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 (FceR) 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. IL-4-10 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 (IL4-10 FP) in its dimeric form ~70 kDa, and here demonstrate preserved additive function and strong immunosuppressive activity in-vitro, ex-vivo and in-vivo.

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% CO₂ 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 (1×10⁶ cells/ml) in the presence or absence of IL4-10 fusion protein; total volume 200 µl/well, 37°C, 5% CO₂ for 42 h; [³H]-thymidine was added (5 mCi/ml) during the last 18 h. Cells were harvested and [³H]-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 (FcyR) and Fc epsilon (IgE) receptor (FceR) expression was evaluated using labelled antibodies against FcyRI,
FcyRIIa, FcyRIIb, FcyRIII 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 FcyRIIa and FcyRIIb2 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 FcyRIIa, FcyRIIb2 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−△Ct, where △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 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 ([3H]-thymidine incorporation) and CD4
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 Th1 helper type 1 (Th1) and Th17 activity, respectively, were reduced by IL4-10 FP, which effect was comparable to that of IL-4 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 Fcy and Fce receptor expression.
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differentially on monocytes. IL-4 inhibited expression of activating Fcγ receptors I, Ia and III and up-regulated FcεR, whereas IL-10 increased FcγRI, Ia, 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, Ia 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 (FcyR) (I and III) and Fc epsilon receptor (FceR) expression was measured using flow cytometry (n = 4). (b) Expression of the FcyR subclasses and FceR 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 FcyRIIa and inhibitory FcyRIIb was also studied using flow cytometry (n = 4). (d) In an additional six donors, regulation of IL4-10 FP on FcyRIIa and FcyRIIb expression as well as their ratio was measured. Pooled data are shown (n = 10). (e) The effect of IL4-10 FP on FcyRIIa and FcyRIIb 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 < 0·05 and 0·01, respectively.
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(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 Fcy 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 FcR expression. Similar polarization towards Th2 activity due to atopic diseases is associated with a good clinical outcome of diseases such as RA.

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 IL-4-10 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 mIL4–10 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 < 0.05 and 0.01, respectively.

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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. 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; 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.

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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.