Abstract

Objectives. Fibroblast-like synoviocytes (FLSs) are crucial players in the pathogenesis of synovitis in rheumatic diseases. Targeting FLS activation represents an approach to the development of therapeutic strategies. Our aim was to investigate whether the microenvironment of inflamed joints could modulate the expression of IL-22 and IL-22R1 on OA and RA FLSs. We also examined the effect of IL-22 on FLS activation as well as on their IL-17-related responses.

Methods. IL-22 and IL-22R1 expression was studied by RT-PCR and immunoblotting. Proliferation was measured by an ELISA kit. IL-17 receptors, p19IL-23 and alarmins were analysed by RT-PCR. IL-17 receptor expression was evaluated by flow cytometry. MMP1 and IL-23 were measured by ELISA. S100A8/A9 expression was detected by immunofluorescence and ELISA. Signal transducer and activator of transcription 3 (STAT3) phosphorylation was quantified using a cell-based ELISA kit.

Results. IL-22 and IL-22R1 were expressed constitutively in FLSs. We demonstrated that S100A8 and S100A9 were synthesized in FLSs. We reported that inflammatory mediators increased the expression of the IL-22/IL-22R1 axis, amplifying FLS activation. IL-22 enhanced FLS proliferation and up-regulated MMP1 and S100A8/A9 production. STAT3 phosphorylation was induced after IL-22 treatment and the stimulatory effect of IL-22 on S100A8/A9 was reduced after the activities of Janus kinase 2 (JAK2) and JAK3 were blocked. We showed an inhibitory action of IL-22 on IL-23 and IL-17RC expression in RA FLSs and on IL-17RA in OA FLSs.

Conclusion. Therapies based on the pharmacological disruption signalling of IL-22 could be beneficial for the treatment of rheumatic diseases. The restricted expression of IL-22R1 to non-lymphoid cells could lead to a reduction of side effects mediated by immune responses.

Key words: IL-22, IL-22R1, alarmins, synovial fibroblast, osteoarthritis, rheumatoid arthritis.

Introduction

IL-22 belongs to the IL-10 superfamily of cytokines and signals through a heterodimeric receptor complex composed of the IL-22R1 and IL-10R2 chains [1], mainly activating the signal transducer and activator of transcription 3 (STAT3). Unlike the ubiquitous presence of IL-10R2 chain, IL-22R1 expression is restricted to non-immune cells determining them as specific IL-22 targets [2, 3].

The role of IL-22/IL-22R1 interactions in inflammatory disorders is still controversial and either protective or highly pathogenic functions have been described [4]. IL-22 has been shown to be protective during the development of hepatitis [5] and inflammatory bowel disease [6], whereas in psoriasis an inflammatory role has been found [7].

RA is a chronic, inflammatory, autoimmune disease that primary attacks synovial joints [8]. Fibroblast-like
synoviocytes (FLSs) are leading cells in the establishment and progression of arthritis [9]. Their pathogenic behaviour is activated and enhanced in response to multiple pathways, including proinflammatory mediators and Toll-like receptor (TLR) agonists [10].

Although several studies have suggested a correlation between IL-22 and arthritis, its precise involvement in the disease physiopathology remains to be established. In 2005 Ikeuchi et al. [11] described the presence of IL-22 and its specific receptor in synovial tissue and RA FLSs, as well as its role in promoting proliferation and CCL2/MCP-1 production. In addition, increased numbers of Th17 cells are detected in the peripheral blood monocellular cells and joints of RA patients, which are one of the IL-22-expressing cell subsets identified [12–14]. Also, high IL-22 levels in the synovial fluid of RA patients have been found to be correlated with radiographic disease progression [15, 16]. However, recent findings in the study of IL-22 in the mouse CIA model suggest a dual nature for its effector function in inflammatory arthritis. IL-22-deficient mice had a reduced incidence of arthritis and showed less inflammation and joint destruction [17]. In contrast, in the same model, IL-22 administration prior to the onset of arthritis has been shown to be associated with an increase in IL-10 and a reduction in the progression of arthritis severity [18].

In this study we examined the IL-22/IL-22R1 axis in FLSs of OA and RA patients. The hypothesis tested was that the expression of IL-22 and its receptor in FLSs would be modulated by inflammatory cytokines and TLR ligands present in the joint microenvironment. Also, IL-22 stimulates the aggressive behaviour of FLSs and modulates their molecular contribution to the IL-17/IL-23 axis. Thus we analysed the effect of IL-22 on FLS proliferation and MMP production, studying for the first time in FLSs both the constitutive and the IL-22-induced expression of S100A8/A9 alarmins, recently considered as markers of destructive processes in the joint. We further tested the role of IL-22 in the expression of IL-17 receptors (IL-17RA and IL-17RC) and IL-23 in FLSs.

**Methods**

**Patients and FLS cultures**

Synovial tissue samples were obtained from nine patients with RA and nine with OA at the time of knee replacement surgery. All RA patients fulfilled the ACR revised criteria for the diagnosis of RA [19]. The study was performed according to the recommendations of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Hospital 12 de Octubre and the Hospital La Princesa. All the patients signed a written consent to participate in the study.

FLS cultures were established by explant growth in 10% foetal bovine serum/DMEM. The purity of the FLSs used was ≥95% by flow cytometric analysis. FLSs were used between passages 3 and 9.

Lipopolysaccharide (LPS) from *Salmonella enteritidis* (Sigma-Aldrich, St. Louis, MO, USA) was used at 20 μg/ml. Recombinant human IL-22 (rhIL-22) and TNF-α (rhTNF-α) (ImmunoTools, Friesoythe, Germany) were used at 100 ng/ml and 1 nM, respectively. The endotoxin level of the rhIL-22 was measured by a limulus amoebocyte assay [limulus amoebocyte lysate (LAL) colorimetric endpoint assay] and it was smaller than 1 EU/μg, excluding the effects of bacterial contaminants in the assays. Poly I:C, CP-690,550 and AG490 were obtained from InvivoGen (San Diego, CA, USA) and used at 20 μg/ml, 500 nM and 50 μM, respectively.

**Reverse transcription and semi-quantitative real-time PCR assay**

Human IL-22, IL-22R1, IL-17RA, IL-17RC and p19 IL-23 mRNA expression were analysed after 24 h of stimulation with rhIL-22, TNF-α, poly I:C or LPS. For S100A8 and S100A9 mRNA expression analysis, FLSs were pre-incubated for 2 h with CP-690,550 or AG490 before rhIL-22 addition.

Total RNA was obtained using Tri Reagent (Sigma-Aldrich). A total of 2 μg (total RNA) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA).

Semi-quantitative real-time PCR analysis for all molecules tested was performed using SYBR Green PCR Master Mix with the exception of S1A008 and S1A009, which were tested using TaqMan Gene Expression Master Mix (Life Technologies) (supplementary Table S1, available at *Rheumatology* Online). β-Actin was used as an endogenous reference gene. For relative quantification we compared the amount of target mRNA normalized to the endogenous reference using the formula 2−ΔΔCt.

**Western blot analysis of IL-22 and IL-22R1**

FLSs were cultured for 24 h with LPS, poly I:C or TNF-α. Proteins were extracted in ice-cold radio immunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 30 mM NaF, 5 mM EDTA, 1% Triton X-100, 1% NP-40, 0.1% SDS, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate and protease inhibitor cocktail]. Twenty microgram proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-human IL-22R1 (1 μg/ml; ProSci Incorporated, San Diego, CA, USA) and mouse anti-human IL-22 (1 μg/ml; R&D Systems, Minneapolis, MN, USA). Mouse anti-β-actin (1:10000, Sigma-Aldrich) was used as a loading control. Horseradish peroxidase-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used. Proteins were detected using western blotting luminol reagent (Santa Cruz Biotechnology), analysed using the Bio-Rad Quantity One program (Bio-Rad, Hercules, CA, USA) and normalized against β-actin.
ELISA of IL-22, MMP1, MMP3, S100A8/A9 complex (calprotectin) and IL-23 in culture supernatants

FLS-conditioned supernatants were concentrated with Amicon Ultra-0.5 centrifugal filter devices (Millipore, Darmstadt, Germany) for MMP3, IL-23 and calprotectin determination.

Levels of IL-22 in supernatants were measured using two different ELISA kits purchased from R&D Systems and eBioscience (San Diego, CA, USA). The means of the minimum detectable dose for these two kits were 2.7 and 5 pg/mL, respectively.

IL-23 and MMP3 levels in supernatants after 24 h of rhIL-22 stimulation were measured using a Human IL-23 (p19/p40) Platinum ELISA Kit (eBioscience) and a Human MMP3 ELISA Kit (R&D Systems). MMP1 and calprotectin were determined after pre-incubation for 2 h with CP-690,550 or AG490 and rhIL-22 stimulation. An MMP1 ELISA Promokine Kit (PromoCell GmbH, Heidelberg, Germany) and a LEGEND MAX Human MRP8/14 ELISA Kit (BioLegend, San Diego, CA, USA) were used.

Immunocytochemical detection of S100A8 and S100A9

FLSs were cultured onto glass coverslips, fixed with 4% paraformaldehyde, permeabilized and blocked with PBS/5% goat serum. Coverslips were incubated with primary antibody against S100A8 + S100A9 complex (Abcam, Cambridge, UK) or a control isotype. Cells were then incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA), counterstained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) and mounted with Prolong Gold (Invitrogen). Images were acquired with an Olympus DP72 microscope.

Cell-based ELISA for phospho-STAT3

For quantification of the phosphorylation of STAT3 (Y705) we used a cell-based ELISA kit (R&D Systems). FLSs (1 × 10⁴/well) were allowed to grow for 24 h and starved for 16 h. The cells were then stimulated with rhIL-22 for 10 min, fixed with 4% formaldehyde/PBS and stained according to the manufacturer’s recommendations.

FLS proliferation assay

FLSs (1 × 10⁴/0.2 mL/96 wells) were allowed to attach, were starved for 24 h and were stimulated with rhIL-22 for 48 h. A cell proliferation ELISA kit (Roche, Basel, Switzerland) was used.

IL-17RA and IL-17RC expression on FLSs by flow cytometry

FLSs were cultured for 24 h with or without rhIL-22 and detached by Gibco Cell Dissociation Buffer (Life Technologies, Paisley, UK). Cells were labelled with allophycocyanin-conjugated mouse monoclonal anti-human IL-17R (clone 133617) and Alexa Fluor 488 conjugated mouse monoclonal anti-human IL-17RC (clone 309822) antibodies (R&D Systems). FLSs were incubated with isotype-matched control antibodies. Cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using FCS Express v3 software.

Statistical analysis

Results are expressed as the mean ± SEM. The significance of the results was analysed using Student’s two-tailed t-test, Mann-Whitney U-test, and analysis of variance (ANOVA) using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). P-values < 0.05 were considered significant.

Results

Characterization of IL-22 and IL-22R1 expression in FLSs

IL-22 mRNA expression was detected by semi-quantitative real-time PCR in all the lines studied. We showed that IL-22 mRNA was expressed at similar levels in RA and OA FLSs (Fig. 1A). IL-22 protein was determined by western blotting in FLS cultures and no significant differences were observed between both pathologies (Fig. 1B). HeLa cell lysate was used as a positive control. We also tested the potential secretion of IL-22 by cultured FLSs using two different ELISA kits. The levels of IL-22 in supernatants obtained from FLSs without any stimulation or upon treatment with inflammatory mediators were below the detection limits of both kits (data not shown).

We next examined the constitutive expression of IL-22 receptor. Semi-quantitative real-time PCR analysis showed that IL-22R1 mRNA is expressed in both types of FLSs and higher levels of IL-22R1 mRNA were measured in OA FLSs (Fig. 1C). However, this difference was not confirmed at the protein level by western blotting, where we observed similar values (Fig. 1D). HepG2 cell lysate was used as a positive control.

Effect of proinflammatory cytokines and TLR ligands on IL-22 and IL-22R1 expression by FLSs

We next investigated whether TNF-α and TLR ligands, both described in the inflamed joint microenvironment, could affect the expression of IL-22 and its receptor. After TNF-α stimulation, a significant increase in both IL-22 and IL-22R1 transcripts was induced in FLSs (Fig. 1A and C), which was confirmed at the protein level (Fig. 1B and D).

We further studied the effect of TLR3 (poly I:C) and TLR4 (LPS) ligands. IL-22 mRNA expression was induced in response to poly I:C (Fig. 2A), whereas no effect was demonstrated at the protein level (Fig. 2B). A significant increase in IL-22R1 transcripts and protein was detected after poly I:C stimulation (Fig. 2C and D). LPS treatment induced a significant increase in both IL-22 and IL-22R1 mRNA and protein expression (Fig. 3).

Thus IL-22 and IL-22R1 were expressed constitutively in FLSs. The TNF-α, poly I:C and LPS-induced increase of IL-22 transcripts was only confirmed at the protein level after TNF-α and TLR4 engagement. Moreover, the presence of TNF-α, poly I:C and LPS induced the expression of IL-22R1 in cultured FLSs.
IL-22 involvement in joint destruction by FLSs
As proinflammatory and TLR ligands increased the potential of FLSs as IL-22 targets, we investigated the potential role of the IL-22/IL-22R1 axis in FLS activation by studying proliferation, MMP production and alarmin S100A8/A9 expression.

IL-22 effect on FLS proliferation
To explore the effect of IL-22 in FLS expansion, a proliferation assay was used to quantify bromodeoxyuridine (BrdU) uptake into newly synthesized DNA after IL-22 stimulation. IL-22 significantly enhanced FLS proliferation, especially in RA (Fig. 4A), suggesting its contribution to the synovium hyperplasia.

Effect of IL-22 on MMP1 and MMP3 production
The IL-22-induced up-regulation of MMP1 and MMP3 in human primary keratinocytes has been reported [7]. Also, MMP1 and MMP3 expression in FLSs has been correlated with joint destruction [20]. Therefore we measured MMP1 and MMP3 levels by ELISA in the FLS culture supernatants after IL-22 treatment. We showed that IL-22 induced a significant stimulation of MMP1 protein in both OA and RA FLSs (Fig. 4B), whereas no effect of IL-22 on MMP3 production was detected (data not shown).

Constitutive expression of alarmins S100A8 and S100A9 and effect of IL-22 stimulation
We studied the constitutive expression of S100A8 and S100A9. We demonstrated for the first time S100A8 and S100A9 mRNA expression in both OA and RA FLSs (Fig. 4C and D). Stimulation with IL-22 increased S100A8 mRNA expression with a similar intensity in both OA and RA FLSs (Fig. 4C). Regarding S100A9 transcripts, IL-22 did not increase its expression in OA FLSs, whereas in RA S100A9 mRNA expression was 2.6-fold higher than in the unstimulated condition (Fig. 4D).

Fig. 1 IL-22 and IL-22R1 constitutive expression in OA and RA FLSs and after TNF-α stimulation.

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Fig. 2 IL-22 and IL-22R1 expression in OA and RA FLSs after poly I:C stimulation.

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IL-22 (A) and IL-22R1 (C) mRNA expression was measured by semi-quantitative real-time PCR. Values are the mean (s.e.m.) from at least four FLS lines in triplicate, with basal OA as the referent. IL-22 (B) and IL-22R1 (D) proteins were measured by western blotting. Protein bands were densitometrically analysed and normalized against β-actin intensity. Each result is the mean (s.e.m.) of at least three experiments. Pictures are a representative example. Differences between OA basal and RA basal were significant; ###P < 0.001. Differences between basal and TNF-α-stimulated FLSs were significant; *P < 0.05 and ***P < 0.001.

IL-22 (A) and IL-22R1 (C) mRNA expression was measured by semi-quantitative real-time PCR. Values are the mean (s.e.m.) from at least four FLS lines measured in triplicate, with basal OA as the referent. IL-22 (B) and IL-22R1 (D) proteins were measured by western blotting. Protein bands were densitometrically analysed and normalized against β-actin intensity. Each result is the mean (s.e.m.) of at least three experiments. Pictures are a representative example. Differences between basal and poly I:C-stimulated FLSs were statistically significant; *P < 0.05, **P < 0.01 and ***P < 0.001.
Next we studied the intracellular protein expression of the complex S100A8/A9. Immunocytochemistry showed heterodimeric complex S100A8/A9 expression scattered in the cytoplasm of basal OA and RA FLSs (Fig. 4E, which was not observed in the controls (Fig. 4E, insert). To examine whether IL-22 induces S100A8/A9 secretion, supernatants from IL-22-stimulated FLSs were studied via ELISA for this complex. We detected alarmins in the supernatants of unstimulated FLSs. IL-22 induced its secretion both in OA and RA FLSs, approximately twice as often in OA as in RA FLSs (Fig. 5A).

IL-22 induced secretion in OA FLSs, whereas in RA FLSs, IL-22 did not significantly alter S100A8/A9 secretion. To confirm the involvement of the JAK/STAT pathway in IL-22-induced S100A8/A9 secretion, we tested the effect of using two JAK inhibitors, the JAK3 inhibitor CP-690,550 and the JAK2 inhibitor AG490. Neither of these inhibitors, when tested alone, significantly altered the basal levels of MMP1 or S100A8/A9 (data not shown). In contrast, expression of the S100A8/A9 complex in supernatants from IL-22-stimulated FLSs decreased significantly, near basal levels, after the activities of JAK2 and JAK3 were inhibited. These results suggest the involvement of the JAK/STAT signalling pathway in IL-22-induced S100A8/A9 production in FLSs (Fig. 5A).

Effect of IL-22 on IL-17 receptor expression and IL-23 production

The synovium in RA patients, and to a lesser degree in OA patients, is infiltrated by T lymphocytes. Among these, Th17 contributes to arthritis pathogenesis by secreting cytokines such as IL-17 and IL-22 [21]. IL-17A mediates its effects through binding to the receptor complex composed of IL-17RA and IL-17RC subunits, which are expressed in RA FLSs [22]. Also, IL-23 is a heterodimeric cytokine consisting of a unique IL-23p19 subunit paired with a common IL-12/23p40 subunit, which is involved in the maintenance and survival of Th17 cells [23].

Thus we examined the functional involvement of IL-22 in the contribution of FLSs to the IL-17-mediated response in arthritis. To this end, we studied the effect of IL-22 on IL-17RA and IL-17RC mRNA and protein expression and also its influence on the level of IL-23p19 transcripts and on IL-23 protein production. IL-17RA and IL-17RC were constitutively expressed in FLSs. IL-22 stimulation resulted in a significant reduction of IL-17RA transcripts in OA FLSs (Fig. 6A), whereas IL-17RC was down-regulated in RA FLSs (Fig. 6B). Flow cytometry analysis of IL-17RA- and IL-17RC-expressing FLSs confirmed the basal protein expression of both subunits (Fig. 6C and D). IL-17RA was significantly reduced on IL-22-stimulated OA FLSs [6.6% (0.78)] compared with the basal level [10.04% (0.79)], whereas there was no significant differences for IL-17RC expression (Fig. 6C and D and supplementary Table S2, available at Rheumatology Online). In RA FLSs, IL-17RC was significantly down-regulated after IL-22 treatment [10.68% (0.49)] and no differences were observed for IL-17RA expression (Fig. 6C and D and supplementary Table S2, available at Rheumatology Online). These results confirmed the receptor pattern observed at the mRNA level. Finally, we observed that IL-22 significantly decreased IL-23p19 mRNA expression as well as IL-23 protein levels only in RA FLSs (Fig. 6E and F).
Numerous questions concerning IL-22 in rheumatic diseases remain to be elucidated. Although the main source of this cytokine is assumed to be Th cells, other cells of the immune system have been described. In this sense IL-22 expression in synovial tissue and FLSs was previously found in RA patients [11]. Our findings confirm the presence of IL-22 in RA FLSs and demonstrate, for the first time, its constitutive expression in OA FLSs. We also showed that potential stimuli present in the rheumatoid joint, such as TNF-α and LPS, are able to induce IL-22 expression. Previous studies have shown that rheumatoid FLSs may acquire certain characteristics of immune cells, such as the production of inflammatory cytokines and the expression of disease-specific HLA-DR molecules [10]. Thus we propose that FLSs contribute to IL-22 occurrence in the joint microenvironment, with less relevance than that provided by Th cells. Nevertheless, its physiological relevance must be clarified.

Of interest, IL-22 target cells are restricted to non-immune cells that express IL-22R1. In synovial tissue and FLSs from RA patients a basal expression of IL-22R1 has been described [11]. We report herein the constitutive presence of IL-22R1 in both OA and RA FLSs, in agreement with a recent study that demonstrated IL-22R1 expression in FLSs from OA, RA and PsA [24]. Moreover, we noticed that TNF-α and the engagement of TLR3 and TLR4 increased IL-22R1 expression, supporting the involvement of innate immune mechanisms in the joint destruction that occurs in RA and OA [25, 26]. Our results are in agreement with previous studies that reported the TNF-α- and IFN-γ-induced up-regulation of IL-22R1 transcripts in keratinocytes and dermal fibroblasts [27, 28] as well as the LPS-induced expression of this receptor in the liver [29]. Thus TNF-α together with exogenous (bacterial products, cytomegalovirus) and endogenous ligands (hyaluronan and fibronectin fragments, nucleic acids of necrotic debris) of TLR3 and TLR4 could intensify the effect of IL-22 in the joint microenvironment of arthritic patients.

We reported the potential role of IL-22 in joint destruction by means of the increase in FLS proliferation, MMP1 and alarmins production. Alarmins S100A8 and S100A9 form both heterodimer and homodimer active complexes [30], considered mediators involved in regulation of the cytoskeleton, cell migration and inflammation. Recent studies using RA and OA mice models have shown that S100A8/A9 regulates joint inflammation and cartilage destruction [31, 32]. Also, S100A8 has been identified as an endogenous ligand of TLR4 that enhances osteoclastic

**Fig. 4** IL-22 effect on proliferation and MMP1 production. Constitutive expression of S100A8 and S100A9 and the effect of IL-22.

(A) FLS proliferation assessed by BrdU incorporation. Values are the mean (S.E.M.) from at least six lines in triplicate, with basal conditions as the referent. (B) MMP1 ELISA. Values are the mean (S.E.M.) from four OA and RA FLS lines in triplicate. (C) S100A8 and (D) S100A9 mRNA expression. Values are the mean (S.E.M.) from at least four FLS lines in triplicate, with basal OA as the referent. (E) Immunofluorescence staining in FLSs under basal conditions. Insert: control isotype. Differences between basal and IL-22-stimulated FLSs were significant; *P < 0.05, **P < 0.01 and ***P < 0.001.
S100A8 and S100A9 are also ligands of TLR4; it could be TLR4 that enhances the expression of IL-22/IL-22R1 and secretion by both OA and RA FLSs. As LPS is a ligand of also that IL-22 induced the S100A8/A9 heterocomplex secretion, demonstrating that IL-22 enhanced the gene expression of both alarmins and the presence of the heterodimer at protein level. Moreover, our findings express the S100A8 and S100A9 in FLSs, showing the mRNA expression of both alarmins in rheumatic diseases is beginning to be elucidated and future research could yield promising results for therapeutic targets.

In our study we observed that tyrosine phosphorylation of STAT3 was induced in FLSs after IL-22 treatment, as occurs in other cell types [3]. Therefore this pathway would be involved in previously described IL-22-mediated proliferation, given that STAT3 has been implicated in promoting cell survival and growth in many cell types [38]. Using the AG490 or CP-690,550 JAK inhibitors, we showed that interference of the JAK/STAT pathway significantly abrogated IL-22-induced S100A8/A9 production in FLSs. These results are in line with a recent report demonstrating JAK2/STAT3 signalling involvement in RANK ligand up-regulation by IL-22 in RA FLSs [39]. Moreover, our data with the JAK3 inhibitor CP-690,550, which has shown effectiveness in RA treatment, explain its beneficial effect [40].

Concerning the effects of IL-22 on IL-17-related responses in FLSs, we observed an inhibitory action on the IL-17R/IL-23 axis wherein IL-22 down-regulates IL-23 and IL-17RC expression in RA FLSs at both the mRNA and protein levels. Moreover, we reported an inhibition of IL-17RA in OA FLSs after IL-22 treatment. As it is well established that IL-23 is involved in the maintenance of Th17, these data could be interpreted as a negative loop of IL-22 in which, by reducing IL-23 production, it would contribute to hamper Th17 maintenance and survival, perhaps favouring the plasticity to Th1 previously described in advanced RA stages [41]. However, more experiments are needed to clarify the involvement of IL-22/IL-22R1 signalling in the IL-17R/IL-23 axis.

In conclusion, our data demonstrated that inflammatory mediators and TLR ligands present in the joint increased the expression of the IL22/IL-22R1 axis in FLSs, with subsequent amplification of joint inflammation and destruction through the enhancement of MMP1 and S100A8/A9 production. Also, we showed the IL-22 mediated regulation of proliferation and the decrease in IL-23 production as well as the down-regulation of IL-17RC in RA FLSs and IL-17RA in OA FLSs.

Overall, our results point to a proinflammatory action of IL-22. Given that FLSs were obtained from patients in the later stages of OA and RA evolution, our data support the hypothesis that the effect of IL-22 is likely dependent on the stage of synovial inflammation. Indeed, previous studies in RA report an IL-22-induced osteoclastogenesis in the later phases of the disease [39], and a recent work describes the contribution of IL-22 in the later phase of synovial inflammation [42].

Our results show that therapies based on the pharmacological disruption of IL-22 signalling could be beneficial for the treatment of rheumatic diseases. It is also of interest that the restricted expression of IL-22R1 to non-lymphoid cells could lead to a reduction in side effects mediated by immune responses.

Bone resorption through TLR4 activation [33]. In this regard, S100A8 and S100A9 exert a catabolic effect on human chondrocytes that is TLR4 dependent [34]. S100A8/A9 has also been described as a marker of disease activity and joint inflammation in RA, PsA and SpA [35]. Concerning the source of alarmins in the joint, the heterodimer has been described in synovial fluid neutrophils, synovial membrane macrophages [36] and chondrocytes [37] of RA patients. Our results revealed a new origin of S100A8 and S100A9 in FLSs, showing the mRNA expression of both alarmins and the presence of the heterodimer at protein level. Moreover, our findings demonstrated that IL-22 enhanced the gene expression of S100A8 in RA FLSs, as occur in keratinocytes [7], and also that IL-22 induced the S100A8/A9 heterocomplex secretion by both OA and RA FLSs. As LPS is a ligand of TLR4 that enhances the expression of IL-22/IL-22R1 and S100A8 and S100A9 are also ligands of TLR4, it could be suggested that the presence of a positive loop between IL-22 signalling and S100A8/A9 through TLR4 would perpetuate inflammation in the joint. Overall, the role of alarmins in rheumatic diseases is beginning to be elucidated and future research could yield promising results for therapeutic targets.
Inflammatory stimuli and TLR ligands increased the expression of IL-22/IL-22R1 in FLSs.

S100A8 and S100A9 alarmins are expressed in fibroblast-like synoviocytes and up-regulated by IL-22 treatment.

Our results point to a proinflammatory action of IL-22 in FLSs in the later stages of OA and RA evolution.

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**Supplementary data**

Supplementary data are available at Rheumatology Online.

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