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EDITOR'S CHOICE

IL4-10 fusion protein: a novel immunoregulatory drug combining activities of interleukin 4 and interleukin 10

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Summary

The objective of this study was to test the capacity of a newly developed fusion protein of interleukin 4 (IL-4) and IL-10 [IL4-10 fusion protein (FP)] to shift multiple pro-inflammatory pathways towards immune regulation, and to inhibit pro-inflammatory activity in arthritis models. The effects of IL4-10 FP in comparison with IL-4, IL-10 and IL-4 plus IL-10 on pro- and anti-inflammatory mediators, T cells and immunoglobulin (Ig) receptors in favour of immunoregulatory activity were studied. In addition, the capacity of IL4-10 FP to inhibit pro-inflammatory activity in *ex-vivo* and *in-vivo* arthritis models was investigated. IL4-10 FP robustly inhibited pro-inflammatory cytokine [IL-1β, tumour necrosis factor (TNF)-α, IL-6 and IL-8] production in whole blood cultures, mediated by both the IL-10 and the IL-4 moiety. IL4-10 fusion protein induced IL-1 receptor antagonist (IL-1RA) production and preserved soluble TNF receptor (sTNFR) levels, strongly increasing IL-1RA/IL-1β and sTNFR/TNF-α ratios. In addition, IL4-10 FP strongly inhibited T helper (Th) type 1 and 17 cytokine secretion, while maintaining FoxP3 expression and up-regulating Th2 activity. In addition, while largely leaving expression of activating Fc gamma receptor (FcγR)I, III and Fc epsilon receptor (FcεR) unaffected, it significantly shifted the FcγRIIa/FcγRIIb ratio in favour of the inhibitory FcγRIIb. Moreover, IL4–10 FP robustly inhibited secretion of pro-inflammatory cytokines by rheumatoid arthritis synovial tissue and suppressed experimental arthritis in mice, without inducing B cell hyperactivity. IL4-10 fusion protein is a novel drug, signalling cells to induce immunoregulatory activity that overcomes limitations of IL-4 and IL-10 stand-alone therapy, and therefore has therapeutic potential for inflammatory diseases such as rheumatoid arthritis.

Keywords: arthritis, autoinflammatory diseases, cytokines, inflammation, Th1/Th2 cells

Introduction

Lack of efficacy by some biologicals may reflect that targeting one mediator of inflammation is insufficient to overcome redundancy of the immune system. Therefore, a multi-target approach constitutes an attractive therapeutic option. As interleukin 4 (IL-4) and IL-10 attenuate multiple inflammatory processes via distinct, overlapping and complementary mechanisms, both cytokines are promising candidates for treatment of inflammatory diseases [1,2] However, clinical trials with human IL-4 or IL-10 as stand-alone drugs did not fulfil the promise raised in preclinical studies. Patients with rheumatoid arthritis (RA) and psoriasis only modestly improved upon administration of IL-4 or IL-10 in Phase I/II studies, although both cytokines were well tolerated [3,4]

Disappointing results of IL-4 and IL-10 as stand-alone drugs in clinical trials may reflect their poor bioavailability or lack of synergy with other anti-inflammatory cytokines. Indeed, IL-4 and IL-10 synergistically inhibit many inflammatory activities and induce anti-inflammatory mechanisms such as IL-1 receptor antagonist (IL-1RA) induction

[2,5,6] IL-4 and IL-10 also synergize in experimental models for arthritis and, importantly, also mutually neutralize their pro-inflammatory effects, such as up-regulation of activating Fc receptors [2,7–9] Thus, a combination therapy of IL-4 and IL-10 is potentially superior to stand-alone therapy. However, high costs virtually exclude development of a combination therapy. Moreover, a combination therapy does not address the limited bioavailability of cytokines due to their relatively low molecular weight.

To solve these limitations we designed a prototype of a novel class of cytokines: fusion-proteins of two anti-inflammatory and/or regulatory cytokines, combining activities of these cytokines into one molecule. We generated a glycosylated IL4-10 fusion protein (IL4-10 FP, patent application no. 61/556,843 [10]) in its dimeric form ~70 kDa, and here demonstrate preserved additive function and strong immunosuppressive activity *in-vitro*, *ex-vivo* and *in-vivo*.

Methods

Additional methods and used materials are described in the Supporting information.

Cell and tissue cultures

Blood from healthy human controls was obtained from our in-house donor service. Synovial tissue was obtained from RA patients who underwent total knee arthroplasty. The study was approved by the medical ethics review board of the UMCU and all patients and healthy volunteers gave informed consent.

LPS-stimulated whole blood culture. Heparinized blood obtained from healthy volunteers was diluted 1 : 10 in RPMI-1640 medium, supplemented with penicillin/streptomycin (P/S). Lipopolysaccharide (LPS) was added at 10 ng/ml. IL4-10 fusion protein and controls recombinant human IL-4 and IL-10 were simultaneously added and titrated at equimolar concentrations, ranging from 0·01 to 3 nM. The concentration of 1·47 nM of IL4-10 fusion protein (50 ng/ml) was chosen for further experiments. Receptor blocking antibodies against IL-4 receptor (anti-IL-4R) or IL-10 receptor (anti-IL-10R) were added at 10 μg/ml and 20 μg/ml, respectively. After 18 h incubation at 37°C, 5% CO_2, supernatant was collected and stored at –80°C. The mouse whole blood assay was performed in the same way, using mouse blood from euthanized BALB/c control mice.

SEB-stimulated PBMC culture. Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors were isolated with Ficoll-Paque, cultured in RPMI GlutaMAX with 10% fetal bovine serum (FBS) and 1% P/S, in addition to staphylococcal enterotoxin-B (SEB) (0·1 ng/ml) and 1·47 nM IL-4, IL-10, the combination or IL4-10 fusion protein. Final culture conditions were 1×10^6 cells/ml, 0·5 ml/well, 37°C, 5% CO_2 for 42 h. Supernatant was collected and stored at –80°C.

Monocyte culture. Monocytes were isolated from healthy donor PBMCs using $CD14^+$ AutoMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) purification, cultured in RPMI GlutaMAX with 10% FBS and 1% P/S. To study Fc receptor expression, cells were cultured unstimulated, in addition to 1·47 nM IL-4, IL-10, the combination or IL4-10 fusion protein. Dose-dependent inhibition of tumour necrosis factor (TNF)-α by IL4-10 FP (0·01–3 nM) was studied using LPS (10 ng/ml) stimulated cells. Final culture conditions: 1×10^6 cells/ml, 0·5 ml/well, 37°C, 5% CO_2 for 42 h (and 18 h for titration curves of IL4-10 FP). For flow cytometry (to study FcR expression), cells were put on ice 30 min before harvesting. For quantitative polymerase chain reaction (qPCR), cell lysates were prepared using RLTplus lysis buffer. Supernatants were collected and stored at –80°C.

Synovial tissue culture. Weighed macroscopically identical synovial tissue pieces obtained from knee joints of RA patients were cultured for 72 h at 37°C, 5% CO_2 in the presence or absence of 1·47 nM IL4-10 fusion protein in RPMI GlutaMAX, with 10% human AB serum (hABS) and 1% P/S. Supernatant was collected and stored at –80°C.

Proliferation assay

PBMCs were stimulated with SEB (0·1 ng/ml) and cultured (1×10^6 cells/ml) in the presence or absence of IL4-10 fusion protein; total volume 200 μl/well, 37°C, 5% CO_2 for 42 h; [^{3}H]-thymidine was added (5 mCi/ml) during the last 18 h. Cells were harvested and [^{3}H]-thymidine incorporation measured by liquid scintillation counting. Incorporated tritium was counted and expressed in counts per minute.

Cytokine and antibody assessments

Supernatants were analysed for TNF-α, IL-1β, interferon (IFN)-γ, IL-5, IL-6, IL-8, IL-17, IL-1RA and soluble TNF receptor (sTNFR2) by Multiplex (Luminex, Austin, TX, USA) and/or enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Mice sera were analysed for immunoglobulin (Ig)G and IgE using ELISA kits, according to the manufacturer's instructions.

Flow cytometry

Viable monocytes were gated based on forward-/side-scatter and a viability dye. Fc gamma (IgG) receptor (FcγR) and Fc epsilon (IgE) receptor (FcεR) expression was evaluated using labelled antibodies against FcγRI,

FcγRIIa, FcγRIIb, FcγRIII and FcεRII. Forkhead box protein 3 (FoxP3) and Ki-67 staining was performed using an intracellular staining kit. Percentages of positive/negative cells were based on set markers using isotype controls. Cell acquisition was performed using the FACSCanto (BD Biosciences, San Diego, CA, USA) and analysed using FlowJo (Tree Star Inc., Ashland, OR, USA).

Gene expression by quantitative PCR

Expression of FcγRIIa and FcγRIIb2 genes in relation to glucuronidase beta (GUSB) as housekeeping gene was evaluated. Total ribonucleic acid (RNA) extraction from the cultured monocytes (as described above) was performed with the AllPrep Kit according to the manufacturer's instructions. Qubit (Thermo Fisher Scientific, Waltham, MA USA) was used to quantify the amount of total RNA extracted. RNA was reverse-transcribed with the Superscript IV kit, according to the manufacturer's instructions. Duplicate real-time PCR reactions were performed in the QuantStudio system (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) using specific FcγRIIa, FcγRIIb2 and GUSB primers with SyBR Select Master Mix, according to the manufacturer's instructions. Reactions were performed with the following thermal cycling profile: 2 min at 50°C followed by 5 min at 95°C, plus 40 cycles of 15 s at 95°C and 1 min at 60°C. Real-time qPCR data were normalized to the expression of GUSB and the relative gene expression of messenger RNA (mRNA) was calculated by using the formula: $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{mean}$ sample $- Ct_{mean}$ GUSB.

Proteoglycan-induced arthritis (PGIA)

Human proteoglycan (PG) was dissolved at a concentration of 2·5 mg/ml in PBS and emulsified in an equal volume of the synthetic adjuvant dimethyl-dioctadecyl ammonium bromide (DDA; Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS). Twenty-four-week old female BALB/c wild-type mice (Charles River Laboratories Inc., Wilmington, MA, USA) were used for immunization. The experiment was performed in accordance with the guidelines of the animal ethical committee. All mice were immunized intraperitoneally (i.p.) with 200 µl of the emulsion (250 µg PG and 2 mg DDA); on day 21 mice were boosted with 200 µl i.p. injection of the same emulsion. Mice were examined for onset and severity of disease in a blinded manner. Arthritis symptoms were graded by the following scoring system: grade 0, normal appearance; grade 1, slight erythema/oedema (1–3 digits); grade 2, erythema/oedema in more than three digits or mild swelling in ankle/wrist joint; and grade 3, erythema/oedema in entire paw; grade 4, massive erythema/oedema of entire paw extending into proximal joints, ankylosis and loss of function. Each limb was graded, giving a maximum possible score of 16 per mouse. Arthritis incidence and severity was assessed from day 21 onwards and was defined by a single affected paw of at least 1 point. Only upon onset of arthritis at day 28, mice were included in the study and were divided into two groups on the basis of arthritis scores. Mice were treated with intravenous injections of 200 ng mouse IL4-10 fusion protein or PBS as a control. Injections were given every day for a period of 4 days, starting at day 28. Mice were euthanized on day 35. Blood was retrieved and processed for measurement of IgG and IgE in sera of all mice.

Statistical analysis

Cytokine concentrations in supernatants, Fc receptor expression, proliferation and expression of FoxP3 were analysed by the Wilcoxon signed-rank test, comparing the (stimulated) medium condition to the IL4-10 FP condition. Statistical analysis of the mIL4-10 FP effect on PGIA was analysed by an unpaired *t*-test, as data were normally distributed (D'Agostino–Pearson normality test).

Results

IL4-10 fusion protein robustly suppresses pro-inflammatory cytokine production and alters the balance between pro-inflammatory cytokines and their naturally occurring inhibitors

Recombinant human IL4-10 fusion protein dose-dependently inhibited TNF-α production in LPS-stimulated whole blood cultures (Fig. 1a). Monocytes are assumed to be the main targets in our LPS-stimulated whole blood assay. Here we demonstrate that IL4-10 FP inhibited TNF-α production equally effectively by isolated monocytes as in whole blood (Fig. 1b). In addition, prolonged exposure of LPS-stimulated whole blood to IL4-10 FP (18 *versus* 42 h) resulted in stronger inhibition of TNF-α production (Fig. 1b). The effect of IL4-10 fusion protein on TNF-α production was abolished by receptor-blocking antibodies against the IL-4-receptor (anti-IL-4R) or the IL-10 receptor (anti-IL-10R), indicating that the effect of IL4-10 FP is mediated by both moieties of the protein (Fig. 1c). IL4-10 FP also strongly inhibited IL-1β production (Fig. 1d). Importantly, IL4-10 fusion protein left activation-induced production of IL-1RA and sTNFR, naturally produced antagonists of IL-1β and TNF-α, largely unaffected, strongly increasing IL-1RA/IL-1β and sTNFR/TNF-α ratios (Fig. 1d).

IL4-10 fusion protein skews Th1/Th17 activity towards Th2 activity and sustains regulatory T cells

Superantigen SEB induced IFN-γ, IL-17 and IL-5 production, lymphocyte ([^{3}H]-thymidine incorporation) and CD4

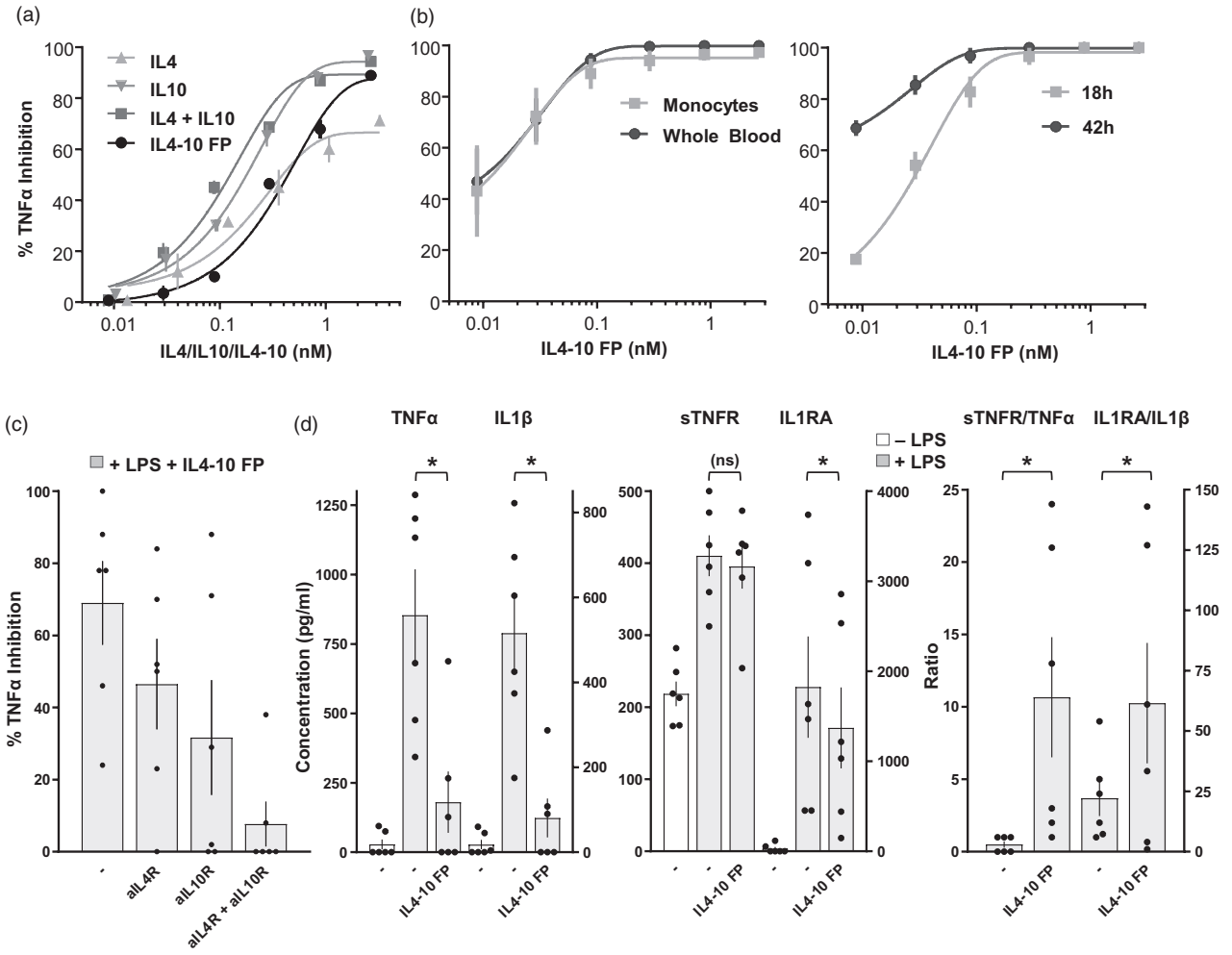

Fig. 1. IL4-10 fusion protein (IL4-10 FP) protein alters the balance of tumour necrosis factor (TNF)-α and IL-1β and their naturally occurring inhibitors *in-vitro*. (a) Diluted (1 : 10 v/v) heparinized blood from healthy volunteers was incubated for 18 h with lipopolysaccharide (LPS) to induce TNF-α release. The capacity of different concentrations of human IL4-10 FP to inhibit TNF-α production in LPS-stimulated whole blood cell cultures was compared to IL-4 and IL-10 ($n = 4$). (b) The potency of IL4-10 FP to inhibit LPS-induced TNF-α production by whole blood and isolated monocytes ($n = 4$) was compared. In addition, the capacity of prolonged incubation (42 h) to enhance the potency of IL4-10 FP to inhibit LPS-induced TNF-α production in whole blood was studied ($n = 2$). (c) To indicate the requirement of both the IL-4 and IL-10 moiety IL4-10 FP-mediated inhibition (1·47 nM) of TNF-α production in whole blood was tested upon blockade of the IL-4 and IL-10 receptor (IL-4R and IL-10R) using monoclonal antibodies. (d) Next to TNF-α, the potency of IL4-10 FP to regulate LPS-induced IL-1β production as well as naturally occurring inhibitors IL-1RA and soluble TNFR in whole blood was studied. In addition, the potency to skew the balance of IL-1 receptor agonist (RA)/IL-1β and soluble TNF receptor (sTNFR)/TNF-α was studied. Bars (containing individual data points) and graphs represent mean and standard error of the mean (s.e.m.). *Statistically significant difference of $P < 0{\cdot}05$.

T cell proliferation (Ki-67 expression) in PBMC cultures, which was associated with increased numbers of FoxP3-expressing regulatory T cells (Fig. 2). Production of the pro-inflammatory cytokines IFN-γ and IL17, indicative of T helper type 1 (Th1) and Th17 activity, respectively, were reduced by IL4-10 FP, which effect was comparable to that of IL-10 alone or the combination of IL-4 and IL-10 (Fig. 2a). Opposite to this down-regulation, IL-5 production, indicative of Th2 activity, was up-regulated by IL-4 alone and by IL4-10 FP. Pooled data demonstrated that IL4-10 FP significantly inhibited antigen-induced Th1 and Th17 cytokine secretion (IFN-γ, IL-17) and up-regulated Th2 activity (IL-5) (Fig. 2b). In addition, IL4-10 FP sustained proliferation and the number of FoxP3-expressing regulatory T cells (Fig. 2c).

IL4-10 fusion protein minimally affects IgG and IgE receptor levels

Consistent with the literature, we found that IL-4 and IL-10 regulate Fcγ and Fcε receptor expression

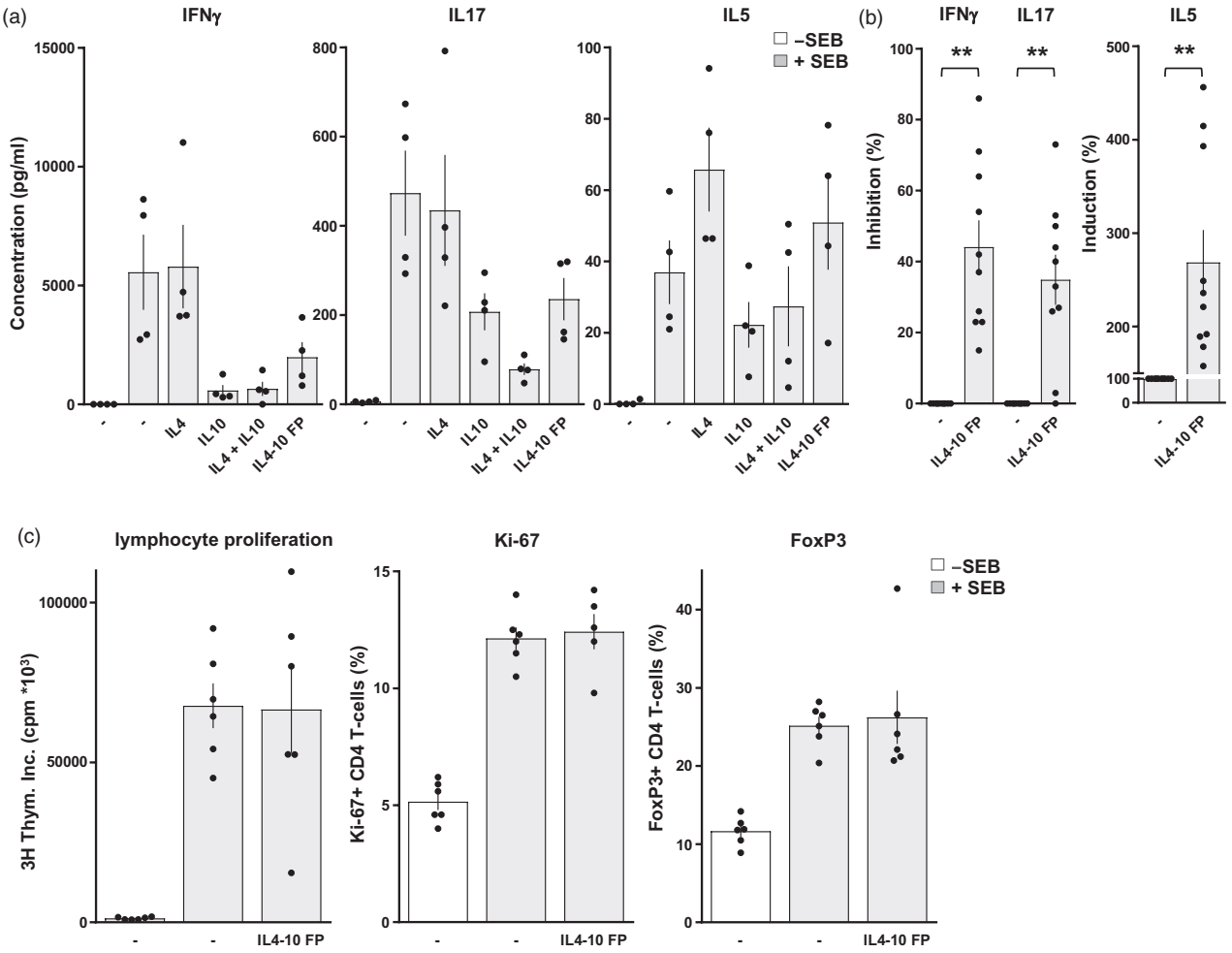

Fig. 2. IL4-10 FP skews T helper (Th) type 1 and 17 activity towards Th2 activity and sustains regulatory T cells. Blood mononuclear cells were cultured with superantigen staphylococcal enterotoxin B (SEB) (42 h) in the presence of IL-4, IL-10, the combination or IL4-10 FP (all 1·47 nM). (a) IL4-10 FP-induced regulation of Th1-, Th2- and Th17-associated cytokines [interferon (IFN)-γ, IL-5 and IL-17, respectively] was studied (n = 4). (b) T cell cytokine production by SEB-activated peripheral blood mononuclear cells (PBMCs) upon IL4-10 FP was measured in an additional six donors. Pooled data (n = 10), expressed as % change *versus* medium-only cultures are shown. (c) In addition, the effect of IL4-10 FP on proliferation of lymphocytes ([^{3}H]-thymidine incorporation) and CD4 T cells (percentage of Ki-67-expressing cells) and on the percentage of regulatory T cells [percentage of forkhead box protein 3 (FoxP3)-expressing cells, n = 6] was studied. Bars (containing individual data points) represent mean and standard error of the mean (s.e.m.). **Statistically significant difference of $P < 0{\cdot}01$.

differentially on monocytes. IL-4 inhibited expression of activating Fcγ receptors I, IIa and III and up-regulated FcεR, whereas IL-10 increased FcγRI, IIa, IIb and III, not affecting FcεR (Fig. 3a,c). The combination of IL-4 and IL-10 largely normalized Fcγ and FcεRs, confirming previous literature showing that IL-4 controls IL-10 up-regulated FcRs and vice versa. Pooled data revealed that IL4-10 FP did not significantly alter the expression of monocyte activating FcγRI, IIa and IIb and minimally increased FcγRIII and FcεR expression compared to control cultures (Fig. 3b,d). Interestingly, IL4-10 FP significantly changed the ratio of FcγRIIb/IIa in favour of the inhibitory FcγRIIb (Fig. 3d). This was confirmed by measuring mRNA levels of either receptor (Fig. 3e).

IL4-10 fusion protein inhibits *ex-vivo* cytokine production by synovial tissue of arthritic patients and inhibits experimental arthritis

In line with the inhibition of IL-1β and TNF-α (Fig. 1), human IL4-10 FP strongly inhibited secretion of pro-inflammatory cytokines IL-6 and IL-8 in whole blood cultures triggered by LPS (Fig. 4a). In addition, spontaneous production of IL-6 and IL-8 by RA synovial tissue *ex-vivo* was markedly inhibited by IL4-10 FP (Fig. 4b). Finally, to further explore the therapeutic potential of IL4-10 fusion protein we assessed its potency to inhibit experimental proteoglycan-induced arthritis. For *in-vivo* mouse studies, a mouse recombinant IL4-10 fusion protein

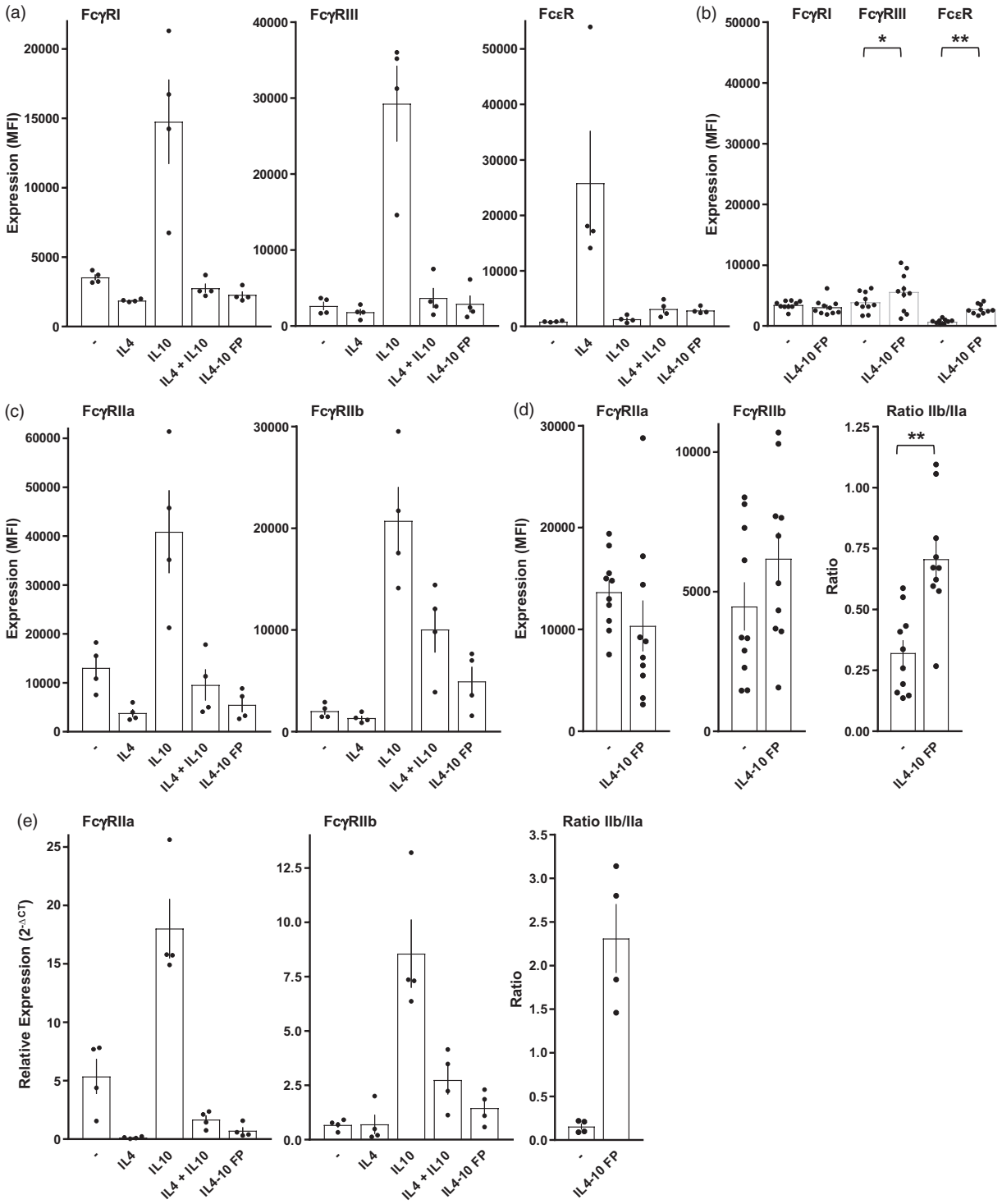

Fig. 3. IL4-10 FP sustains normal expression of immunoglobulin receptors on monocytes. Isolated monocytes were cultured (42 h) in the presence of IL-4, IL-10, the combination or IL4-10 FP (all 1·47 nM). (a) The capacity of IL4-10 FP and the individual and combined cytokines to (differentially) regulate different subclasses of Fc gamma receptor (FcγR) (I and III) and Fc epsilon receptor (FcεR) expression was measured using flow cytometry ($n = 4$). (b) Expression of the FcγR subclasses and FcεR on monocytes upon IL4-10 FP was measured in an additional six donors. Pooled data are shown ($n = 10$). (c) The effect of IL4-10 FP, the individual and combined cytokines on the activating FcγRIIa and inhibitory FcγRIIb was also studied using flow cytometry ($n = 4$). (d) In an additional six donors, regulation of IL4-10 FP on FcγRIIa and FcγRIIb expression as well as their ratio was measured. Pooled data are shown ($n = 10$). (e) The effect of IL4-10 FP on FcγRIIa and FcγRIIb gene expression as well as their ratio was additionally measured using quantitative polymerase chain reaction (PCR) ($n = 4$). Bars (visualizing individual data points) represent mean and standard error of the mean (s.e.m.). ***Statistically significant differences of $P < 0{\cdot}05$ and $0{\cdot}01$, respectively.

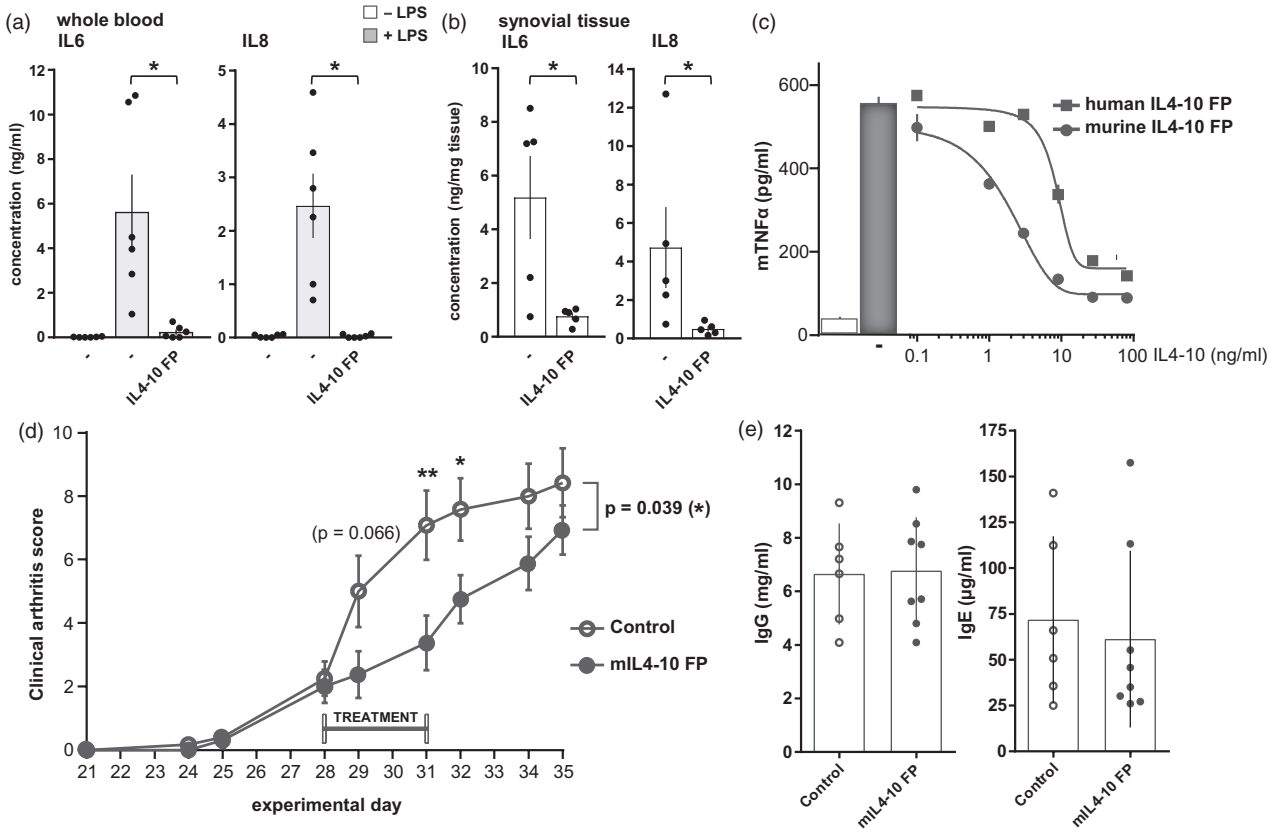

Fig. 4. IL4-10 FP inhibits inflammatory activity of *ex-vivo* cultured rheumatoid arthritis (RA) synovial tissue and suppresses established experimental arthritis. (a) Lipopolysaccharide (LPS)-induced secretion of interleukin (IL)-6 and IL-8 in whole blood cultures was measured upon treatment with IL4-10 FP ($n = 6$). (b) Furthermore, IL4-10 FP effects on spontaneous release of IL-6 and IL-8 from RA synovial tissue explants were studied ($n = 5$). (c) For *in-vivo* mouse studies, a mouse recombinant IL4-10 fusion protein (mIL4-10 FP) was developed. The potency of mIL4-10 FP and human IL4-10 FP to inhibit tumour necrosis factor (TNF)-α release in a LPS-stimulated mouse whole blood assay was investigated. (d) The ability of mIL4-10 FP upon intravenous injection to inhibit arthritis severity in established proteoglycan-induced arthritis in mice was studied (mIL4-10 FP was administered on 4 subsequent days, starting from day 28). (e) mIL4-10 FP effects on B cell activity were studied by measuring serum immunoglobulin (Ig)G or IgE levels using enzyme-linked immunosorbent assay (ELISA) [$n = 6$ for control/phosphate-buffered saline (PBS) and $n = 8$ for the mI-L4–10 FP group]. Bars (visualizing individual data points) and graphs represent mean and standard error of the mean (s.e.m.). *,**Statistically significant differences of $P < 0{\cdot}05$ and 0·01, respectively.

(mIL4–10 FP) was developed to avoid induction of neutralizing antibodies against the human fusion protein. Mouse recombinant IL4-10 FP dose-dependently inhibited TNF-α production in mouse whole blood assays, and turned out to be more potent than human IL4-10 FP (Fig. 4c). mIL4-10 fusion protein significantly suppressed disease severity in established experimental arthritis in mice (Fig. 4d), without inducing B cell hyperactivity, as measured by IgG and IgE serum levels that were not changed upon IL4-10 FP treatment (Fig. 4e).

Discussion

In this study we show that IL4-10 fusion protein inhibits the production of a number of key pro-inflammatory cytokines in LPS-stimulated whole blood cultures. It also inhibits production of superantigen-induced pro-inflammatory Th1/Th17 activity, while preserving regulatory T cell activities. IL4-10 FP also shifts the balance of IL-1β and TNF-α towards their naturally occurring antagonists IL-1RA and sTNFR, and largely preserves normal Fcγ receptor expression by monocytes. Moreover, IL4-10 FP decreases pro-inflammatory cytokine production from *ex-vivo* synovium cultures of RA patients and inhibits established experimental arthritis.

While inhibiting production of multiple monokines and Th1/Th17 cytokines, IL4-10 fusion protein *in-vitro* clearly skewed T cell activity towards Th2 activity, as witnessed by increased levels of IL-5 (and IL-13, data not shown) and Fc·R expression. Similar polarization towards Th2 activity due to atopic diseases is associated with a good clinical outcome of diseases such as RA

[11] It is unclear whether these beneficial *in-vitro* effects of IL4-10 FP can be extrapolated to *in-vivo* conditions. Although obvious care should be taken with respect to induction of atopic responses, apparently the generation of allergic reactions requires additional triggers, as in clinical trials with IL-4 or IL-10 no allergic-type responses were induced [11], not even upon high doses of IL-4 [12] In line with this, mIL4-10 FP inhibits experimental arthritis without inducing B cell hyperactivity, and in particular IgE concentrations are not significantly altered. The exact mechanisms behind this lack of B cell stimulation is unclear, but might be related to the activity of IL-10 to inhibit IgG and IgE production by (IL-4/T cell activated) B cells if in the context of antigen-presenting cells such as monocytes, as has previously been demonstrated [13].

IL4-10 fusion protein may be used to target Th1/Th17 driven inflammatory diseases such as RA and spondyloarthropathies, but also to target inflammation in conditions associated with monokine production (IL-1β, TNF-α, IL-6) such as osteoarthritis, and many more. Essentially, all diseases considered for IL-10 treatment in the past may be targets for IL4-10 fusion protein. Compared with IL-10, IL4-10 FP has several advantages. First, some adverse effects of IL-10 are prevented by the IL-4 moiety of the cytokine. For example, activating FcγRs are up-regulated by IL-10, which enhances immune complex-mediated events in IL-10-treated RA patients [9,14] IL-4 counteracts the increased expression of FcγRs by IL-10 [2] This counteracting effect results in constant levels of FcγRs [2] as we also observed for IL4-10 FP. In addition, IL4-10 FP skews the balance of activating FcγRIIa to inhibitory FcγRIIb. Secondly, IL4-10 fusion protein combines the activities of two different anti-inflammatory cytokines in a single molecule, including synergistic activities that have been demonstrated previously [2,5] Therefore, IL4-10 FP may have more potential than stand-alone IL-4 or IL-10 in some of these diseases, for example in arthritis [2,5,8,15] Thirdly, IL4-10 fusion protein has an improved bioavailability compared to their natural counterparts due to a larger molecular weight. Proteins with a molecular weight < 60 kDa are filtered through the basal membrane in the glomeruli and are cleared rapidly by the kidney. Following intravenous administration, recombinant human IL-10 has a terminal phase half-life of 2·7–4·5 h [16] Due to its longer peptide chain the molecular weight of the IL4-10 fusion protein is larger than that of IL-10 (~70 kDa). Glycosylation of IL4-10 FP further adds to its molecular weight. We recently performed a pharmacokinetic study in rats. Human IL4-10 FP was shown to have an increased half-life compared to human IL-10 (C. Steen-Louws, manuscript in preparation), demonstrating an increased bioavailability. To demonstrate the feasibility of IL4-10 fusion protein, we recently tested its potential in animal models for inflammatory and neuropathic chronic pain. IL4-10 FP strongly relieved hyperalgesia in all models, and inhibited pain superior to IL-4 or IL-10 mono or combination therapy [10] This emphasizes the potential of IL4-10 fusion protein to inhibit immunopathology and pain in inflammatory and degenerative diseases.

IL4-10 fusion protein is a new drug that signals cells to induce immunoregulatory activity and overcomes the limitations of IL-4 and IL-10 stand-alone therapy, and therefore has therapeutic potential for inflammatory diseases such as rheumatoid arthritis. Recent data suggest an even broader potential as, next to prevention of pain [10], IL4-10 FP suppresses articular cartilage damage in models for osteoarthritis [17].

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Disclosures

The authors declare no conflicts of interest.

Author contributions

C. S.-L. performed the experiments, analysed the data and wrote the paper; S. A. Y. H. performed the experiments, analysed the data and wrote the paper; J. P.-C. performed the experiments; A. P. L. performed the experiments; M. B. M. de S. performed the experiments; N. E. performed the experiments; F. P. J. G. Lafeber designed the study; C. E. H. designed the study and wrote the paper; J. A. G. van R. designed the study, analysed the data and wrote the paper.

References

1 Chomarat P, Banchereau J, Miossec P. Differential effects of interleukins 10 and 4 on the production of interleukin-6 by blood and synovium monocytes in rheumatoid arthritis. Arthritis Rheum 1995; **38**:1046-54.

2 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 Rheum 2001; **44**:3-12.

3 van den Bosch F, Russel A, Keystone EC *et al.* rHuIL-4 in subjects with active rheumatoid arthritis: a phase I dose escalating safety study [abstract]. Arthritis Rheum 1998; **41**(Suppl. 9):S56.

4 Maini RN, Paulus H, Breedveld FC *et al*. rHuIL-10 in subjects with active rheumatoid arthritis: a phase I and cytokine response study [abstract]. Arthritis Rheum 1997; **40**(Suppl. 9):S224.
5 Powrie F, Menon S, Coffman RL. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity *in vivo*. Eur J Immunol 1993; **23**:2223-9.
6 Marie C, Pitton C, Fitting C, Cavaillon JM. IL-10 and IL-4 synergize with TNF-alpha to induce IL-1ra production by human neutrophils. Cytokine 1996; **8**:147-51.
7 Pricop L, Redecha P, Teillaud JL *et al*. Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. J Immunol 2001; **166**:531-7.
8 Joosten LA, Lubberts E, Durez P *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 Rheum 1997; **40**:249-60.
9 van Roon JA, Wijngaarden S, Lafeber FP, Damen C, van de Winkel J, Bijlsma JW. Interleukin 10 treatment of patients with rheumatoid arthritis enhances Fc gamma receptor expression on monocytes and responsiveness to immune complex stimulation. J Rheumatol 2003; **4**:648-51.
10 Eijkelkamp N, Steen-Louws C, Hartgring SA *et al*. IL4-10 fusion protein is a novel drug to treat persistent inflammatory pain. J Neurosci 2016; **36**:7353-63.
11 van Roon JA, Bijlsma JW, Lafeber FP. Suppression of inflammation and joint destruction in rheumatoid arthritis may require a concerted action of Th2 cytokines. Curr Opin Investig Drugs 2002; **3**:1011-6.
12 Atkins MB, Vachino G, Tilg HJ *et al*. Phase I evaluation of thrice-daily intravenous bolus interleukin-4 in patients with refractory malignancy. J Clin Oncol 1992; **10**:1802-9.
13 Punnonen J, de Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. J Immunol 1993; **151**:1280-89.
14 te Velde AA, de Waal Malefijt R, Huijbens RJ, de Vries JE, Figdor CG. IL-10 stimulates monocyte Fc gamma R surface expression and cytotoxic activity. Distinct regulation of antibody-dependent cellular cytotoxicity by IFN-gamma, IL-4, and IL-10. J Immunol 1992; **149**:4048-52.
15 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 Rheum 1996; **39**:829-35.
16 Huhn RD, Radwanski E, Gallo J *et al*. Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. Clin Pharmacol Ther 1997; **62**:171-80.
17 Steen-Louws C, Popov-Celeketic J, Mastbergen SC *et al*. IL4-10 fusion protein has chondroprotective, anti-inflammatory and potentially analgesic effects in the treatment of osteoarthritis. Osteoarthritis Cartilage 2018; **26**:1127-35.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Supplementary File. Additional Methods & Materials.

