

RESEARCH ARTICLE

Canine IL4-10 fusion protein provides disease modifying activity in a canine model of OA; an exploratory study

E. M. van Helvoort^{1*}, J. Popov-Celeketic¹, N. Eijkelkamp^{2,3}, K. Coeleveld¹, M. A. Tryfonidou⁴, C. D. Wijne¹, C. E. Hack², F. P. J. G. Lafeber¹, S. C. Mastbergen¹

1 Department of Rheumatology & Clinical Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, **2** Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, **3** Laboratory of Neuroimmunology and Developmental Origins of Disease, University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, **4** Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

* E.M.vanHelvoort-3@umcutrecht.nl



OPEN ACCESS

Citation: van Helvoort EM, Popov-Celeketic J, Eijkelkamp N, Coeleveld K, Tryfonidou MA, Wijne CD, et al. (2019) Canine IL4-10 fusion protein provides disease modifying activity in a canine model of OA; an exploratory study. PLoS ONE 14 (7): e0219587. <https://doi.org/10.1371/journal.pone.0219587>

Editor: Chuen-Mao Yang, Chang Gung University, TAIWAN

Received: February 5, 2019

Accepted: June 27, 2019

Published: July 11, 2019

Copyright: © 2019 van Helvoort et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: The study was funded by the Dutch Arthritis Society project NR12-2-202 and LLP9. MT is supported by funding from the Dutch Arthritis Society (LLP22).

Competing interests: FL and CEH are inventors on the patent WO2013070076 owned by UMC Utrecht Holdings. This does not alter our adherence to

Abstract

Objective

An ideal disease modifying osteoarthritis drug (DMOAD) has chondroprotective, anti-inflammatory, and analgesic effects. This study describes the production and characterization of a canine IL4-10 fusion protein (IL4-10 FP) and evaluates its *in vivo* DMOAD activity in a canine model of osteoarthritis (OA).

Design

The canine Groove model was used as an *in vivo* model of degenerative knee OA. Six weeks after OA induction dogs were intra-articularly injected weekly, for ten weeks, with either IL4-10 FP or phosphate buffered saline (PBS). In addition to the use of human IL4-10 FP, canine IL4-10 FP was developed and characterized *in vitro*, and tested *in vivo*. Force plate analysis (FPA) was performed to analyze joint loading as a proxy measure for pain. After ten weeks dogs were euthanized and cartilage and synovial tissue samples were analyzed by histochemistry (OARSI scores) and biochemistry (cartilage proteoglycan turnover).

Results

Repetitive intra-articular injections with human IL4-10 FP led to antibody formation, that blocked its functional activity. Therefore, a canine IL4-10 FP was developed, which completely inhibited LPS-induced TNF α production by canine blood cells, and increased proteoglycan synthesis of canine cartilage *in vitro* ($p = 0.043$). *In vivo*, canine IL4-10 FP restored the, by OA impaired, joint loading ($p = 0.002$) and increased cartilage proteoglycan content ($p = 0.029$).

PLOS ONE policies on sharing data and materials. JP, NE, and CEH are shareholders of Synerkine Pharma BV, a spin-off of the UMC Utrecht licensing the patents on the IL4-10 fusion protein.

Conclusions

This first study on the potential DMOAD activity upon prolonged repeated treatment with IL4-10 FP demonstrates that a species-specific variant has anti-inflammatory and chondroprotective effects *in vitro* and chondroprotective and analgesic effects *in vivo*. These data warrant further research on the DMOAD potential of the IL4-10 FP.

Introduction

Osteoarthritis (OA) is characterized by changes in all (peri-)articular tissues, such as cartilage, (subchondral) bone, synovial tissue, ligaments, and muscles[1, 2]. Currently, OA treatment is mostly symptomatic and mainly focused on pain reduction. If conservative treatments (e.g. physical therapy), analgesic drugs (e.g. acetaminophen), and/or anti-inflammatory drugs (e.g. non-steroidal anti-inflammatory drugs) fail, limited joint preserving surgical options are available (e.g. joint distraction), and finally a total joint replacement becomes inevitable[2]. Therefore, there is still a great need for joint preserving conservative drug treatments. Such treatments should ideally be chondroprotective (regenerative), anti-inflammatory, and analgesic, and should be preferably combined in one drug. At present such treatments do not exist, although multiple drugs are studied as potential disease modifying OA drug (DMOAD). Intra-articular (i.a.) administration of fibroblast growth factor-18 (FGF18) has demonstrated chondroprotective effects in a bovine *in vitro* model[3], in an *in vivo* rat model[4] and in a randomized, double-blind, placebo-controlled trial, although pain relief was not achieved[5, 6].

Transforming growth factor-beta1 (TGF- β 1) producing human chondrocytes were able to generate cartilage with hyaline-like characteristics in two different animal models[7] and demonstrated clinical benefits in multiple clinical trials[8–12] with small, not statistically significant but encouraging chondroprotective activity[8, 12, 13], and anti-inflammatory activity [13]. SM04690, a Wnt pathway inhibitor, induced chondrocyte differentiation, reduced cartilage catabolism *in vitro*, and showed chondroprotective activity in a rat model of OA[14], and in humans[15]. None of these studies demonstrated chondroprotective, anti-inflammatory, and analgesic effects in combination.

Interleukin 4 (IL4) and IL10, two anti-inflammatory cytokines, are suggested to have therapeutic potential in OA: synovial cells and chondrocytes express IL4 and IL10 receptors (IL4R, IL10R)[16–18]. IL4R signaling changes mechano-transduction in chondrocytes linked to matrix turnover in OA[19] and variants in the IL4R alpha gene are associated with susceptibility to osteoarthritis[20]. Besides, IL10 and IL4 inhibit chondrocyte apoptosis and cartilage breakdown[21, 22] and reduce synovial inflammation by reversing prostaglandin E₂ production by OA synovial fibroblasts[23].

IL4 and IL10 do have overlapping and complementary activities[24] and combined administration showed promising effects in experimental models of arthritis[25, 26]. Recently, we developed a human fusion protein of IL4 and IL10 (hIL4-10 FP)[27] that has DMOAD properties in multiple models of osteoarthritis[28]. Human OA cartilage tissue explants were demonstrated to express elevated levels of IL4R and IL10R, rendering OA cartilage more sensitive to the IL4-10 FP.

Moreover, two i.a. injections of IL4-10 FP in dogs with experimental OA were analgesic and *in vitro* IL4-10 provided chondroprotective and anti-inflammatory activity in human cartilage and synovial tissue explants. These findings warranted further *in vivo* evaluation on the potential disease modifying properties of this novel IL4-10 fusion molecule. As such we

designed an *in vivo* study using the canine Groove model of osteoarthritis to study the chondroprotective, anti-inflammatory, and analgesic activity of the IL4-10 FP upon repeated i.a. injections.

Material and methods

Production and characterization of IL4-10 FP

hIL4-10 FP was produced as previously published[29]. Canine IL4-10 FP (cIL4-10 FP) was produced by transient transfection of HEK293F cells with pcDNA3.1-neo expression vector (Invitrogen; Carlsbad, CA) with dual CMV promotor. The vector contained two transgenes: cDNA coding for cIL4-10 FP and cDNA coding beta-galactoside-2, 3-sialyl-transferase to optimize glycan capping with sialic acid. To enable purification, a hexa-histidine affinity tag was cloned at the N-terminus of cIL4-10 FP. Cells were cultured in GIBCO FreeStyle 293 Expression Medium (Invitrogen; Carlsbad, CA). Cells were subcultured three to four times prior to transfection and transfected with 293fectin (Invitrogen; Carlsbad, CA) at the cell viability of at least 90%.

The cIL4-10 FP was purified using Ni-NTA agarose (Qiagen; Hilden) according to manufacturer protocol. Purified canine protein was dialyzed overnight against 2L of phosphate buffered saline (PBS; pH = 7.4), sterile filtered and stored at -80°C. Purity of cIL4-10 FP batches was evaluated by Coomassie-stained 12% SDS-PAGE gel and HP-SEC analysis.

For SDS-PAGE samples were diluted 1:1 in 2x Leammli Sample Buffer (Bio-Rad; Hercules, CA) containing 100mM dithiothreitol (Sigma-Aldrich; Saint Louis, MO), incubated ten minutes at 95°C, and loaded on a 12% polyacrylamide gel (Mini-PROTEAN-TGX; Bio-Rad; Hercules, CA). Electrophoresis was performed at 150V for 1,5 hours, under reducing conditions (Tris/glycine/SDS buffer; Bio-Rad; Hercules, CA). Protein bands were visualized by InstantBlue protein stain (Expedeon; Cambridge).

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-Blot Turbo system, Bio-Rad; Hercules, CA). Membranes were blocked in 5% milk (Elk; Campina; Zaltbommel) in PBST (phosphate buffer saline with 0.1% Tween-20, Merck; Darmstadt) and incubated overnight with a monoclonal mouse anti-canine IL4 or monoclonal mouse anti-canine IL10 (R&D systems; Minneapolis, MN) in PBST containing 1% milk. Membranes were subsequently incubated with goat anti-mouse IgG-HRP (Santa Cruz Biotechnology; Dallas, TX) for one hour at room temperature. To visualize the bands ECL Western Blotting Substrate was added according to manufacturer protocol (Pierce, Thermo Fisher Scientific; Waltham, MA).

Bioactivity check of cIL4-10 FP *in vitro*

To assess biological activity of the cIL4-10 FP two *in vitro* experiments were performed, before *in vivo* testing. To evaluate anti-inflammatory activity of cIL4-10 FP, heparinized canine blood was diluted 1:10 in RPMI1640 medium (Gibco, Thermo Fisher Scientific; Waltham, MA) supplemented with 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific; Waltham, MA), and incubated with lipopolysaccharide (LPS; Sigma-Aldrich; Saint Louis, MO) at 100ng/ml and cIL4-10 FP, recombinant canine IL4 (R&D systems; Minneapolis, MN) or recombinant canine IL10 (R&D systems; Minneapolis, MN), both as controls, at 0.001-3nM for 18 hours at 37°C, 5% CO₂. Culture supernatants were then assayed for canine Tumor Necrosis Factor alpha (TNFα; R&D systems; Minneapolis, MN) using Enzyme linked immuno sorbet assay (ELISA). The inhibition of TNFα production (by cIL4-10 FP, cIL4 or cIL10) was calculated according to the formula: Inhibition (%) = $(1 - (A - B) / (C - B)) \times 100$, where A = TNFα levels in

LPS-stimulated cultures treated with cIL4-10 FP, cIL4 or cIL10; B = TNF α levels in unstimulated culture; and C = TNF α levels in LPS-stimulated culture.

To assess chondroprotective activity *in vitro*, surplus cartilage of five healthy dogs used for unrelated studies was used to determine cartilage proteoglycan (PG) synthesis. Per dog, 16 cartilage explants were harvested and cultured in a 96-wells plate (Thermo Fisher Scientific; Waltham, MA) in culture medium (Gibco, Thermo Fisher Scientific; Waltham, MA) containing TNF α (10ng/mL) to impair chondrocyte activity with or without cIL4-10 FP (100ng/mL) (n = 8 for each condition). Samples were cultured for four days at 37°C and chondrocyte PG synthesis was determined as described before[30]. In short, after one hour of pre-culture, 20 μ CI Na₂³⁵SO₄ (Dupont, NEX-041-H, carrier free; Wilmington, DE) in DMEM was added to each sample. After four hours of labeling, samples were washed with PBS and digested with papain for two hours at 65°C. Glycosaminoglycans (GAGs) were precipitated by addition of cetylpyridium chloride (CPC). ³⁵SO₄²⁻-labelled GAGs were measured by liquid scintillation on a TRI-CARB 2800TR (PerkinElmer; Waltham, MA). Values were normalized to specific activity of the pulse medium, pulse time, and wet weight of the explants and expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage (nmol.h⁻¹.g⁻¹). Data of eight samples were averaged for each cartilage donor (dog).

In vivo canine model of OA

The study was approved by the animal ethical committee of the Utrecht University (DEC 2014.III.09.081). For logistical reasons the experiment was split in two sets of eight dogs (four treated with IL4-10 FP and four with PBS).

Skeletally matured female mixed breed dogs (46 \pm 31 months, 30.4 \pm 17.4 kg; Marshall BioResources; North Rose, NY) were matched on weight and walking pattern and then split into two comparable groups. Dogs were housed in pairs and let out on a patio in large groups for at least 2 hours a day. They were fed a standard diet and had water *ad libitum*. OA was induced in the right leg according to the Groove model[31]. Dexdomitor (0.02 mg/kg i.m.) and Ketamin (0.5 mg/kg i.m.) were used as pre-medication before induction with thiopental (20mg/kg i.v.). Anesthesia was done with Sufentanil (0.0225 mg/kg/h i.m.) and Isofluran. Six to ten grooves of 2-3mm depth were made in the femoral cartilage as described in detail previously. Dogs were treated with painkillers (Carprofen; 4mg/kg p.o.) and antibiotics (Cefazolin; 35mg/kg i.v. pre-surgery and Synulox; 15mg/kg p.o. twice a day post-surgery) one day before surgery and during five days after surgery. During the first week after surgery regular wound checks were performed. Starting 2 days after surgery dogs were let out on the patio again. General control of animals and housing were performed during the whole study period. Based on previous experiments it was not expected that clinical symptoms (e.g. infection due to OA induction) would be of such degree that the human endpoint would be reached and euthanasia would be inevitable.

The Groove model is known to be an intrinsic cartilage degenerative model demonstrating progressive cartilage degradation[30, 32]. The model has been specifically designed to test chondroprotective treatments because treatment is not counteracted by a permanent trigger (like joint instability) or by a dominating inflammatory component (like chemically induced models). As such this model is explicitly suitable for testing DMOAD activity but not mainly anti-inflammatory activity. After six weeks of OA development, dogs were treated weekly with i.a. injections in their affected knee for ten successive weeks. Dogs in the treatment group were injected with IL4-10 FP (10 μ g in 500 μ L) whilst dogs in the control group were injected with PBS (500 μ L). The left contralateral knee of each dog served as an internal control. Periodically during treatment blood was drawn and force plate analyses were performed providing data on joint loading as a proxy for pain. After the treatment period of ten weeks, dogs were

ethanized and cartilage and synovial tissue samples were harvested. Natriumpentobarbital i.v. was used as euthesate. Ten minutes before euthanasia, dogs were injected with Dexdomitor (0.02 mg/kg i.m.) and Ketamin 0.5 mg/kg i.m.)

Experimental set-up

The first part of the canine *in vivo* experiment was performed treating four dogs i.a. with human IL4-10 FP and four dogs with i.a. PBS as a control group.

As human IL4-10 FP turned out to be immunogenic in dogs after multiple i.a. injections, the canine IL4-10 FP was produced, characterized and tested for *in vitro* bioactivity as described above. Subsequently, the second part of the canine *in vivo* experiment was performed using this canine IL4-10 FP, treating four dogs i.a. with canine IL4-10 FP and four with i.a. PBS as a control group (Fig 1)

Immunogenicity assessment for IL4-10 FP

ELISA was used to determine Immunoglobulin G (IgG) titers in serum of injected dogs. Serum was drawn before start of treatment and after every i.a. injection. In case of antibody detection after the tenth injection, intermediate time points were analyzed as well to identify when antibody formation was initiated.

After demonstrating the formation of Immunoglobulin G (IgG) in the hIL4-10 FP injected dogs, whole blood assays were performed to evaluate the neutralizing effects of these antibodies. Two 96-wells plate were coated with hIL4-10 FP. One plate was covered with dilutions of serum containing possible IgGs against hIL4-10 FP (after ten injections) and one plate was covered with dilutions of serum without IgGs against hIL4-10 FP (before ten injections). Subsequently TNF α inhibiting capacity of the hIL4-10 FP was determined.

Force plate analysis

In order to evaluate pain, force plate analysis (FPA) was performed to determine joint (un)loading as a proxy for pain. Vertical peak force (Fz), braking force (Fy+), and propulsive force

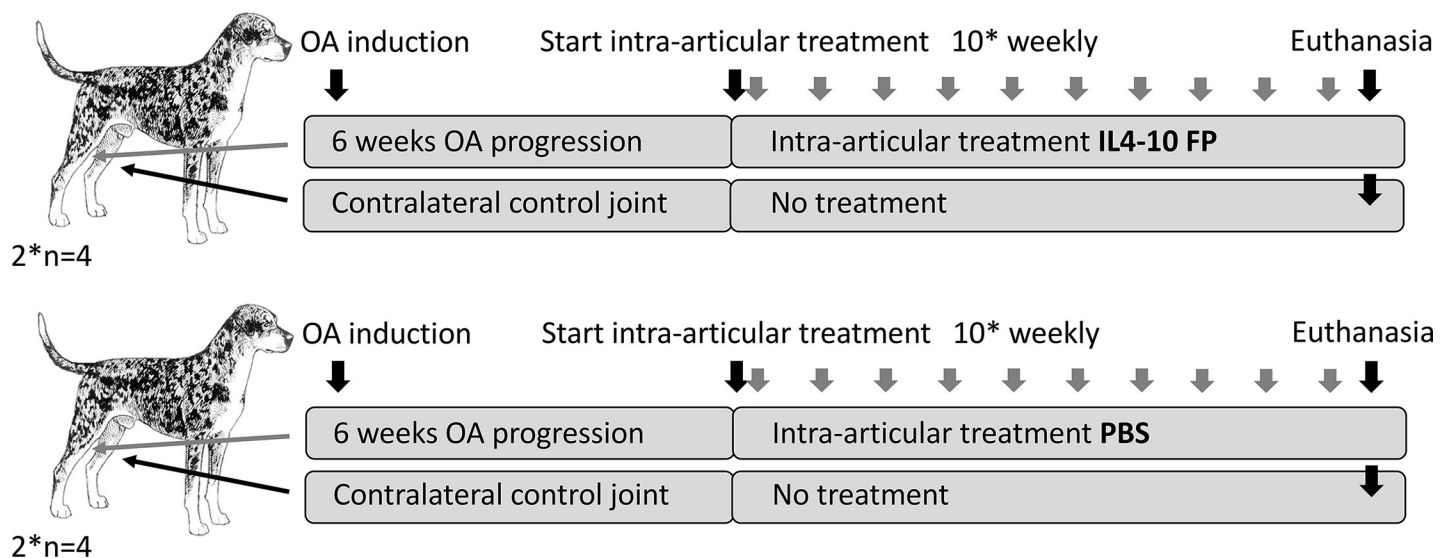


Fig 1. Experimental set-up of the canine *in vivo* experiment. The first part of the canine *in vivo* experiment was performed treating four dogs with i.a. human IL4-10 FP and four dogs with i.a. PBS as a control group. Because of IL4-10 FP neutralizing antibody formation the second part was performed treating four dogs with i.a. canine IL4-10 FP and four dogs with i.a. PBS.

<https://doi.org/10.1371/journal.pone.0219587.g001>

(Fy-) were determined. The ratio of the affected hind leg over the contralateral control hind leg was calculated for each dog for each time point. FPA was performed before Groove surgery to determine baseline values and during the treatment period before and 24 hours after the 1st, 6th and 9th injection.

Assessment of synovial inflammation

Macroscopic scoring of synovial tissue samples was done on digital photographs of the synovial fat pad according to OARSI (range 0–5)[33]. Knee synovial tissue samples were embedded in Tissue Tek (Sakura; Torrance, CA) and stored at -80°C. Sections of 5µm were cut and stained with hematoxylin-eosin (Mayer's hemalum solution; Merck, Darmstadt) and number of cell layers, lining characteristics and cell infiltration were scored from 0–6 (total score ranged from 0–18) according to OARSI[33]. Sections were analyzed by two observers blinded for treatment, and an average of six sections (three sections per observer) was calculated for each knee.

Assessment of cartilage degeneration

Macroscopic scoring of cartilage tissue samples was done on digital photographs of the tibial plateau according to OARSI[33]. Also histologic cartilage degeneration was evaluated in tibial cartilage only, because grooves were applied in the femoral cartilage. Four samples of tibial cartilage were used. Cartilage samples were fixed in formalin and embedded in paraffin. Sections of 5 µm were cut and stained with Safranin-O-fast-green (Saf-O). Sections were scored for cartilage structure, chondrocyte pathology and PG staining from 0–12 (total score ranged from 0–36) according to OARSI[33]. All sections were stained in the same Safranin-O baths. Each sample (four per knee) was scored by two observers blinded for treatment. An average of eight scores was used for statistical evaluation. For one dog in the cIL4-10 FP group all four pieces of the right tibia could not be evaluated due to technical issues. Data was imputed by taking the mean change of the remaining three dogs and adding this value to the value of the contralateral knee.

Cartilage proteoglycan (PG) turnover

The surgically untouched tibial cartilage was used for determining *ex vivo* PG turnover, as described before[30]. For determining PG content the glycosaminoglycans (GAGs) in a papain digest of the tissue samples were precipitated and stained with Alcian Blue dye solution[34]. This staining was quantified photometrically by change in absorbance at 620nm. Chondroitin sulphate (Sigma-Aldrich C4383; Saint Louis, MO) was used as a reference. Values were normalized to wet weight of the cartilage explants (mg/g). For total release of PGs, GAGs in the culture medium were precipitated from the medium, stained with Alcian blue dye solution and quantified photometrically with chondroitin sulphate as a reference. The total amount of GAGs released (blue staining) is expressed as a percentage of the original tissue content (% total PG release).

Determination of PG synthesis is described above (under *in vitro* bioactivity experiments). Release of newly formed PGs was determined as a measurement of retention of newly formed PGs. The release of $^{35}\text{SO}_4^{2-}$ -labelled PGs in the medium during three days was determined. After labelling, the samples were rinsed three times for 45 minutes in 1.5mL culture medium and then incubated in 200µL fresh culture medium without sulphate label. After three days released GAGs in the culture medium were precipitated by Alcian Blue dye solution. $^{35}\text{SO}_4^{2-}$ -labelled GAGs were measured by liquid scintillation analysis and values were normalized to wet weight of the explants. Release of newly formed PGs is normalized to total PG synthesis rate and expressed as percentage release of newly formed PGs (% release newly formed PGs).

An average of eight samples taken from predefined locations, identical for contralateral control and experimental joints, of the tibial cartilage was used as representative value for each joint.

Previous research shows that values of contralateral (left) knees are comparable to baseline values of experimental (right) knees.

Statistical analysis

Statistical analyses were done using SPSS Statistics 25 (IBM; Armonk, NY). A Wilcoxon-signed rank test was used to analyze the *in vitro* bioactivity. For FPA, a linear mixed model, correcting for time, baseline values, and pre-injection values was used to evaluate the effects on joint loading 24 hours after i.a. injection. For inflammation and cartilage degeneration parameters mean differences between experimental knees and contralateral knees were determined and considered to represent the effect of OA induction and treatment with either IL4-10 FP or PBS within the experimental knees. The effects within the experimental knees were compared between PBS and IL4-10 FP group using Mann-Whitney U tests. P-values <0.05 were considered as statistically significant for all tests.

Results

Canine IL4-10 FP production and characterization

Human IL4-10 FP was immunogenic in dogs upon repeated i.a. injections, leading to the formation of antibodies after only three repetitive i.a. injections (Fig 2B). Whole blood assays evaluating the TNF α -inhibition of hIL4-10 FP after adding serum of hIL4-10 FP injected dogs after ten injections showed a complete loss of activity of the hIL4-10 FP, demonstrating a neutralizing effect of the IgGs (Fig 2C). Moreover, the immunogenicity caused severe synovial inflammation (Fig 2A), visible as diffuse discoloration due to neovascularization as well as tissue proliferation with villi formation.

Therefore, a canine variant of the IL4-10 FP was developed. In Fig 3A schematic representation is shown of the cIL4-10 FP and its amino acid sequence, including the linker sequence and seven predicted glycosylation sites. Purified cIL4-10 FP was detected on Coomassie stained SDS gel as a smear composed of multiple protein bands corresponding to a molecular mass of 37 – 50kDa (Fig 3B). A smeared protein band was also detected on a Western Blot by anti-IL4 and anti-IL10 antibodies, while after deglycosylation of cIL4-10 FP with PNGaseF only one sharp protein band of 30kDa was detected, as expected based on amino acid sequence (Fig 3C), indicating the apparent smear represented different glycoforms of cIL4-10 FP. HP-SEC analysis showed that cIL4-10 FP preparations mainly consisted of non-covalently linked dimers with a mass of ~70 kDa, as well as some monomers of ~35 kDa (Fig 3D). Both monomer and dimer have been demonstrated to be biologically active.

Bioactivity of canine IL4-10 FP *in vitro*

The bioactivity of cIL4-10 FP was evaluated *in vitro* by its ability to diminish TNF α production in an LPS-stimulated canine whole blood culture. cIL4-10 FP dose-dependently inhibited TNF α production, with maximal inhibition at 3.0 nM. Bioactivity of the canine IL4-10 FP was comparable to that of canine IL-10, whilst IL-4 was less efficient (Fig 4A).

To test the chondroprotective activity of cIL4-10 FP, PG synthesis in TNF α -stimulated canine cartilage explants was evaluated *in vitro*. cIL4-10 FP statistically significantly improved PG synthesis in TNF α compromised cartilage explants as compared to untreated, TNF α compromised cartilage explants (4.0 (95%CI: 2.2–5.7) vs 2.0 (95%CI: 1.5–2.5), $p = 0.043$, Fig 4B).

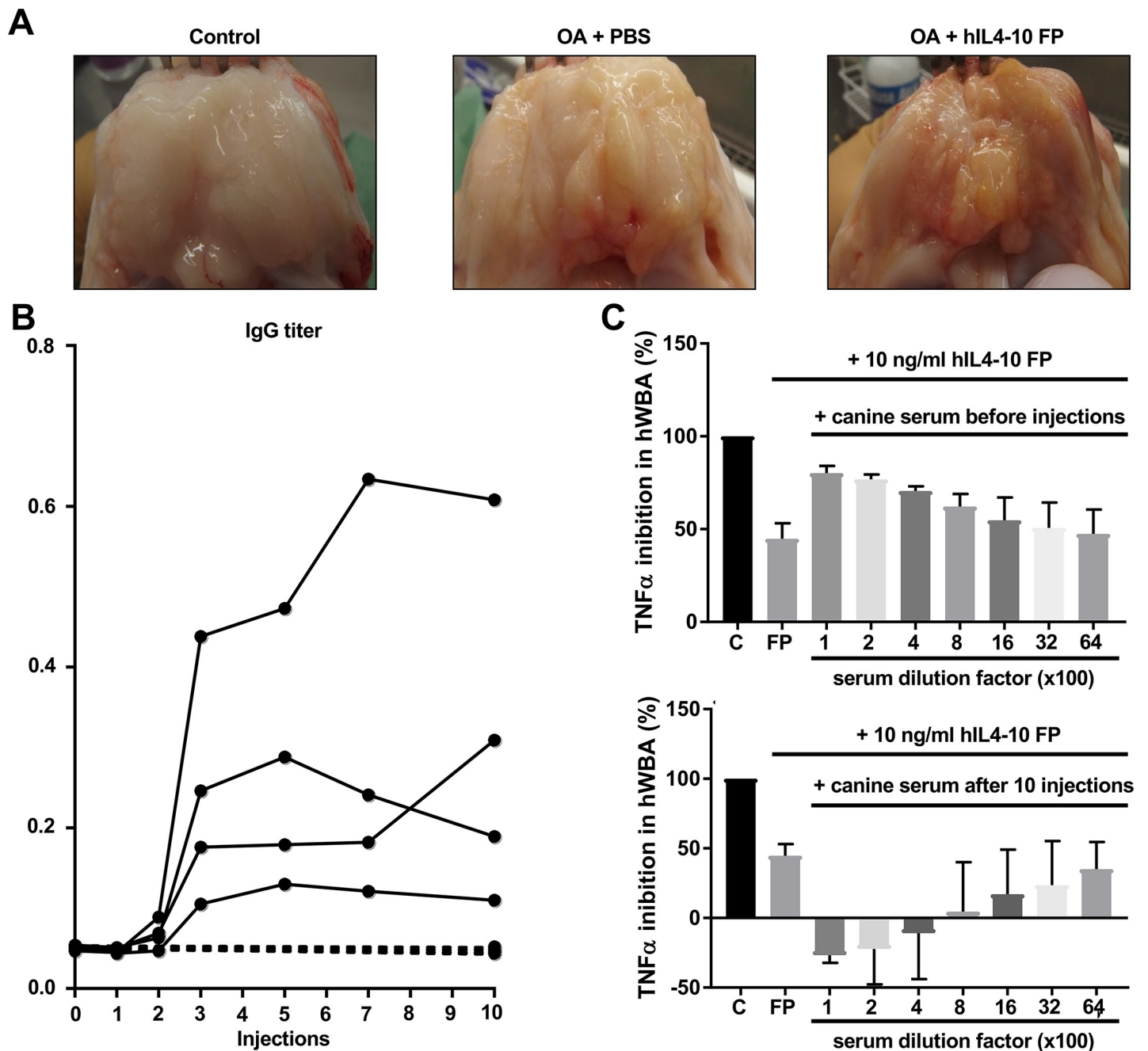


Fig 2. Immune response against hIL4-10 FP. A. Macroscopic pictures of synovial fatpad of control, PBS injected and hIL4-10 FP injected knees. B. IgG titers in serum of PBS injected dogs (dotted line) and hIL4-10FP injected dogs (continuous line). C. TNF α inhibition of hIL4-10 FP after adding serum taken before i.a. injections (above) and after i.a. injections with hIL4-10 FP (below). TNF α inhibition is expressed as percentage of inhibition in serum of non-injected dogs.

<https://doi.org/10.1371/journal.pone.0219587.g002>

Canine IL4-10 FP in the canine Groove model of OA

Immunogenicity. After ten injections of cIL4-10 FP, OD450 values of serum in the ELISA for IgG antibodies against cIL4-10 FP were comparable to values obtained with baseline samples, showing that ten repeated i.a. injections with species-specific protein did not lead to antibody formation against cIL4-10 FP (Fig 5C). Intermediate time points and neutralizing effects were not evaluated any further.

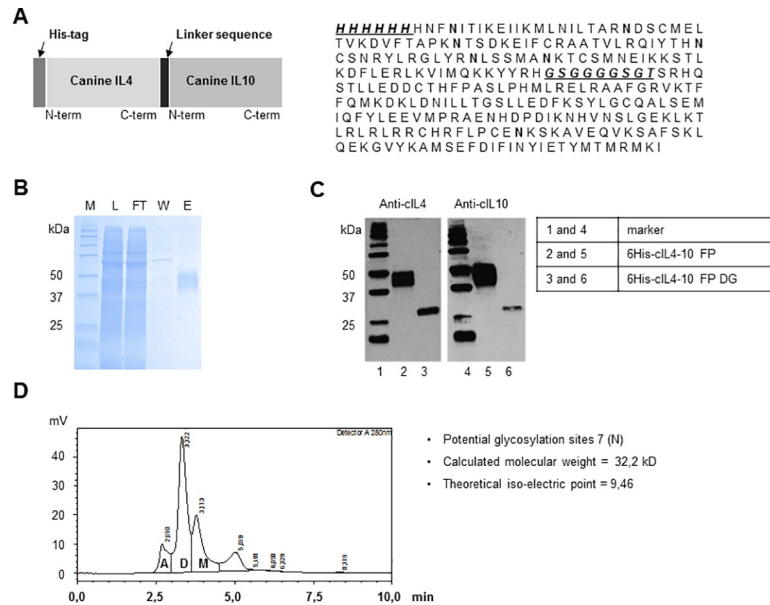


Fig 3. Molecular characterization of cIL4-10 FP. A. Schematic overview of the cIL4-10 FP. B. Coomassie Brilliant Blue stained SDS gel in Ni-NTA protein purification steps; M: Marker, L: Load, FT: Flow-through, W: Wash, E: Elution. C. Western blot analysis of purified cIL4-10 FP (untreated and deglycosylated; DG). D. HP-SEC profile showing; A: Aggregate, D: Dimer, M: Monomer.

<https://doi.org/10.1371/journal.pone.0219587.g003>

Synovial inflammation. The Groove model is characterized by minimal signs of synovial inflammation [30, 32]. Macroscopic and histologic OARSI scoring of synovial inflammation showed indeed only minimal inflammation (Fig 5A representative macroscopic images and

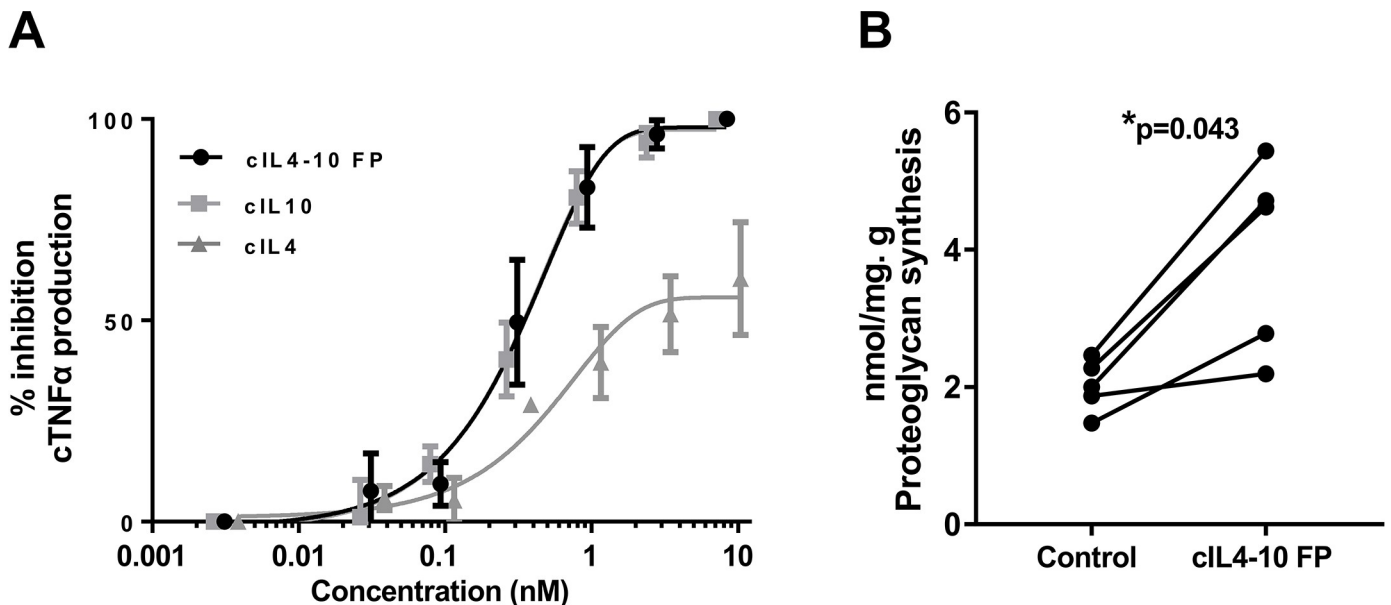


Fig 4. In vitro activity of cIL4-10 FP. A. Anti-inflammatory activity of cIL4-10 FP. Whole canine blood was stimulated with LPS with or without IL4-10 FP, IL10, or IL4 for 18 hours. Canine TNFα was measured using ELISA. B. Chondroprotective activity of cIL4-10 FP. Healthy canine cartilage explants were cultured in TNFα containing medium with or without cIL4-10 FP for four days. Proteoglycan synthesis rate was measured using liquid scintillation of ³⁵SO₄²⁻ labelled GAGs. Values are expressed per dog and are the mean of eight tissue samples per dog. Mean proteoglycan synthesis rate was 2.0 and 4.0 for controls and cIL4-10 FP treated conditions, respectively.

<https://doi.org/10.1371/journal.pone.0219587.g004>

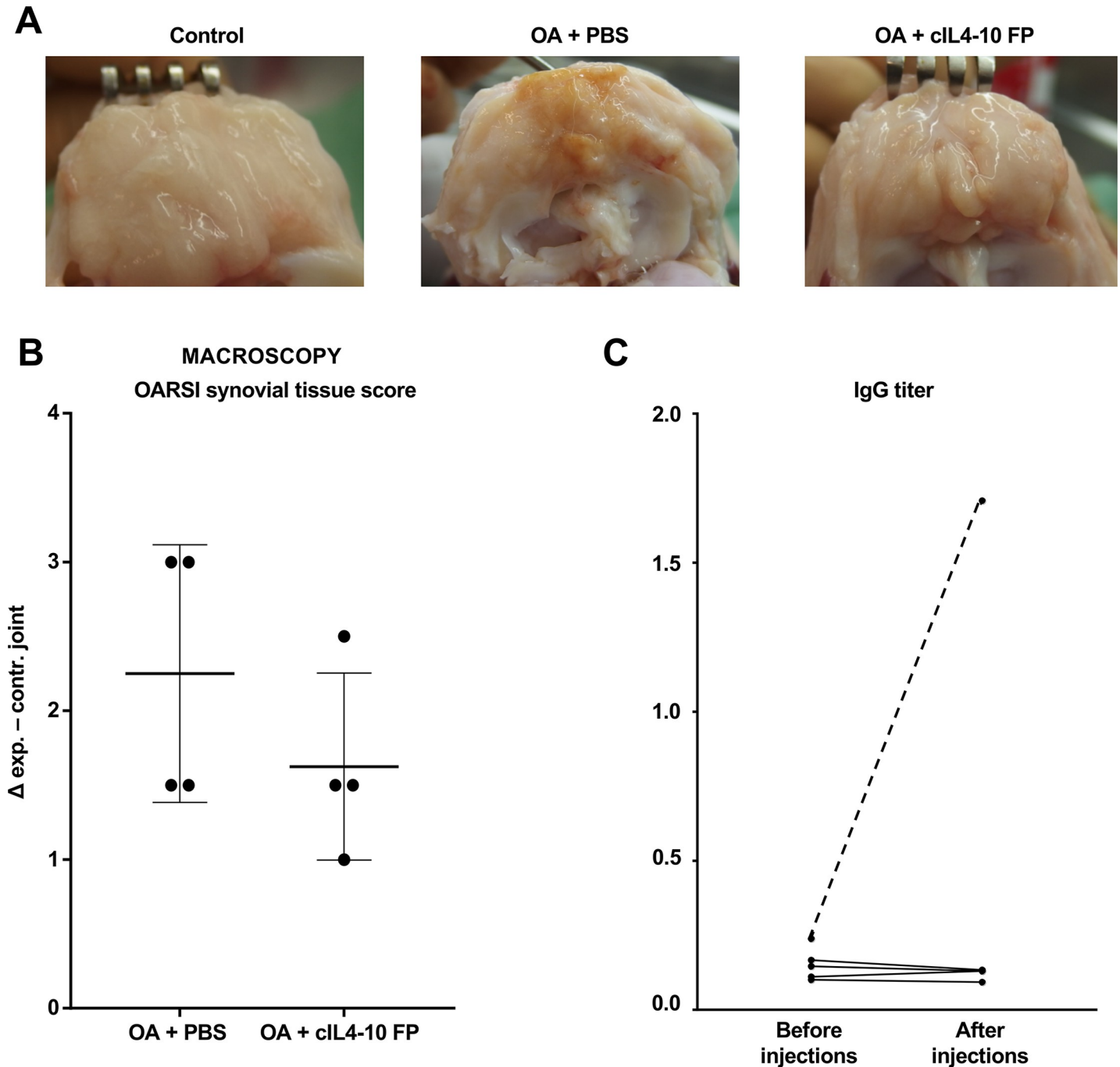


Fig 5. Immunogenicity of cIL4-10 FP in the canine Groove model of OA after injections with PBS or cIL4-10 FP. A. Macroscopic pictures of synovial fat pad of control, PBS injected and cIL4-10 FP injected knees. B. Differences in macroscopic synovial inflammation (OARSI score) between experimental and contralateral joint, representing a change from baseline to end of the study for the experimental joint. Values are expressed per dog (dots) and as median \pm IQR (bars). Mean macroscopic OARSI scores in contralateral joints were 0.5 (95%CI: -0.2–1.2) and 0.4 (95%CI: -0.4–1.1) in PBS group and cIL4-10 FP group, respectively. Mean macroscopic OARSI scores in experimental joints were 2.8 (95%CI: 0.9–4.6) and 2.0 (95%CI: 1.4–2.7) in PBS group and cIL4-10 FP group, respectively. C. IgG titers in serum of cIL4-10 FP injected dogs (continuous line) and, as a positive control, a hIL4-10FP injected dog (dotted line).

<https://doi.org/10.1371/journal.pone.0219587.g005>

Fig 5B scores of the individual animals). As such analyzing anti-inflammatory activity was limited to the canine *in vitro* assays. Mean changes in histologic inflammation grade were -1.2 (95%CI: -3.7–1.3) and 0.3 (95%CI: -2.1–2.7) out of 18 for PBS injected dogs and cIL4-10 FP

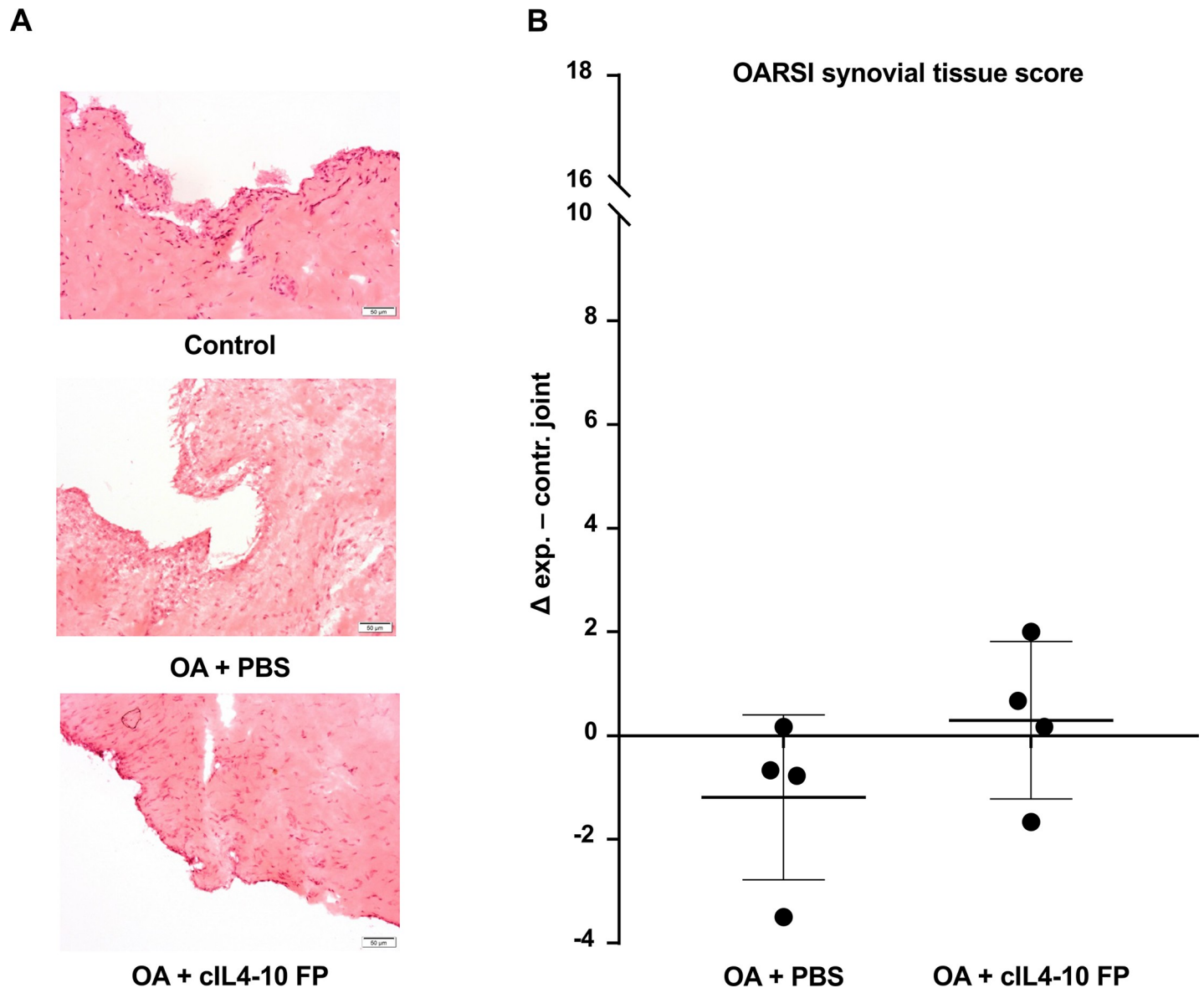


Fig 6. Histologic synovial inflammation in the canine Groove model of OA after injections with PBS or cIL4-10 FP. A. Representative images of Haematoxylin-eosin stained synovial tissue sections of control (without Groove surgery), PBS injected and cIL4-10 FP injected knees. Samples were collected at the end of the experiment; 16 weeks after Groove surgery and after ten weekly i.a. injections. B. Differences in histologic synovial inflammation (OARSI score) between experimental and contralateral joint, representing a change from baseline to end of the study for the experimental joint. Values are expressed per dog (dots) and as median \pm IQR (bars). Mean histologic OARSI scores in contralateral joints were 3.0 (95%CI: 1.7–4.3) and 1.3 (95%CI: -0.1–2.6) in PBS group and cIL4-10 FP group, respectively. Mean histologic OARSI scores in experimental joints were 1.8 (95%CI: -0.8–4.5) and 1.5 (95%CI: -0.8–3.8) in PBS group and cIL4-10 FP group, respectively.

<https://doi.org/10.1371/journal.pone.0219587.g006>

injected dogs, respectively (Fig 6). None of these changes were statistically significant (Table 1).

Force plate analysis. Groove surgery induced a reduction in stance (Fz) and propulsive force (Fy+) in both groups. After 10 i.a. injections with cIL4-10 FP Fz and Fy+ were restored to pre OA values. Overall, cIL4-10 FP increased Fz and Fy+ 24 hours after i.a. injections compared to PBS injected dogs ($p = 0.002$ and $p = 0.01$ for Fz and Fy+, respectively, Fig 7). Similarly, the breaking force (Fy-) trended to a decrease after surgery and a recovery after i.a. cIL4-10 FP injections, although not statically significant.

Table 1. Mean values of 4 dogs with p-values for all parameters of synovial inflammation and cartilage damage.

Parameter	PBS			cIL4-10 FP			p-value
	Contr.	Exp.	Δ Exp.-Contr.	Contr.	Exp.	Δ Exp.-Contr.	
Force plate analysis							
Vertical peak force (Fz)							0.002
Braking force (Fy+)							0.010
Propulsive force (Fy-)							0.067
Synovial inflammation							
Macroscopy	0.5 (-0.2-1.2)	2.8 (0.9-4.6)	2.3 (0.9-3.6)	0.4 (-0.4-1.1)	2.0 (1.4-2.7)	1.6 (0.6-2.6)	0.343
Histology	3.0 (1.7-4.3)	1.8 (-0.8-4.5)	-1.2 (-3.7-1.3)	1.3 (-0.1-2.6)	1.5 (-0.8-3.8)	0.3 (-2.1-2.7)	0.886
Cartilage damage							
Macroscopy	0.5 (0.2-0.8)	1.8 (0.1-3.4)	1.3 (-0.1-2.6)	0.4 (0.1-0.6)	1.6 (1.2-1.9)	1.2 (0.6-1.8)	0.886
Histology	4.2 (1.2-7.2)	9.2 (5.7-12.7)	5.1 (-1.3-11.4)	6.0 (1.7-10.3)	7.2 (2.8-11.5)	1.2 (-4.1-6.4)	0.343
- Cartilage structure	1.3 (-0.4-2.5)	3.8 (3.0-4.5)	2.5 (0.6-4.4)	1.4 (0.0-2.8)	2.1 (0.4-3.8)	0.7 (-0.6-2.0)	0.057
- Chondrocyte pathology	0.5 (-0.2-1.1)	2.0 (-0.7-4.7)	1.5 (-1.7-4.8)	2.3 (-0.3-4.8)	1.9 (-0.6-4.4)	-0.4 (-4.5-3.6)	0.686
- PG staining	2.5 (1.2-3.8)	3.5 (2.2-4.8)	1.0 (-1.3-3.3)	2.3 (1.2-3.7)	2.6 (1.9-3.3)	0.3 (-1.2-1.8)	0.486
Cartilage biochemistry							
- PG synthesis	3.5 (-0.2-7.2)	1.4 (0.1-2.7)	-2.1 (-5.4-1.2)	6.1 (-1.7-13.9)	3.5 (-3.4-10.3)	-2.7 (-9.3-4.0)	0.686
- Release of newly formed PGs	79.7 (61.6-97.7)	92.0 (84.4-99.6)	12.4 (-10.8-35.6)	75.7 (50.2-101.2)	89.2 (74.4-104.0)	13.5 (-6.6-33.6)	0.886
- PG content	37.8 (20.4-55.2)	27.8 (24.6-31.0)	-10.0 (-25.3-5.3)	30.7 (27.3-34.1)	34.1 (28.3-39.9)	3.4 (-2.6-9.4)	0.029
- Release of PGs	10.5 (9.6-11.4)	12.0 (10.7-13.5)	1.6 (-0.4-3.5)	12.6 (12.0-13.2)	13.8 (10.9-16.7)	1.2 (-1.1-3.6)	0.886

Values are expressed as a mean of four dogs (95% CI). P-values represent results of Mann-Whitney U tests to compare changes within the group (Δexp.-contr.) between both groups.

<https://doi.org/10.1371/journal.pone.0219587.t001>

Cartilage damage. Evaluation of Saf O stained cartilage sections showed a small increase in OARSI cartilage structural damage score in experimental (OA) knees compared to contralateral control knees (2.5 (95%CI: 0.6-4.4) points out of 12 points) in the PBS treated group, which was normalized in the cIL4-10 FP treated group (0.7 (95%CI: -0.6-2.0) points), at borderline significance (p = 0.057, Fig 8B). The other subscales of the OARSI score, chondrocyte pathology and proteoglycan staining, as well as total OARSI score, showed similar trends, although not statistically significant (Table 1).

The protective effect on cartilage was corroborated by PG content. This PG content was decreased in the OA joints injected with PBS (-10.0 (95%CI: -25.3-5.3) mg/g; p = 0.068) whereas in the cIL4-10 FP injected OA joints there was no difference in PG content compared to the contralateral control joint (+3.4 (95%CI: -2.6-9.4) mg/g; p = 0.144). cIL4-10 FP significantly normalized the reduced PG content; p = 0.029, compared to the PBS treated group (Fig 8C). Changes in PG synthesis, release, and retention did not statistically significant differ between both treatment groups although differences were mostly in favor of the cIL4-10 FP group (Table 1).

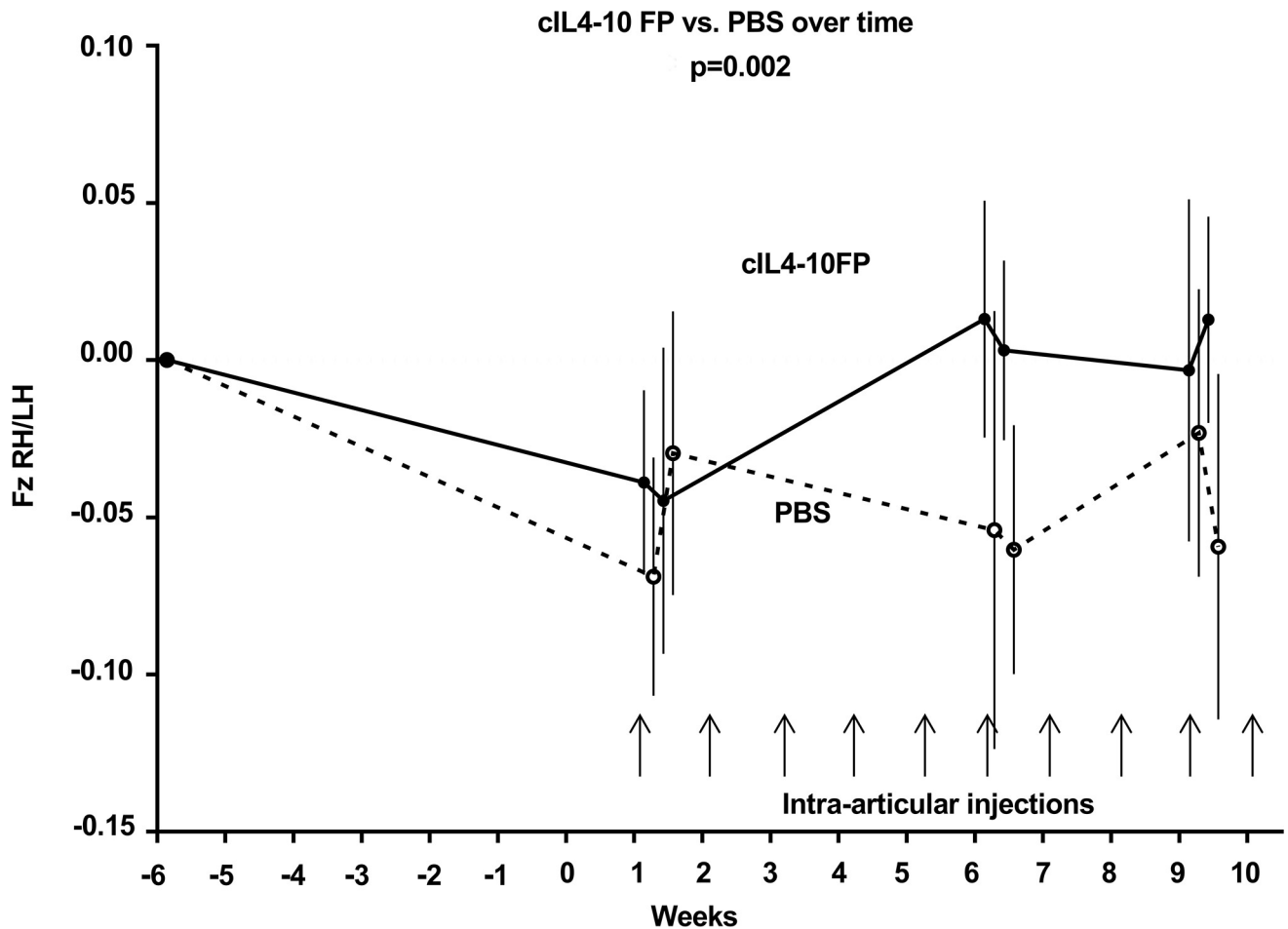


Fig 7. Analgesic effects of cIL4-10 FP in the canine Groove model of OA. Six weeks after Groove surgery dogs received weekly i.a. injections with cIL4-10 FP or PBS for ten weeks. Objective force plate analysis was used to determine joint loading as a proxy for pain. After OA induction, Fz RH/LH was clearly reduced. After treatment the Fz RH/LH statistically significant increased in the cIL4-10 FP treated group (continuous line, n = 4) compared to the PBS injected group (dotted line, n = 4); p = 0.002 over the whole time span. Values are expressed as a mean of four dogs \pm SEM.

<https://doi.org/10.1371/journal.pone.0219587.g007>

Discussion

In a previous explorative *in vivo* study, no antibody formation was observed after two repeated injections of human IL4-10 FP in dogs[28]. This encouraged us to test the efficacy of multiple repeated injections of human IL4-10 FP in this canine model. However, ten weekly, i.a. injections with human IL4-10 FP induced significant IL4-10 FP neutralizing antibody formation that prevented evaluation of the effects of human IL4-10 FP. Although apparently obvious, this demonstrates that testing a human protein for longer periods in an animal model may induce unwanted antibody formation that may prevent therapeutic efficacy.

Therefore, in this study we designed a canine variant of the IL4-10 FP and tested this for its DMOAD activity in the *in vivo* canine Groove model.

A clear limitation of this study is the number of dogs treated with the cIL4-10 FP and, as a result, the lack of power. Due to ethical approval and budget reasons it was not possible to extend the number of dogs per group to the originally intended n = 8. The study also lacks a healthy control group. The use of the contralateral knee as an internal control is reported to be valid[31, 32] but has its limitations as altered loading or systemic alterations by development of the OA as well as the treatment may have been of influence.

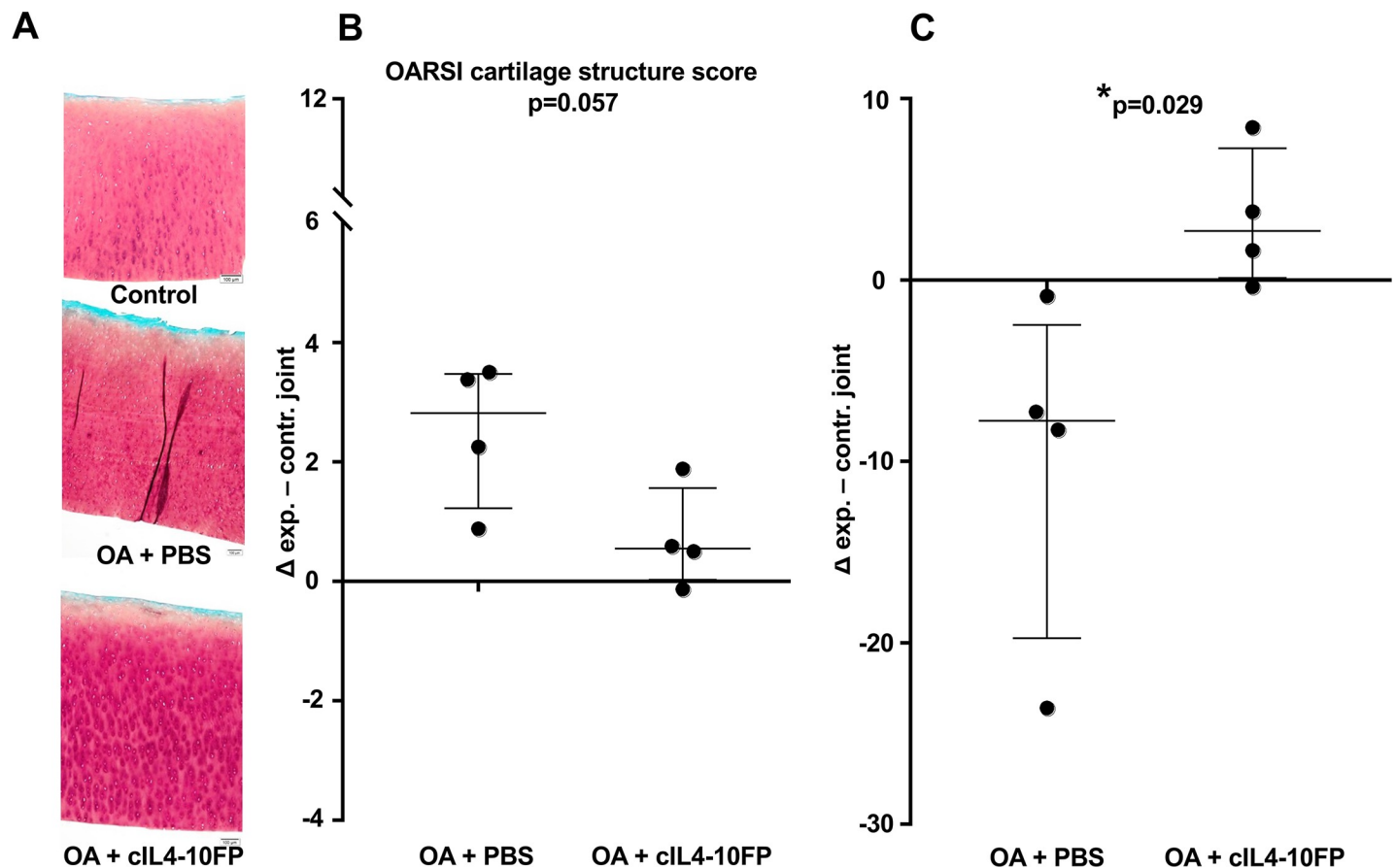


Fig 8. Cartilage damage in the canine Groove model of OA after injections with PBS or cIL4-10 FP. A. Representative images of Safranin-O stained tibial cartilage section of control (without Groove surgery), PBS injected and cIL4-10 FP injected knees. Samples were collected at the end of the experiment; 16 weeks after Groove surgery and after ten weekly i.a. injections. B. Differences in tibial cartilage structure (part of the OARSI score) between experimental and contralateral joint, representing a change from baseline to end of the study for the experimental joint. Values are expressed per dog (dots) and as median \pm IQR (bars). Mean scores in contralateral joints were 1.3 (95%CI: -0.4–2.5) and 1.4 (95%CI: 0.0–2.8) in PBS group and cIL4-10 FP group, respectively. Mean histologic OARSI scores in experimental joints were 3.8 (95%CI: 3.0–4.5) and 2.1 (95%CI: 0.4–3.8) in PBS group and cIL4-10 FP group, respectively. C. Differences in proteoglycan content in tibia of injected knees. Values are expressed per dog (dots) and as median \pm IQR (bars). Mean proteoglycan content in contralateral joints were 37.8 (95%CI: 20.4–55.2) and 30.7 (95%CI: 27.3–34.1) in PBS group and cIL4-10 FP group, respectively. Mean proteoglycan content in experimental joint were 27.8 (95%CI: 24.6–31.0) and 34.1 (95%CI: 28.3–39.9) in PBS group and cIL4-10 FP group, respectively.

<https://doi.org/10.1371/journal.pone.0219587.g008>

Nevertheless, the value of using a large animal model with the financial and ethical limitations but significantly higher translational value to the human situation may be appreciated.

To ensure comparable pharmacological activity, the design of the canine IL4-10 FP, was similar to that of human IL4-10 FP and the *in vitro* bioactivity was evaluated in the same type of species-specific assays. Either fusion protein consisted of full length species-specific IL4 and IL10 sequences, connected with the same flexible glycine-serine-rich linker sequence. Indeed, cIL4-10 FP showed anti-inflammatory and chondroprotective activity in canine *in vitro* models, similar to the activity of hIL4-10 FP in human *in vitro* models [27, 28, 35]. In addition, cIL4-10 FP showed analgesic and chondroprotective effects in a *in vivo* canine model of OA, although the second part of the study was limited to four dogs per group. Importantly, canine IL4-10 FP did not induce antibody formation upon multiple i.a. injections in this canine model. The human variant of the IL4-10 FP has been demonstrated to be stable under different conditions and not delink. As such the delinking of the canine variant is not anticipated, due to its same construction, although not studied in detail.

According to previous findings, in this OA model only minimal synovial inflammation is observed in PBS injected knees, as well as in cIL4-10 FP injected knees, underlining the non-immunogenic nature of the species-specific IL4-10 FP. The low grade synovial inflammation in this model was an advantage in demonstrating potential i.a. immunogenic responses as well as inflammation independent chondroprotective effects, but more or less ruled out the evaluation of the anti-inflammatory activity of the cIL4-10 FP. Nevertheless, *in vitro* cIL4-10 FP had anti-inflammatory properties, shown by the ability to reduce LPS-induced TNF α production in canine whole blood. Moreover, hIL4-10 FP lowered the release of inflammatory cytokines from cartilage and synovial tissue, and decreased cartilage destructive properties of inflamed synovial tissue[27, 28, 35].

In addition to the potential anti-inflammatory effects, we here demonstrated that cIL4-10 FP is also chondroprotective *in vivo*. These findings are in line with the *in vitro* chondroprotective effect of cIL4-10 FP, normalizing the hampered PG synthesis rate of compromised OA canine cartilage. Moreover, hIL4-10 FP normalizes PG release and PG synthesis, as well as PG content in human *in vitro* OA cartilage experiments[28, 35].

In the present study, joint loading was used as a proxy for OA pain, induced by Groove surgery. A reduction in FPA parameters represent an objective outcome measure for evaluation of lameness, and to some extent of the magnitude of pain. However these measures do not mean that it tells us exactly how much pain dogs experience or, translating it to the human situation, how it affects quality of life[36]. It might be possible that dogs adapt their joint loading over time despite persisting pain. Irrespectively, we demonstrated a significant improvement in the OA induced reduction in joint loading over the injection period. These data corroborate our previous finding that two i.a. injections with hIL4-10 FP improved joint loading in the canine Groove model[28] prior to established systemic and local immunogenic response due to a species-mismatch. Overall these findings indicate that IL4-10 FP has analgesic properties. Indeed, the IL4-10 FP also inhibited pain in an *in vivo* rat Groove model[37] and two mouse models, confirming the analgesic capacity of the IL4-10 FP[27, 28, 38].

Altogether, the above-mentioned anti-inflammatory, chondroprotective, and analgesic properties clearly warrant further research to develop the IL4-10 FP as a DMOAD. In treatment of the larger joints, intra-articular treatment may be preferred above systemic treatment since it needs less (expensive) drug in comparison to systemic application, has a lower risk of systemic side effects, and targets the non-vascularized articular cartilage directly. This would favor patients and healthcare systems. However, application in generalized OA including more and smaller joints will be a challenge. As such, treatment at first focusses on local administration in the larger joints. However, a few issues need to be addressed before it can be brought into a clinical trial. Firstly, injecting patients every week is not feasible in clinical care. To overcome this problem, it is essential to find a way to enhance bioavailability in the joint. Currently, different molecule characteristics and slow release systems are being tested. Secondly, phenotypic differences between OA patients may warrant phenotypic treatment approaches (personalized medicine). IL4-10 FP consists of two anti-inflammatory cytokines, therefore future *in vivo* studies are needed to evaluate whether or not an inflammatory phenotype is the most responsive to this FP.

In conclusion, human IL4-10 FP is immunogenic in dogs upon multiple repeated i.a. injections. Canine IL4-10 FP can be produced and diminishes canine blood cell inflammatory activity and improves PG synthesis of canine cartilage. In addition, the canine IL4-10 FP shows promising DMOAD activity *in vivo* in the canine Groove model. Further research to develop the IL4-10 FP as a DMOAD is clearly warranted.

Supporting information

S1 ARRIVE checklist.

(PDF)

S1 Safranin O Results. OARSI scoring.

(DOCX)

Acknowledgments

We thank A. Doornenbal (department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University) for his help with performing the Force Plate measurements.

Author Contributions

Conceptualization: E. M. van Helvoort, J. Popov-Celeketic, N. Eijkelkamp, C. E. Hack, F. P. J. G. Lafeber, S. C. Mastbergen.

Data curation: E. M. van Helvoort, K. Coeleveld.

Formal analysis: E. M. van Helvoort, C. D. Wijne, F. P. J. G. Lafeber, S. C. Mastbergen.

Funding acquisition: M. A. Tryfonidou, C. E. Hack, F. P. J. G. Lafeber, S. C. Mastbergen.

Investigation: K. Coeleveld.

Methodology: J. Popov-Celeketic, N. Eijkelkamp, M. A. Tryfonidou, C. D. Wijne, C. E. Hack, F. P. J. G. Lafeber, S. C. Mastbergen.

Supervision: J. Popov-Celeketic, N. Eijkelkamp, C. E. Hack, F. P. J. G. Lafeber.

Writing – original draft: E. M. van Helvoort.

Writing – review & editing: J. Popov-Celeketic, N. Eijkelkamp, K. Coeleveld, M. A. Tryfonidou, C. D. Wijne, C. E. Hack, F. P. J. G. Lafeber, S. C. Mastbergen.

References

1. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. *Arthritis and rheumatism*. 2012; 64(6):1697–707. <https://doi.org/10.1002/art.34453> PMID: 22392533
2. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. *Lancet (London, England)*. 2011; 377(9783):2115–26.
3. Reker D, Kjelgaard-Petersen CF, Siebuhr AS, Michaelis M, Gigout A, Karsdal MA, et al. Sprifermin (rhFGF18) modulates extracellular matrix turnover in cartilage explants ex vivo. *Journal of translational medicine*. 2017; 15(1):250. <https://doi.org/10.1186/s12967-017-1356-8> PMID: 29233174
4. Moore EE, Bendele AM, Thompson DL, Littau A, Waggie KS, Reardon B, et al. Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2005; 13(7):623–31.
5. Lohmander LS, Hellot S, Dreher D, Krantz EF, Kruger DS, Guermazi A, et al. Intraarticular sprifermin (recombinant human fibroblast growth factor 18) in knee osteoarthritis: a randomized, double-blind, placebo-controlled trial. *Arthritis & rheumatology (Hoboken, NJ)*. 2014; 66(7):1820–31.
6. Eckstein F, Wirth W, Guermazi A, Maschek S, Aydemir A. Brief report: intraarticular sprifermin not only increases cartilage thickness, but also reduces cartilage loss: location-independent post hoc analysis using magnetic resonance imaging. *Arthritis & rheumatology (Hoboken, NJ)*. 2015; 67(11):2916–22.
7. Song SU, Cha YD, Han JU, Oh IS, Choi KB, Yi Y, et al. Hyaline cartilage regeneration using mixed human chondrocytes and transforming growth factor-beta1-producing chondrocytes. *Tissue engineering*. 2005; 11(9–10):1516–26. <https://doi.org/10.1089/ten.2005.11.1516> PMID: 16259606

8. Ha CW, Noh MJ, Choi KB, Lee KH. Initial phase I safety of retrovirally transduced human chondrocytes expressing transforming growth factor-beta-1 in degenerative arthritis patients. *Cytherapy*. 2012; 14(2):247–56. <https://doi.org/10.3109/14653249.2011.629645> PMID: 22242865
9. Lee MC, Ha CW, Elmallah RK, Cherian JJ, Cho JJ, Kim TW, et al. A placebo-controlled randomised trial to assess the effect of TGF-ss1-expressing chondrocytes in patients with arthritis of the knee. *The bone & joint journal*. 2015; 97-b(7):924–32.
10. Cherian JJ, Parvizi J, Bramlet D, Lee KH, Romness DW, Mont MA. Preliminary results of a phase II randomized study to determine the efficacy and safety of genetically engineered allogeneic human chondrocytes expressing TGF-beta1 in patients with grade 3 chronic degenerative joint disease of the knee. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2015; 23(12):2109–18.
11. Ha CW, Cho JJ, Elmallah RK, Cherian JJ, Kim TW, Lee MC, et al. A Multicenter, Single-Blind, Phase IIa Clinical Trial to Evaluate the Efficacy and Safety of a Cell-Mediated Gene Therapy in Degenerative Knee Arthritis Patients. *Human gene therapy Clinical development*. 2015; 26(2):125–30. <https://doi.org/10.1089/humc.2014.145> PMID: 25760423
12. Kim MK, Ha CW, In Y, Cho SD, Choi ES, Ha JK, et al. A Multicenter, Double-Blind, Phase III Clinical Trial to Evaluate the Efficacy and Safety of a Cell and Gene Therapy in Knee Osteoarthritis Patients. *Human gene therapy Clinical development*. 2018; 29(1):48–59. <https://doi.org/10.1089/humc.2017.249> PMID: 29641281
13. Guerhazi A, Kalsi G, Niu J, Crema MD, Copeland RO, Orlando A, et al. Structural effects of intra-articular TGF-beta1 in moderate to advanced knee osteoarthritis: MRI-based assessment in a randomized controlled trial. *BMC musculoskeletal disorders*. 2017; 18(1):461. <https://doi.org/10.1186/s12891-017-1830-8> PMID: 29145839
14. Deshmukh V, Hu H, Barroga C, Bossard C, Kc S, Dellamary L, et al. A small-molecule inhibitor of the Wnt pathway (SM04690) as a potential disease modifying agent for the treatment of osteoarthritis of the knee. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2018; 26(1):18–27.
15. Yazici Y, McAlindon TE, Fleischmann R, Gibofsky A, Lane NE, Kivitz AJ, et al. A novel Wnt pathway inhibitor, SM04690, for the treatment of moderate to severe osteoarthritis of the knee: results of a 24-week, randomized, controlled, phase 1 study. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2017; 25(10):1598–606.
16. Demaziere A, Leek R, Athanasou NA. Histological distribution of the interleukin-4 receptor (IL4R) within the normal and pathological synovium. *Revue du rhumatisme et des maladies osteo-articulaires*. 1992; 59(3):219–24. PMID: 1319084
17. Iannone F, De Bari C, Dell'Accio F, Covelli M, Cantatore FP, Patella V, et al. Interleukin-10 and interleukin-10 receptor in human osteoarthritic and healthy chondrocytes. *Clinical and experimental rheumatology*. 2001; 19(2):139–45. PMID: 11332442
18. van Meegeren ME, Roosendaal G, Jansen NW, Wenting MJ, van Wesel AC, van Roon JA, et al. IL-4 alone and in combination with IL-10 protects against blood-induced cartilage damage. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2012; 20(7):764–72.
19. Millward-Sadler SJ, Khan NS, Bracher MG, Wright MO, Salter DM. Roles for the interleukin-4 receptor and associated JAK/STAT proteins in human articular chondrocyte mechanotransduction. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2006; 14(10):991–1001.
20. Forster T, Chapman K, Loughlin J. Common variants within the interleukin 4 receptor alpha gene (IL4R) are associated with susceptibility to osteoarthritis. *Human genetics*. 2004; 114(4):391–5. <https://doi.org/10.1007/s00439-004-1083-0> PMID: 14745651
21. John T, Muller RD, Oberholzer A, Zreiqat H, Kohl B, Ertel W, et al. Interleukin-10 modulates pro-apoptotic effects of TNF-alpha in human articular chondrocytes in vitro. *Cytokine*. 2007; 40(3):226–34. <https://doi.org/10.1016/j.cyto.2007.10.002> PMID: 18023359
22. Salter DM, Nuki G, Wright MO. IL-4 inhibition of cartilage breakdown in bovine articular explants. *The Journal of rheumatology*. 1996; 23(7):1314–5. PMID: 8823723
23. Alaaeddine N, Di Battista JA, Pelletier JP, Kiansa K, Cloutier JM, Martel-Pelletier J. Inhibition of tumor necrosis factor alpha-induced prostaglandin E2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. *Arthritis and rheumatism*. 1999; 42(4):710–8. [https://doi.org/10.1002/1529-0131\(199904\)42:4<710::AID-ANR14>3.0.CO;2-4](https://doi.org/10.1002/1529-0131(199904)42:4<710::AID-ANR14>3.0.CO;2-4) PMID: 10211885
24. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. *Arthritis and rheumatism*. 2001; 44(1):3–12. [https://doi.org/10.1002/1529-0131\(200101\)44:1<3::AID-ANR2>3.0.CO;2-U](https://doi.org/10.1002/1529-0131(200101)44:1<3::AID-ANR2>3.0.CO;2-U) PMID: 11212172
25. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, et al. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. *Arthritis and rheumatism*. 1997; 40(2):249–60. PMID: 9041936

26. van Roon JA, van Roy JL, Gmelig-Meyling FH, Lafeber FP, Bijlsma JW. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. *Arthritis and rheumatism*. 1996; 39(5):829–35. PMID: [8639180](#)
27. Eijkelkamp N, Steen-Louws C, Hartgring SA, Willemen HL, Prado J, Lafeber FP, et al. IL4-10 Fusion Protein Is a Novel Drug to Treat Persistent Inflammatory Pain. *The Journal of neuroscience: the official journal of the Society for Neuroscience*. 2016; 36(28):7353–63.
28. Steen-Louws C, Popov-Celeketic J, Mastbergen SC, Coeleveld K, Hack CE, Eijkelkamp N, et al. IL4-10 fusion protein has chondroprotective, anti-inflammatory and potentially analgesic effects in the treatment of osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2018; 26(8):1127–35.
29. Prado J, Popov-Celeketic J, Steen-Louws C, Raof R, Hack E, Eijkelkamp N. Development of Recombinant Proteins to Treat Chronic Pain. *Journal of visualized experiments: JoVE*. 2018; 11(134).
30. Mastbergen SC, Marijnissen AC, Vianen ME, van Roermund PM, Bijlsma JW, Lafeber FP. The canine 'groove' model of osteoarthritis is more than simply the expression of surgically applied damage. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2006; 14(1):39–46.
31. Marijnissen AC, van Roermund PM, TeKoppele JM, Bijlsma JW, Lafeber FP. The canine 'groove' model, compared with the ACLT model of osteoarthritis. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2002; 10(2):145–55.
32. Marijnissen AC, van Roermund PM, Verzijl N, Tekoppele JM, Bijlsma JW, Lafeber FP. Steady progression of osteoarthritic features in the canine groove model. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2002; 10(4):282–9.
33. Cook JL, Kuroki K, Visco D, Pelletier JP, Schulz L, Lafeber FP. The OARSI histopathology initiative—recommendations for histological assessments of osteoarthritis in the dog. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society*. 2010; 18 Suppl 3:S66–79.
34. Lafeber FP, Vander Kraan PM, Van Roy JL, Huber-Bruning O, Bijlsma JW. Articular cartilage explant culture; an appropriate in vitro system to compare osteoarthritic and normal human cartilage. *Connective tissue research*. 1993; 29(4):287–99. PMID: [8269705](#)
35. van Vulpen LFD, Popov-Celeketic J, van Meegeren MER, Coeleveld K, van Laar JM, Hack CE, et al. A fusion protein of interleukin-4 and interleukin-10 protects against blood-induced cartilage damage in vitro and in vivo. *Journal of thrombosis and haemostasis: JTH*. 2017; 15(9):1788–98. <https://doi.org/10.1111/jth.13778> PMID: [28696534](#)
36. Conzemius MG, Evans RB. Caregiver placebo effect for dogs with lameness from osteoarthritis. *Journal of the American Veterinary Medical Association*. 2012; 241(10):1314–9. <https://doi.org/10.2460/javma.241.10.1314> PMID: [23113523](#)
37. de Visser HM, Weinans H, Coeleveld K, van Rijen MH, Lafeber FP, Mastbergen SC. Groove model of tibia-femoral osteoarthritis in the rat. *Journal of orthopaedic research: official publication of the Orthopaedic Research Society*. 2017; 35(3):496–505.
38. Popov-Celeketic J, De Visser HM, Coeleveld K, Steen-Louws C, Hack CE, Weinans H, et al. IL4-10 fusion protein as a disease modifying therapy for osteoarthritis evaluated in a rat Groove model *in vivo*. 2018; 26:S292.