

Osteoarthritis and Cartilage



IL4-10 fusion protein has chondroprotective, anti-inflammatory and potentially analgesic effects in the treatment of osteoarthritis

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SUMMARY

Objective: Effective disease-modifying drugs for osteoarthritis (DMOAD) should preferably have chondroprotective, anti-inflammatory, and analgesic activity combined in a single molecule. We developed a fusion protein of IL4 and IL10 (IL4-10 FP), in which the biological activity of both cytokines is preserved. The present study evaluates the chondroprotective, anti-inflammatory, and analgesic activity of IL4-10 FP in *in vitro* and *in vivo* models of osteoarthritis.

Methods: Human osteoarthritic cartilage tissue and synovial tissue were cultured with IL4-10 FP. Cartilage proteoglycan turnover and release of pro-inflammatory, catabolic, and pain mediators by cartilage and synovial tissue were measured. The analgesic effect of intra-articularly injected IL4-10 FP was evaluated in a canine model of osteoarthritis by force-plate analysis.

Results: IL4-10 FP increased synthesis ($P = 0.018$) and decreased release ($P = 0.018$) of proteoglycans by osteoarthritic cartilage. Release of pro-inflammatory IL6 and IL8 by cartilage and synovial tissue was reduced in the presence of IL4-10 FP (all $P < 0.05$). The release of MMP3 by osteoarthritic cartilage and synovial tissue was decreased ($P = 0.018$ and 0.028) whereas TIMP1 production was not significantly changed. Furthermore, IL4-10 FP protected cartilage against destructive properties of synovial tissue mediators shown by the increased cartilage proteoglycan synthesis ($P = 0.0235$) and reduced proteoglycan release ($P = 0.0163$). Finally, intra-articular injection of IL4-10 FP improved the deficient joint loading in dogs with experimentally induced osteoarthritis.

Conclusion: The results of current preliminary study suggest that IL4-10 FP has DMOAD potentials since it shows chondroprotective and anti-inflammatory effects *in vitro*, as well as potentially analgesic effect in a canine *in vivo* model of osteoarthritis.

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Introduction

Osteoarthritis (OA) is a highly prevalent chronic degenerative joint disease and a growing socioeconomic problem, imposing

enormous costs and challenges to healthcare. It is clinically characterized by pain and functional disability of the affected joints. Clinical management of OA is mainly symptomatic and includes non-pharmacological, pharmacological, and surgical approaches^{1,2}. Currently available treatments are inefficient to slow down OA progression, leaving a clear need for development of disease modifying therapy^{3,4}.

OA is characterized by structural changes that include the damage and the loss of articular cartilage, peri-articular bone changes, and synovial inflammation. Importantly, these three tissues are functionally interconnected and their interplay influences the pathogenesis of OA. These structural changes result from multiple anabolic and catabolic factors that act upon several joint

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tissues. For example, cartilage and synovial tissue release multiple mediators and breakdown products that mutually affect these tissues⁵; subchondral bone affects the (calcified) cartilage in OA through neurovascular invasion^{6,7}; synovial inflammation contributes to osteophyte formation⁸ and subchondral bone marrow lesions⁹. However, the exact sequence of pathological events in these tissues and their exact relation to pain remains elusive, and may even differ among distinct OA subtypes^{1,10,11}.

The ideal Disease-Modifying OA Drug (DMOAD) presumably should combine three basic pharmacological effects: relief of pain, reduction of synovial inflammation, and protection against, and repair of, cartilage damage. None of the existing pharmacological therapies for OA combines these effects; neither do the various novel therapeutic approaches of OA. In principle, approved and experimental novel pharmacological treatments of OA target only one of the disease components, such as mediators of cartilage tissue turnover (e.g., MMP inhibitors¹²), bone turnover (e.g., bisphosphonates and strontium ranelate¹³), synovial inflammation (e.g., the more recent attempts with methotrexate or anti-TNF¹⁴), or pain (like the debated anti-NGF studies^{15,16}). Therefore, there is still an unmet medical need for DMOAD.

Interleukin 4 (IL4) and in particular IL10, have shown impressive results in preclinical models for inflammatory disease^{17,18}. However, clinical evaluation of their anti-inflammatory potential in inflammatory conditions like rheumatoid arthritis (RA), inflammatory bowel disease, and psoriasis has been disappointing^{19–22}. These disappointing results may reflect poor bioavailability of the wild-type cytokines as well as their use as stand-alone drug. Various studies suggest a therapeutic potential of IL4 and IL10 in OA also: IL4 and IL10 receptors (IL4R, IL10R) are expressed on synovial tissue cells as well as on chondrocytes^{23–25}. IL4R signalling alters mechano-transduction in chondrocytes linked to matrix turnover in OA²⁶ and genetic variants of the IL4R gene increase susceptibility to OA²⁷. IL10 and IL4 inhibit chondrocyte apoptosis and cartilage breakdown^{28,29}. IL4 and IL10 also reduce synovial inflammation as they reverse TNF α -induced production of prostaglandin E₂ by OA synovial fibroblasts³⁰. Moreover, IL4 and IL10 have partially overlapping and complementary activities¹⁷. In fact, combined administration of both cytokines showed promising, synergistic effects in experimental models of arthritis^{31,32}. However, such a combination therapy of IL4 and IL10 has never been evaluated in clinical trials.

We have designed a IL4–10 fusion protein, *IL4–IL10 FP* (patent application No 61/556,843), which combines the activity of IL4 and IL10 in a single molecule. In several *in vitro* models IL4–10 FP inhibits the production of multiple pro-inflammatory cytokines while preserving the production of cytokine inhibitors and antagonists³³. Furthermore, recently published studies show the chondroprotective and analgesic activity of IL4–10 FP: the fusion protein limits blood induced cartilage damage *in vitro* and *in vivo* in haemophilic-arthropathy models³⁴ and it attenuates pain in mouse models of chronic inflammatory and neuropathic pain³⁵. So far, data in experimental models of OA is lacking. In the present study, we evaluated potential chondroprotective, anti-inflammatory, and analgesic effects of this novel molecule in various human *in vitro* models and in a canine *in vivo* model^{36–38} of OA.

Materials and methods

Production and purification of IL4–10 FP

Human IL4–10 FP was produced and purified according to the protocol published by Eijkelkamp *et al.*³⁵. In short, IL4–10 FP was produced by transient transfection of HEK293 cells (U-Protein Express BV, Utrecht, the Netherlands) with pUPE expression vector

carrying cDNA sequence coding the IL4–10 FP. To optimize glycan capping with sialic acid, cells were co-transfected with beta-galactoside-2,3-sialyl-transferase construct. IL4–10 FP was purified by cation exchange chromatography. Purified protein was stored in sterile PBS, pH 7.4, at -80°C until use.

Ex vivo immunohistochemistry

Cartilage samples obtained from OA knee joints ($n = 8$; 62 ± 5 years) and healthy shoulder joints ($n = 8$; 53 ± 4 years) were frozen in Tissue-Tec. In general, tissue with a Mankin grade 4–6 is collected representing full thickness clearly fibrillated cartilage³⁹. Cryo-sections ($6 \mu\text{m}$) were incubated with the primary antibody overnight at 4°C (mouse anti-human IL4R α , R&D systems or rabbit anti-human IL10R α , LifeSpan BioSciences) (no primary antibody for the negative control). Subsequently, sections were incubated for 30 min at room temperature with the secondary antibody (Pierce and Warriner Ready-to-use HRP-Anti-Mo/Ra/Rb-IgG, Immunologic). The tissue sections were counterstained with 0.1% kernechtrot (Sigma Aldrich) in 5% Al₂(SO₄)₃. The number of cells expressing IL4R or IL10R over the total number of cells at three different locations (superficial, middle, and deep zone) per section was microscopically determined. The percentage of positive cells for either IL4R or IL10R was calculated. A cell was regarded positive if a positively stained nucleus was identified in association with appropriate receptor staining.

In vitro cartilage cultures

Human OA cartilage tissue was harvested from patients ($n = 7$, 60 ± 4 years) undergoing total knee arthroplasty. Healthy human shoulder cartilage tissue as a control ($n = 7$ donors, 58 ± 13 years) was harvested *post-mortem*. Full-thickness cartilage pieces were cut aseptically and sliced into square pieces, weighted aseptically (range 5–15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round bottomed microtiter plates in 200 μl medium with or without addition of IL4–10 FP. Cartilage culture medium consisted of Dulbecco's modified Eagle's medium, supplemented with glutamine (2 mmol/l), penicillin (100 U/ml), and 10% heat inactivated pooled human male AB serum. IL4–10 FP was added at 20 ng/ml final concentration. The concentration of 20 ng/ml was chosen as optimum concentration based on previous dose response studies^{32,34}. Samples were cultured for 4 days at 37°C , 5% CO₂ in air. Proteoglycan synthesis rate and release as a measure of cartilage matrix turnover were determined. Part of the cartilage culture supernatants were collected and stored at -80°C for further analysis.

In vitro synovial tissue cultures

Synovial tissue was obtained from OA patients ($n = 6$, age 66 ± 11 years) undergoing total knee arthroplasty. Ten pieces of about 30 mg wet weight (range 20–40 mg, accuracy ± 0.1 mg) were cut aseptically and cultured individually in 24-wells flat bottom plates in culture medium with or without addition of 20 ng/ml IL4–10 FP. Synovial tissue culture medium consisted of RPMI Glutamax (Invitrogen, Life Technologies), supplemented with 10%, v/v, human AB serum and 1%, w/v, penicillin/streptomycin. Samples were cultured for 24 h at 37°C , 5% CO₂ in air. Culture supernatants were collected and stored in aliquots at -80°C for further analysis.

In vitro evaluation of cartilage destructive properties of OA synovial tissue

Human OA synovial tissue explants ($n = 8$ donors, age 67 ± 11 years) were cultured in presence or absence of IL4–10 FP (20 ng/ml)

for a fixed culture time (varying between 1 and 3 days between experiments). Culture supernatants were harvested and subsequently from each experiment added (25% v/v) to fresh healthy human cartilage cultures. After 4 days of culture proteoglycan turnover of the cartilage samples was analysed. For glycosaminoglycan (GAG) release, one outlier data point was removed as the value was above (Mean + 2 × SD).

Proteoglycan turnover

As measure of proteoglycan synthesis, the rate of sulphate incorporation was determined by use of $^{35}\text{SO}_4^{2-}$ pulse labelling of GAGs⁴⁰. After the 4-day culture period, newly formed GAGs were labelled for 4 h with $^{35}\text{SO}_4^{2-}$. Subsequently cartilage explants were washed with cold phosphate-buffered saline and digested for 2 h at 65°C with papain buffer. GAGs were precipitated with cetylpyridinium chloride (CPC) and the $^{35}\text{SO}_4^{2-}$ -labelled GAGs were measured by liquid scintillation analysis. Proteoglycan synthesis is expressed as nanomoles of sulphate incorporated per hour per gram wet weight of cartilage tissue (nmol/h g). To measure release of GAGs from the explants as a measure of proteoglycan release, GAGs in the culture medium were precipitated and stained with Alcian blue dye solution, as described previously³⁹. Alcian blue staining of the medium was quantified photometrically by the change in absorbance at 620 nm with chondroitin sulphate (Sigma C4384) as a reference. GAG release is expressed as mg GAGs/g wet weight of cartilage tissue released in 4 days (mg/g). To minimize the influence of biological (focal) variation between cartilage samples, at least eight cartilage specimens were randomly collected from each donor and tested individually for proteoglycan release and turnover. Results of these eight specimens were averaged, and the average value was used as the outcome of an individual donor. Values of different donors were used for statistical analysis.

Cytokine and other mediator assessments

Supernatants of cartilage and synovium cultures were analysed for matrix-metalloproteinase (MMP)1 and MMP3, tissue-inhibitor-of-metalloproteinases (TIMP1), and pain-related mediators nerve-growth-factor (NGF) and vascular-endothelial-growth-factor (VEGF) using multiplex assays⁴¹. Some MMP3 concentrations measured were above the highest concentration of the standard. As the reproducibility of the assay for these high concentrations is good (coefficient of variation <3% for the three highest concentrations of the standard curve), these data could still be used in the analyses. The cytokines IL6 and IL8 were measured by ELISA (CytoSet ELISA Kit's; Invitrogen), according to manufacturer's instructions. All values were corrected for tissue weight and culture volume.

In vivo canine Groove model

In an exploratory set-up ($n = 4$ only), analgesic effects of the IL4-10 FP were tested in an *in vivo* canine model of OA. Four skeletally mature mixed breed dogs (mongrels, females, 0.8 ± 0.1 years, 20.5 ± 0.7 kg; animal laboratory Utrecht University) were housed in cages (approximately $3 \times 3 \text{ m}^2$) in groups of two dogs (randomly divided), with at least 1 h a day on a patio (approximately $7 \times 6 \text{ m}^2$) to move freely. They were fed a standard diet and water *ad libitum*. Joint degeneration was induced in the right knee according to the Groove model^{36–38}. This model is characterized by its limited inflammatory component as compared to other models of OA. Ten longitudinal and diagonal grooves, depth 0.5 mm, were made on the weight-bearing parts of the femoral condyles, while preventing bleeding and soft tissue damage as much as possible to avoid induction of inflammation. After surgery, synovium, fasciae and skin

were sutured. The contralateral healthy knee served as a control. In order to obtain information on potential dose dependency, two doses of IL4-10 FP were tested – 1 µg and 10 µg per knee with a washout period of 2 weeks in-between. Five weeks after induction of joint damage, when surgery related pain is resolved and OA related pain presents, IL4-10 FP (1 µg in 1 ml) was injected intra-articularly in the OA joint. A second injection of IL4-10 FP (10 µg in 1 ml) was given in week 7. The contralateral healthy knee was injected with 1 ml PBS at both time points. Joint loading was measured as a surrogate measure of pain, using force plate analysis (FPA)⁴². Longitudinal changes in vertical stance ground reaction forces (GRFs) were evaluated for each leg by FPA (each dog served as its own control). A force-plate (FP), flush-mounted with the surface of an 11-m walkway, sampled (100 Hz) peak GRFs. Forces were normalized by body weight and time, and expressed in N/kg. A dedicated technician guided the dogs by leash over the FP, at a walking pace of constant speed ($1 \pm 0.2 \text{ m/s}$). A successful run consisted of sequential, distinct paw strikes of the right front and hind paw or the left front and hind paw, respectively. Ten valid runs were collected for each side of the dog and GRFs were averaged for each of the four legs. FPA was performed at several time points: data at baseline (pre-surgery) and subsequently prior and for 3 days after injection with IL4-10 FP, twice, starting at week 5 and week 7 are presented. The data is expressed as the ratio of OA/contralateral knee joint of each animal. For all procedures (surgery, intra-articular injections and pain measurements) dogs were treated in a random order. The experiment was approved by animal ethical committee of the University Utrecht (DEC 2011.III.12.123).

Statistical analysis

The expression of IL4R and IL10R (percentage of positive cells; per cartilage zone) in cartilage of OA patients and healthy controls was compared using the Mann–Whitney *U* test (as percentages could not be assumed normally distributed). Comparison of proteoglycan synthesis rate and GAG release rate in cartilage after exposure to medium with/without IL4-10 FP and comparison of release of inflammatory, catabolic and pain-related mediators by OA cartilage after exposure to medium with/without IL4-10 FP was tested using the Wilcoxon signed rank test. To evaluate the effect of synovial culture supernatants (with/without IL4-10 FP) on proteoglycan synthesis and GAG release in cartilage, correcting for baseline levels of proteoglycan synthesis and GAG release we used a mixed effects analysis because the synovium of eight donors was tested on cartilage of two other donors. We have corrected for cartilage donor (and thus baseline level of proteoglycan synthesis and GAG release) in a mixed effects model analysis with condition (synovial culture supernatant vs synovial culture supernatant + IL4-10 FP) and cartilage donor as fixed effects and a random intercept at the level of synovium donor to account for the assessments within patients. The effect of intra-articular injections of IL4-10 FP on pain was analysed by paired *t* test (data from FPA measurements are generally normally distributed). For each animal, the post-treatment values were compared to baseline values. For data analysis SPSS statistical software (SPSS Statistics 21, IL, USA) and SAS version 9.4 were used. (Semi)continuous variables were described with mean (SD) or median with interquartile range (IQR: Q1–Q3) depending on the distribution of the data.

Results

The expression of IL4R and IL10R is higher in human knee OA cartilage compared to healthy human shoulder cartilage

The number of chondrocytes expressing IL4R and IL10R was higher in OA cartilage compared to healthy cartilage. The median

percentage of IL4R positive cells in superficial, middle and deep zone of OA cartilage was 67 (IQR: 59–73)%, 75 (IQR: 62–80)%, and 66 (IQR: 60–78)%, respectively. This was significantly higher as compared to 40 (IQR: 35–47)%, 36 (IQR: 30–44)%, and 50 (IQR: 42–57)% positive cells in healthy cartilage ($P = 0.0006$, $P = 0.0003$ and $P = 0.038$, respectively) [Fig. 1(A)]. The median percentage of IL10R positive chondrocytes was significantly higher in the superficial and middle zone of OA cartilage with 69 (IQR: 66–73)% and 73 (IQR: 60–80)% positive cells in each zone compared to 49 (IQR: 34–62)% positive cells in superficial and 53 (IQR: 35–67)% in middle zone of healthy cartilage ($P = 0.021$ and $P = 0.038$, respectively). In the deep zone difference was less pronounced with 68 (IQR: 61–72)% positive cells in OA vs 61 (IQR: 45–76)% positive cells in healthy cartilage ($P = 0.959$) [Fig. 1(B)].

IL4-10 FP beneficially affects proteoglycan turnover in OA cartilage *in vitro*

Proteoglycan synthesis rate of OA cartilage tissue explants was increased when cultured with IL4-10 FP. The median increase of 28 (IQR: 21–60)% by IL4-10 FP was statistically significant compared to control culture ($P = 0.018$) [Fig. 2(A)]. IL4-10 FP had reduced the proteoglycan release measured by release of GAGs by 6 (IQR: –17–(–5))% compared to control ($P = 0.018$) [Fig. 2(B)]. In the case of healthy cartilage tissue explants no significant effect of IL4-10 FP on proteoglycan turnover was seen *in vitro* (Fig. 2(A), $P = 0.866$ for proteoglycan synthesis and Fig. 2(B), $P = 0.799$ for GAG release).

IL4-10 FP reduces release of inflammatory, catabolic and pain-related mediators from human OA cartilage and synovial tissue *in vitro*

In the presence of IL4-10 FP, release of the inflammatory cytokines IL6 and IL8 by OA cartilage explants was reduced by 94 (IQR: –99–(–66))% and 79 (IQR: –92–(–59))%, respectively (both $P = 0.018$) compared to controls [Fig. 3(A)]. The release of MMP3, a protease involved in degradation of extracellular matrix proteins, was significantly reduced ($P = 0.018$) while release of MMP1, also involved in degradation of extracellular matrix, tended to increase but was not statistically significantly changed ($P = 0.091$). The release of TIMP1, an inhibitor of MMPs, by OA cartilage tissue was not significantly changed by IL4-10 FP ($P = 0.176$; Fig. 3(B)).

IL4-10 FP also influenced the release of different mediators by human OA synovial tissue. The amount of inflammatory cytokines IL6 and IL8 released into the culture medium was significantly reduced (–77 (IQR: –81–(–70))%, $P = 0.028$ and –80 (IQR: –93–(–71))%, $P = 0.028$, respectively) compared to control [Fig. 3(A)]. Moreover, the release of MMP1 and MMP3 by OA synovial tissue was reduced by 48 (IQR: –74–(–46))% ($P = 0.028$) and 79 (IQR: –94–(–66))% ($P = 0.028$), respectively. The release of TIMP1 was not significantly changed ($P = 0.249$; Fig. 3(B)). No significant effect of IL4-10 FP was seen on IL1RA or TNFR1I release by synovial tissue (data not shown). The absence of the effect on IL1RA is intriguing, however similar to the effect observed in LPS stimulated whole blood culture where IL4-10 FP only minimally altered IL-1RA concentrations while strongly reducing IL1beta levels (data published elsewhere).

The release of VEGF and NGF, pain-related mediators associated with neurovascular invasion, by OA synovial tissue samples was significantly inhibited by IL4-10 FP *in vitro* as well. The release of VEGF was decreased by 48 (IQR: –67–(–22))% ($P = 0.028$) compared to control culture. NGF was measurable in five out of six donors and in those was significantly reduced in the presence of IL4-10 (–61%; $P = 0.031$, data not shown). The decrease in release of VEGF from cartilage tissue explants was also observed although it was less pronounced (–7 (IQR: –19–2)%; $P = 0.310$) [Fig. 3(A)]. NGF was not detectable in OA cartilage culture supernatants.

IL4-10 FP reduces cartilage destructive properties of OA synovial tissue *in vitro*

Exposure of healthy cartilage explants to culture supernatant of OA synovial tissue reduced proteoglycan synthesis rate by 60 (IQR: –72–(–14))% and increased the GAG release by 6 (IQR: –11–25)% compared to control (healthy cartilage cultured in medium only) [Fig. 4(B) and (C) respectively]. In contrast, medium from synovial tissue (25% v/v) cultured with IL4-10 FP when added to healthy cartilage (not susceptible to IL4-10 FP at a fourfold concentration itself) did not affect proteoglycan turnover of the cartilage. The proteoglycan synthesis rate was comparable to control and significantly higher than that of cartilage samples incubated with medium from synovial tissue cultured without IL4-10 FP ($P = 0.0235$, Fig. 4(B)). Similarly, GAG release by healthy cartilage explants exposed to medium from synovial tissue cultured with IL4-10 FP was 22% significantly lower compared to those exposed to

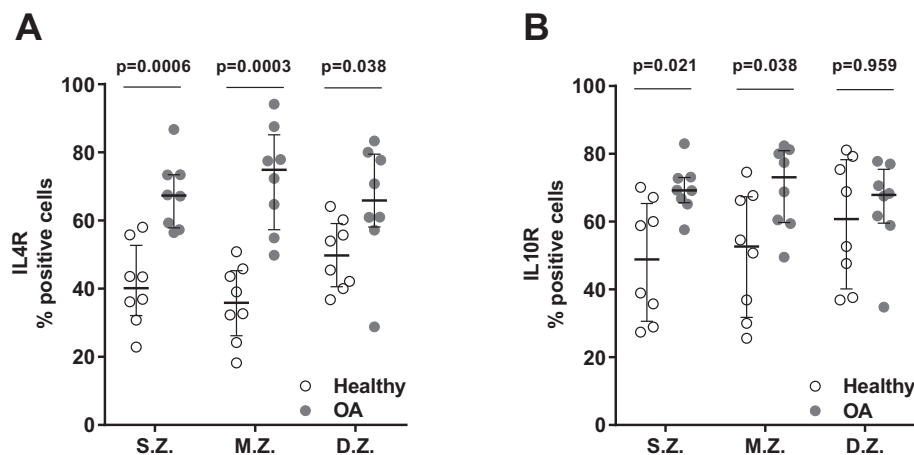


Fig. 1. Expression of IL4 receptor (IL4R) (A), and IL10 receptor (IL10R) (B) on chondrocytes in healthy shoulder and Osteoarthritis (OA) knee cartilage. Data are expressed as percentage positive cells in the superficial (S.Z.), middle (M.Z.) and deep zone (D.Z.) of the cartilage. Median values with interquartile range are shown for healthy cartilage ($n = 8$) and OA cartilage ($n = 8$).

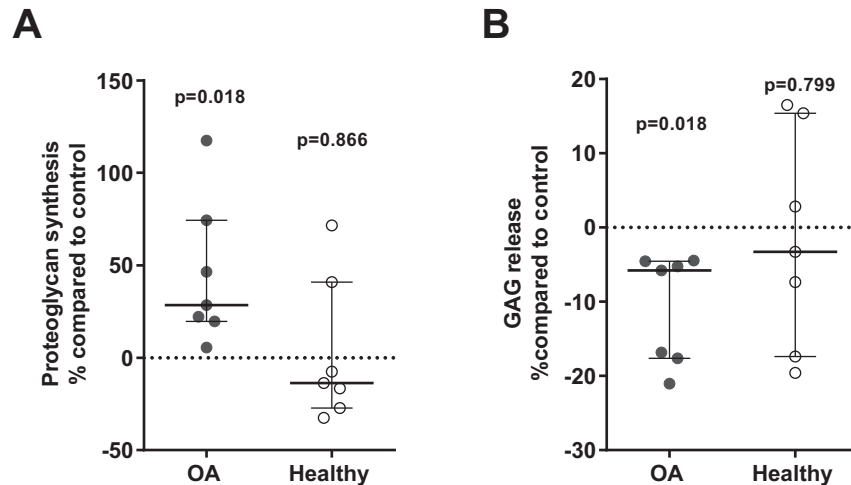


Fig. 2. The effect of IL4-10 fusion protein (IL4-10 FP) on proteoglycan synthesis rate (A) and glycosaminoglycan (GAG) release (B) in Osteoarthritis (OA) and healthy cartilage. Healthy and OA cartilage pieces were cultured individually in the medium with or without addition of IL4-10 FP. After 4 days of culture, proteoglycan synthesis rate and release as a measure of cartilage matrix turnover were determined. Data are expressed as percentage change compared to control cultures (dotted lines, proteoglycan synthesis rate of control cultures was on average 2.1 nmol/h g wet weight of tissue; GAG release of control cultures was on average 5.5 mg/g wet weight of tissue released in 4 days). Median values with interquartile range ($n = 7$ for OA cartilage, $n = 7$ for healthy cartilage) are shown.

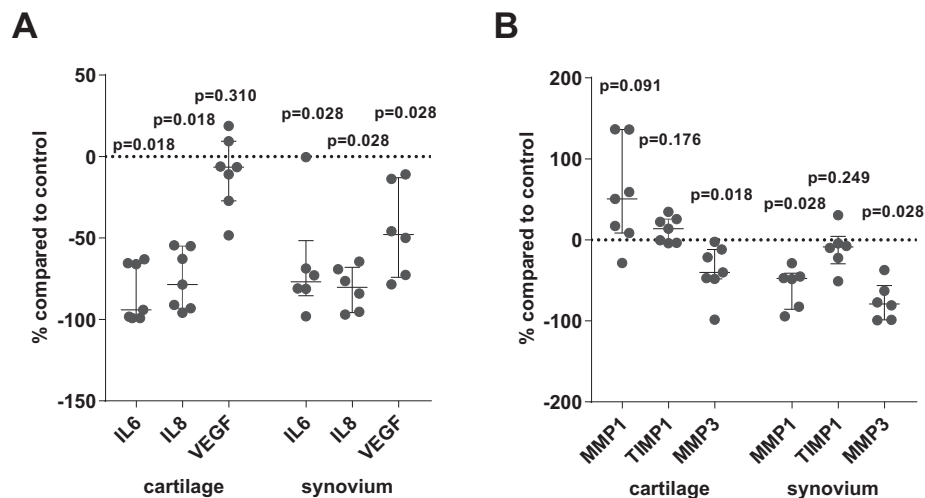


Fig. 3. IL4-10 fusion protein (IL4-10 FP) influences the release of pro-inflammatory (A) and catabolic (B) mediators from Osteoarthritis (OA) cartilage and synovial tissue *in vitro*. All parameters were measured in culture supernatants of cartilage and synovial tissue samples without IL4-10 FP addition (control cultures) or with addition of IL4-10 FP. Data are expressed as percentage change compared to control cultures. Median values with interquartile range ($n = 7$ for cartilage, $n = 6$ for synovial tissue) are shown. Control cultures median values of the mediators released from cartilage: IL6 – 217.3 pg/ml, IL8 – 131.9 pg/ml, vascular-endothelial-growth-factor (VEGF) – 202.5 pg/ml, matrix-metalloproteinase 1 (MMP1) – 6.0 ng/ml, tissue-inhibitor-of-metalloproteinase 1 (TIMP1) – 23.1 ng/ml, matrix-metalloproteinase 3 (MMP3) – 143.6 ng/ml. Median baseline values of mediators released from synovial tissue: IL6 – 5.1 ng/ml, IL8 – 8.9 ng/ml, VEGF – 759.5 pg/ml, MMP1 – 62.1 ng/ml, TIMP1 – 22.7 ng/ml, MMP3 – 221.0 ng/ml.

conditioned medium without IL4-10 FP (-16 (IQR: -22 – (-7))% and 6 (IQR: -11 – 25)% respectively, $P = 0.0163$, Fig. 4(C)).

IL4-10 FP has potential to reduce OA pain *in vivo*

The analgesic effect of IL4-10 FP was measured in the canine Groove model of OA where significant decrease in stance force, a surrogate measure of pain, continues for at least 12 weeks after OA induction⁴². Also in the present experiment, stance force decreased after induction of OA (ratio OA/control knee joint) (week 5: $88 \pm 8\%$ of baseline, $P = 0.054$), indicating pain of the affected joint (Fig. 5). Upon intra-articular injection of $1 \mu\text{g}$ of IL4-10 FP, stance force returned towards baseline in the experimental joint reaching the maximum effect at 3 days after the injection ($93 \pm 7\%$ of baseline). At 2 and 3 days after injection stance force of

the affected joint was not significantly different from baseline anymore ($P = 0.051$ and $P = 0.118$ respectively). Before the second injection unloading had increased again (increase in pain), demonstrated by a statistical significant level compared to baseline (week 7: $89 \pm 6\%$ of baseline; $P = 0.037$), demonstrating the transient effect of the injection. The analgesic effect of IL4-10 FP could be repeated upon a second injection, with a 10 fold concentration resulting again in an increase in joint loading (decrease in pain) as compared to pre-treatment values, increasing up to $93 \pm 5\%$ already at the first day after injection. At 1 and 2 days after injection stance force of the affected joint was not statistically significant different from baseline anymore. At day 3 after injection stance force of the affected joint decreased again demonstrating the transient effect of the injection ($P = 0.009$ compared to pre-OA baseline conditions).

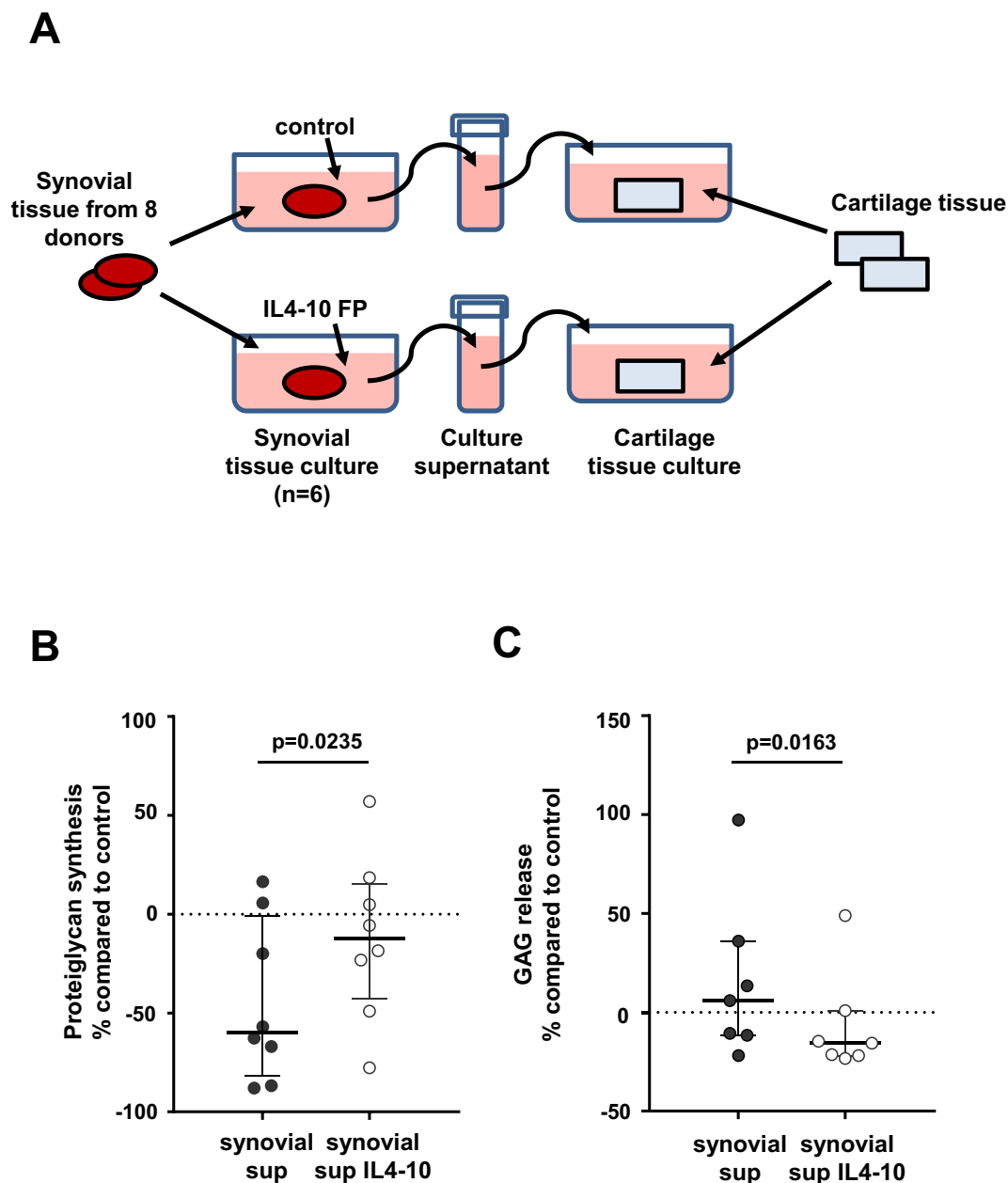


Fig. 4. IL4-10 fusion protein (IL4-10 FP) reduces cartilage destructive properties of Osteoarthritis (OA) synovial tissue *in vitro*. Schematic representation of the experimental design: For each synovial tissue donor, synovial tissue was cultured without (control) and with IL4-10 FP for a fixed time period. Subsequent, culture supernatants were harvested and tested on healthy cartilage tissue (A). The effect of synovial tissue culture supernatant (with and without IL4-10 FP) on proteoglycan synthesis rate (B) and glycosaminoglycan (GAG) release by healthy cartilage samples (C). Healthy human cartilage tissue cultured in culture medium only was used as control (dotted lines). Data are expressed as percentage change compared to control cultures (dotted lines). Median values with interquartile range are shown. PG synthesis ($n = 8$), GAG release ($n = 7$).

Discussion

IL4 and IL10, as stand-alone or combination therapy, showed chondroprotective effects in models of inflammatory joint disease like RA^{17,32} and more degenerative joint disease like haemophilic arthropathy^{25,43}. Moreover, these cytokines modulate inflammation, and contribute to remodelling of articular cartilage extracellular matrix^{44,45}. Cytokines in essence exert their effects in a network of other cytokines, which probably explains why combination therapy of IL4 and IL10 is more efficient than stand-alone treatment in diseases like arthritis. For that reason, we have designed a fusion protein of IL4 and IL10 (IL4-10 FP), which can be developed as a single biologic with combined activity of two

cytokines. In the present study we evaluated IL4-10 FP as a potential Disease-Modifying Drug for the treatment of OA (DMOAD). We show that IL4-10 FP combines chondroprotective, anti-inflammatory, and analgesic effects in human OA tissue *in vitro* and in canine OA *in vivo*.

Remodelling of articular cartilage extracellular matrix by IL4-10 FP requires the presence of receptors for either cytokine on chondrocytes. Chondrocytes produce IL4 and IL10 and the receptors for both cytokines are expressed on their cellular surface^{24,46}. Several studies support a role of IL4 and IL10 in OA. For example, reduced IL4 expression in OA cartilage is associated with decreased overall anti-catabolic activity in OA cartilage⁴⁷. Secretion of IL10 by T regulatory cells isolated from OA patients is reduced compared to non-OA

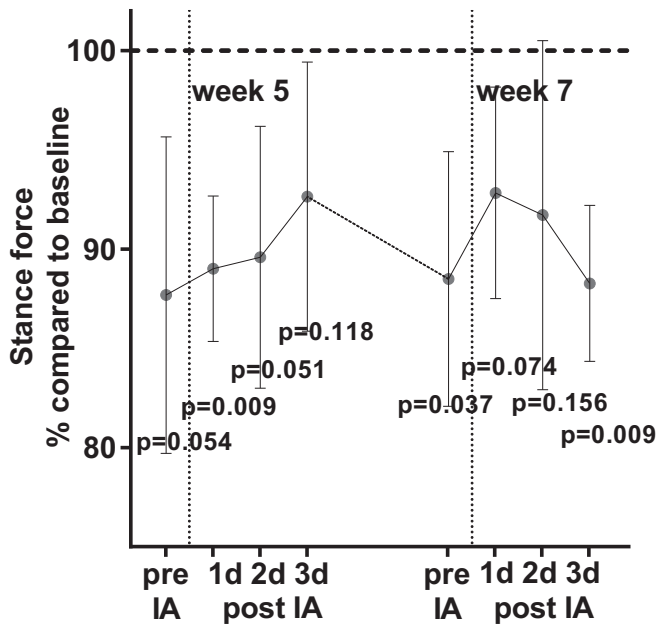


Fig. 5. The effect of intra-articular (IA) injection of IL4-10 fusion protein (IL4-10 FP) on stance force in the canine Groove model for Osteoarthritis (OA). These exploratory data are expressed as percentage change in ratio of osteoarthritic over contralateral control knee joint compared to baseline ratio (before OA induction, horizontal dashed line). IL4-10 FP was injected at week 5 and week 7 after OA induction, indicated with vertical dotted lines. Mean values \pm standard deviation are shown, $n = 4$. IA – intra-articular, d – days.

controls⁴⁸ while the production of IL10 by OA chondrocytes is not changed. We here show that expression of both IL4 and IL10-receptors is higher in knee OA cartilage compared to healthy shoulder cartilage. This extends findings on enhanced gene expression of both receptors in models of blood induced cartilage damage where an increase in expression of IL4R and IL10R was induced in healthy shoulder cartilage *in vitro*²⁵. Although source differences for receptor expression cannot be ruled out, this observation supports that damage of articular cartilage is accompanied by increased expression of IL4 and IL10-receptors. The mechanism causing increased expression remains elusive. As up-regulation of the receptors was predominantly found on chondrocytes in the superficial layer, mediators from the synovial fluid are likely involved. We also show in our study that the fusion protein composed of IL4 and IL10 beneficially affects proteoglycan turnover in human OA cartilage *in vitro* while proteoglycan turnover in healthy cartilage was not affected. The differential effect on OA vs healthy cartilage reflects the increased receptor expression by OA cartilage.

Even though OA is not predominantly driven by inflammation, it can be accompanied by synovial inflammation in subsets of OA patients. IL4-10 FP not only has direct beneficial effect on cartilage proteoglycan turnover, but also silences synovial inflammation. The anti-inflammatory properties of IL4 and IL10 have extensively been discussed in literature and studies have shown their additive and/or synergistic effect when applied in combination. Here we show that IL4-10 FP significantly decreases release of inflammatory markers from human OA synovial and cartilage tissue *in vitro*. Additionally, cartilage destructive properties of OA synovial tissue were inhibited when synovium was pre-treated with IL4-10 FP *in vitro*, suggesting that IL4-10 FP has also indirect chondroprotective effects.

Pain is a dominant clinical feature of OA. Therefore, a DMOAD should have analgesic activity in addition to chondroprotective and anti-inflammatory activity. OA related pain is not well understood.

In general, pain perception in OA does not reflect the extent of cartilage damage. Only severely damaged cartilage in OA becomes innervated at the bone cartilage interface^{6,7}. Hence, synovial inflammation and bone damage more likely are involved in pain sensation. Here, we show that in dogs with induced OA, intra-articular injection of human IL4-10 FP increases joint loading. Although the effect with a higher dose was reached quicker, based on only two injections in this limited number of animals it can only be concluded that the fusion protein has analgesic properties. To determine degree and time dependency of the effect, further studies are needed. IL4-10 FP reduces synovial inflammation, which is known to contribute to increased responsiveness of peripheral nociceptive neurons and therefore increased pain sensitivity in OA^{49,50}. As such, the analgesic effect may (in part) be dependent on its anti-inflammatory effect. Additionally, the analgesic effects of intra-articular IL4-10 FP may result from its inhibitory effects on the release of pain promoting mediators VEGF and NGF by OA synovial tissue, as was observed in the human tissues. Such, and other, mechanisms may explain the analgesic effect of IL4-10 FP in the Groove model.

Local application of IL4-10 FP via intra-articular injection is a treatment option for larger joints affected by OA as it provides high concentration of IL4-10 FP in treated joints with limited or no systemic exposure. Although in the present study the analgesic effect with its maximum between 1 and 3 days seems to lag behind an anticipated bioavailability of a few hours^{51,52} effects are relatively short and demand repeated injections over time. We are currently investigating various approaches to prolong bioavailability of IL4-10 FP in the joint cavity, including administration via controlled release systems.

In summary, the results of this preliminary study show that IL4-10 has DMOAD potentials since it shows cartilage protective, and anti-inflammatory effects *in vitro*, as well as potentially analgesic activity in a canine OA model. This combined activity in a single molecule has to our knowledge never been described before. Further studies on its therapeutic potential in osteoarthritis seem justified and needed.

Contributors

All authors provided substantial contributions to conception and design, or analysis and interpretation of data and drafting the article or revising it critically for important intellectual content and all gave final approval of the version to be published.

Competing interests

None.

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Patient consent

The University Medical Utrecht medical ethical committee approved the use of human post-mortem materials and of residual tissue upon joint replacement surgery upon informed consent which was obtained.

Ethics approval

The Utrecht University Committee for Experiments on Animals approved the study according to Dutch law (DEC nr: 2011.III.12.123).

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