important. Although the effects of Cpd43 were prevented by a FPR2 antagonist, the possibility of other non-FPR-dependent effects of this compound has not been excluded. Also, parallel effects of Cpd43 on other aspects of immune activation relevant to RA, such as T and B cell-dependent autoimmunity, have not yet been studied; our present findings may open such avenues for investigation.

Our study demonstrated that deficiency of AnxA1 in mice resulted in augmentation of arthritis severity in the K/BxN model, indicating a protective role of endogenous AnxA1. These findings are at odds with those of a recent study in which increased arthritis severity in AnxA1$^{-/-}$ mice was not observed in this model (Patel *et al.*, 2012), although these authors did observe reduced efficacy of the glucocorticoid dexamethasone in the absence of AnxA1. In that study, arthritis was induced with a higher dose of K/BxN serum than that used in the current study. Here, conscious of the notion that a more severe model might mask the effects of AnxA1 deficiency, we used a lower dose of K/BxN serum that was still sufficient to induce joint inflammation. Our results suggest that, under these conditions, endogenous AnxA1 slows the development of clinical joint disease, and reduces histopathological evidence of joint inflammation.

In conclusion, we have demonstrated that the low MW FPR agonist Cpd43 exerted significant anti-inflammatory effects on a severe experimental model of RA, in which endogenous AnxA1, the natural ligand for FPR2, also exerted inhibitory effects. In addition, Cpd43 reduced RANKL-mediated osteoclastogenesis and inhibited the production of IL-6 by macrophages, as well the activation of human RA FLS, through effects that appeared to be dependent on interaction with FPR2. These findings suggest that therapeutic agonists of FPR may have important beneficial actions on inflammation and bone loss in RA.

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Author contributions

Y. H. Y., W. K., R. G., Y. J., H. F., X. W. and J. H. performed the experiments. Y. H. Y., E. F. M. and J. Q. designed the experiments. Y. H. Y., W. K., R. G., Y. J., H. F., X. W., Z. Z. and J. H. analysed the data. Y. H. Y., E. F. M., J. H. and J. Q. wrote the manuscript. W. K. and R. G. contributed equally. Y. H. Y. and E. F. M. contributed equally to this work as senior authors.

Conflict of interest

The authors have no conflicting financial interest.

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