Antibody-based targeted delivery of interleukin-4 synergizes with dexamethasone for the reduction of inflammation in arthritis
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Anja Sophie Schmid¹, Teresa Hemmerle², Francesca Pretto², Anja Kipar³ and Dario Neri¹

Abstract

Objectives. We have previously reported that F8-IL4, a fusion protein consisting of the F8 antibody specific to the alternatively-spliced extra domain A of fibronectin and of murine IL-4, cures mice with established arthritis, when used in combination with dexamethasone (DXM). The goal of this study was to assess whether other therapeutic agents, besides DXM, could induce cures in combination with F8-IL4 and to elucidate which leucocytes are most affected by the pharmacological treatment.

Methods. We performed therapy experiments in mice with CIA, using intravenous administrations of F8-IL4 in combination with DXM, MTX, murine cytotoxic T-lymphocyte-associated protein 4 fused to the fragment crystallizable portion of murine IgG2a, as well as mAbs to murine IL17A or the p40 subunit of murine IL12/IL23. Histology and immunohistochemistry for the identification of the various leucocytes were performed on the paws of mice euthanized at different therapy time points.

Results. Only the use of F8-IL4 in combination with DXM induced complete remissions, while all other combinations did not lead to cures. The light microscopical evaluation of paws with arthritis revealed a predominant infiltration of neutrophils, which substantially decreased 24 h after treatment with F8-IL4 and DXM.

Conclusion. The combination of F8-IL4 with DXM promotes a rapid anti-arthritic action by potently inhibiting neutrophil activity. A fully human analogue of F8-IL4 may find clinical utility for the treatment of neutrophil-driven chronic inflammatory conditions.

Key words: collagen-induced arthritis, interleukin-4, immunocytokines, EDA of fibronectin, Type II interleukin-4 receptor, neutrophils

Introduction

The advent of antibody-based products, capable of neutralizing TNF or other cytokine mediators of inflammation, has revolutionized the treatment of patients with RA and other chronic inflammatory conditions [1]. Unfortunately, however, a substantial portion of RA patients does not respond to treatments with various lines of biologics [2, 3]. Furthermore, even for those patients who benefit from treatment with biopharmaceuticals [e.g. TNF blockers, IL6...
receptor blockers, anti-CD20 antibodies and/or fusion proteins of cytotoxic T-lymphocyte-associated protein 4 (CTLA4)], the disease remains incurable and can lead to progressive disability with the destruction of cartilage and bone, systemic complications such as cardiovascular or pulmonary diseases and a higher risk for lymphoma and early death [4]. Thus, there is a need for further improvement in the treatment and management of RA patients.

Mice with CIA have often been used as an animal model to test novel therapeutic modalities [5, 6], thus facilitating subsequent clinical translation activities. In this model, blockade of murine TNF inhibits disease progression, but does not cure the disease [5, 7–10]. Similarly, MTX [11, 12], mouse analogues of abatacept [13, 14], as well as mAbs to murine IL-17A (serving as mimics for the biopharmaceuticals secukinumab and ixekizumab) [15, 16] or the p40 subunit of murine IL12/IL23 (serving as mimics for ustekinumab) [17] have shown activity in the CIA model but no induction of complete and durable remissions.

The antibody-based pharmacodelivery of immunomodulatory cytokines has been proposed as an alternative avenue for the treatment of chronic inflammatory conditions and our laboratory has produced and evaluated immunocytokines based on several immunomodulatory payloads [18, 19]. So far, the only product that could cure mice with established CIA was F8-IL4, a fusion protein consisting of the F8 antibody (which recognizes the alternatively spliced extra domain A of fibronectin) and murine IL4, when used in combination with dexamethasone (DXM) [20, 21]. Interestingly, when used as single agents, neither DXM nor F8-IL4 was able to induce complete remissions. The objective of this study was to discern whether (i) MTX, (ii) murine CTLA4 fused to the Fc portion of murine IgG2a (muCTLA4-Fc) [C4483, Sigma-Aldrich, St Louis, MO, USA] (n = 8), a mAb to murine IL17A (anti-IL17A) (clone 17F3, BioXCell, Lebanon, NH, USA) (n = 8), a mAb to the p40 subunit of murine IL12/IL23 (anti-IL12/IL23) (clone C17.8, BioXCell) (n = 7) or DXM (Dexamethasone Helvepharm, Zentiva, Frauenfeld, Switzerland) (n = 7). On days 1, 4 and 7, the control group received 100 μg PBS i.v. and the treatment groups 100 μg of F8-IL4 i.v. with one of the following: 50 μg MTX (2.5 mg/kg) i.p., 100 μg muCTLA4-Fc i.p., 100 μg anti-IL17A i.p. or 100 μg anti-IL12/IL23 i.p. The clones, dosages and routes of administration were chosen on the basis of previous reports, which had shown efficacy in murine animal models [12–14, 16, 17, 23]. Mice in the combination group with DXM received 100 μg of F8-IL4 i.v. on days 1 and 4 and 100 μg DXM i.p. until the day of sacrifice (the mice were all sacrificed before day 7 for histological analyses). Of all mice the arthritic scores, weights and thicknesses of inflamed paws were monitored daily in a blinded fashion. Mice were sacrificed on day 5 (PBS), day 8 (MTX), day 9 (muCTLA4-Fc) and day 21 (anti-IL12/IL23) due to reaching termination criteria based on arthritic score and weight loss in accordance with local regulations. Mice from the DXM group were sacrificed concomitantly at various time points and mice from the anti-IL17A group were sacrificed on day 28 as this is the maximum amount of time our licence allows us to keep mice after onset and treatment of arthritis if termination criteria are not reached.

Combination therapy

Mice with a new arthritic score of 1–6 were randomly assigned to receive either PBS (n = 8, control group) or a combination of F8-IL4 and MTX (Methotrexate, Pfizer, Zürich, Switzerland) (n = 8), murine CTLA4 fused to the Fc portion of murine IgG2a (muCTLA4-Fc) (C4483, Sigma-Aldrich, St Louis, MO, USA) (n = 8), a mAb to murine IL17A (anti-IL17A) (clone 17F3, BioXCell, Lebanon, NH, USA) (n = 8), a mAb to the p40 subunit of murine IL12/IL23 (anti-IL12/IL23) (clone C17.8, BioXCell) (n = 7) or DXM (Dexamethasone Helvepharm, Zentiva, Frauenfeld, Switzerland) (n = 7). Of all mice the arthritic scores, weights and thicknesses of inflamed paws were monitored daily in a blinded fashion. Mice were sacrificed on day 5 (PBS), day 8 (MTX), day 9 (muCTLA4-Fc) and day 21 (anti-IL12/IL23) due to reaching termination criteria based on arthritic score and weight loss in accordance with local regulations. Mice from the DXM group were sacrificed concomitantly at various time points and mice from the anti-IL17A group were sacrificed on day 28 as this is the maximum amount of time our licence allows us to keep mice after onset and treatment of arthritis if termination criteria are not reached.

Histological and immunohistochemical examinations

Immediately after euthanasia, paws from selected animals [controls on day 1 (n = 2) and day 5 (n = 1) after onset of the disease; F8-IL4 combination group with DXM at 6 h (n = 1),
12 h (n = 1), 24 h (n = 2) and 72 h (n = 1) after the first F8-IL4 treatment and at 24 h (n = 1) and 48 h (n = 1) after the second F8-IL4 treatment] were removed and fixed in 4% paraformaldehyde in PBS (pH 7.4) for ~48 h, then decalcified in EDTA (Biosystems, Muttenz, Switzerland) for 14 days. Decalcified paws were trimmed (sagittal hemisections, comprising the phalanges to the radius and tibia, respectively) and routinely paraffin wax embedded [24]. Serial sections (3–5 μm) were prepared and stained with haematoxylin–eosin for the histological examination, or used for immunohistochemical staining.

Immunohistochemistry was performed to identify neutrophils and neutrophil extracellular traps (NETs), macrophages, T cells and B cells, using the horseradish peroxidase and the avidin–biotin complex method. The following primary antibodies were applied: rat anti-mouse Ly6G (neutrophil marker; clone 1A8, BioLegend, San Diego, CA, USA), rabbit anti-Iba-1 (macrophage marker; antigen: AI1; Wako Chemicals, Zurich, Switzerland), mouse anti-human CD3 (T cell marker; clone F7.2.38, Agilent Technologies, Basel, Switzerland), rat anti-mouse CD45R (B cell marker; clone B220, BD Biosciences, Allschwil, Switzerland) and rabbit anti-histone H3 (citrulline R2 + R8 + R17; NET marker, Abcam, Cambridge, UK). Briefly, after deparaffination, sections underwent antigen retrieval in citrate buffer (pH 6.0, 20 min at 98 °C; for Ly6G, Iba-1 and CD45R) and EDTA buffer (pH 9.0, 20 min at 98 °C; for CD3), followed by blocking of endogenous peroxidase (peroxidase block, S2023, Dako, Baar, Switzerland) for 10 min at room temperature (RT). Slides were then incubated with the primary antibodies (diluted in dilution buffer, Dako) for (i) CD3 and Iba-1 (60 min at RT), followed by a 30 min incubation at RT with the secondary antibody (Envision mouse and rabbit, respectively, Dako) in an autostainer (Dako) and (ii) Ly6G (60 min at RT) and CD45R (overnight at 4 °C), followed by rabbit anti-rat IgG and the avidin–biotin complex kit (both 30 min at RT; Ventana, Tucson, AZ, USA). Staining for histone H3 was undertaken with an autostainer (Discovery XT, Ventana), using citrate buffer, dilution buffer and detection kits provided by the manufacturer. The antibody reaction was visualized with 3,3′-diaminobenzidin and sections counterstained with hemalaun.

Sections of a mouse spleen served as positive controls for the leucocyte markers. For negative controls, the primary antibody was omitted.

All examinations were undertaken by a veterinary pathologist (A.K.) who was blinded to the treatment of the animals and the arthritis scores when assessing the histological and immunohistochemical specimens.

Results

Combination therapy studies in mice with CIA

The therapeutic activity of combination treatments of F8-IL4 with methotrexate, murine CTLA4 fused to the Fc portion of murine IgG2a (an analogue of abatacept), as well as the mAb to murine IL17A and the mAb to the p40 subunit of murine IL12/IL23 was assessed in the collagen-induced model of arthritis in male DBA/1J mice. The combination of F8-IL4 with these drugs reduced the severity of arthritis by decreasing the arthritic score and paw swelling, as compared to the saline control. The combination of F8-IL4 with methotrexate showed the strongest therapeutic effect, with a reduction in the arthritic score of about 50% after 30 days of treatment.

All therapeutics were administered on days 1, 4 and 7 after arthritis onset except dexamethasone, which was given daily during treatment course until sacrifice. Therapeutic efficacy was evaluated daily using (A) the arthritic score expressed as the sum of the scores of all four paws, (B) weight given as percentage of the starting weight on day of enrolment, and (C) paw swelling depicted as the mean of the thickness of all four paws, with the dashed line at 2.26 mm indicating baseline paw thickness of mice without disease (n = 7–8; mean and s.e.m.).
therapy of F8-IL4 with DXM and the therapeutic use of the single agents have been reported previously [20, 21]. The combination therapy was performed again to generate specimens for histological analysis and characterization of the inflammatory process. As previously described, the combined use of DXM and F8-IL4 led to a rapid reduction of arthritic score and paw thickness (Fig. 1). In contrast, none of the other combination modalities led to complete regressions of the disease. Among those regimens, the combination of F8-IL4 with the anti-IL17A antibody yielded the best results with a stabilization of the arthritic score and a decrease in paw thickness, but much more slowly and to a lesser extent compared with F8-IL4 plus DXM (Fig. 1). All other therapeutic combination groups had to be sacrificed before the pre-defined end point at day 28, as the termination criteria were reached.

In situ analysis of the extent and composition of the inflammatory process in the paws in the course of CIA with and without pharmacological intervention

A detailed histological and immunohistochemical examination was undertaken on selected paws of control CIA animals (scores 0–4) to assess the time course of the inflammatory processes and on animals treated with F8-IL4 in combination with DXM. Representative findings are shown in Figs 2–4. Score 0 paws did not exhibit any pathological changes, and no inflammatory cell infiltration. In affected paws a variably intense (increasing with higher scores) neutrophil (Ly6G⁺)-dominated infiltration (with abundant degenerate neutrophils and extensive NET deposition) was observed around tendons and along the long bones, extending into the subcutis and, focally, the epidermis, between skeletal muscle bundles and into the joint spaces. The inflammatory infiltrate also contained a moderate proportion of macrophages (Iba-1⁺) and small numbers of individual T cells (CD3⁺), whereas B cells (CD45R⁺) were almost entirely absent (Figs 2 and 3).

Already after the first administration of F8-IL4 plus DXM, but consistently after the second administration, the inflammatory processes had either entirely resolved or had decreased to a mild or moderate inflammatory infiltration around the joints or along the long bones (Fig. 4; supplementary Figs S1 and S2, available at Rheumatology online). This was still neutrophil dominated (Fig. 4C), with few macrophages, rare T cells and no B cells. The histological findings were in concomitance with a drop of arthritic score.
Treatment with F8-IL4 in combination with DXM induced a substantial reduction of the inflammatory processes in CIA, which was evident shortly after the first administration of the combination therapy.

**Discussion**

All investigated combination partners chosen in this study corresponded to pharmaceutical agents that are often used for the treatment of patients with chronic inflammatory conditions. As previously reported, only the combination of F8-IL4 with DXM was curative in mice with CIA, leading to paws that were grossly indistinguishable from the ones of healthy animals, which highlights the synergistic benefit achieved by the two compounds. Whereas all other combination partners were given three times in a week, DXM was administered daily, as corticosteroid treatment has previously been shown to be beneficial.
when applied for multiple consecutive days [25–27]. The duration of dosing could have influenced the ability to achieve durable remissions with the combination of DXM and F8-IL4; however, the dosing regimen for all therapeutics was chosen based on reports in which positive effects of the agents had been observed in the CIA model [15, 17, 28, 29]. Furthermore, DXM alone does not cure mice and, additionally, it is well tolerated in a daily dosing setting, while this does not apply to all other agents [20].

Our immunohistochemical analysis revealed that neutrophils were by far the dominant leukocytes, and a large proportion of these were found to be degenerate, with strong release of NETs that are known to contribute to the pathogenesis of RA and induce further damage due to the NETosis-associated release of, among others, neutrophil enzymes [30–32]. As we knew from previous experiments that macroscopically a clinical reduction of arthritis score could already be observed 24 h after the first injection of F8-IL4 and DXM, we decided to focus on the earliest phase of treatment, to be able to observe the initial changes in cell populations. Besides the time points presented in Figs 2–4, which already show the treatment effect on neutrophils, an additional three time points were studied (6, 96 and 120 h after the first injection of F8-IL4), demonstrating the same rapid action of the treatment regimen (supplementary Fig. S2, available at Rheumatology online). After treatment with F8-IL4 plus DXM, the composition of the infiltrate did not change substantially; however, it decreased rapidly in its extent and in the majority of paws led to an arthritis score of 0. This confirms that the recruitment of neutrophils drives the inflammation in CIA and, accordingly, the combination of F8-IL4 and DXM effectively dampens the process. In previously published work, regulatory T cells were thought to be the main cell type influenced by the treatment of F8-IL4 plus DXM [21]. However, the aforementioned report also noted reduction in neutrophil counts after therapy. Additionally, IL4 has recently been shown to potently inhibit neutrophil activity, expansion and migration by antagonizing G-CSF and chemokine receptor-mediated signals in a process that crucially depends on the Type II IL4 receptor on neutrophils [33, 34].
The observation that a targeted delivery of IL4 to the site of inflammation potently reduces the extent of neutrophil recruitment and thereby also the damaging effect of their enzymes, which are released with NETs and with their degeneration, suggests that a fully human analogue of F8-IL4 may be useful for the treatment of diseases characterized by extensive neutrophil infiltration and activity. Previous histological analyses of human RA showed that various types of infiltrates can be observed in inflamed joints. In some patients, an abundant perivascular T cell infiltration is observed, while in other subjects macrophages and granulocytes predominate [35–37]. The SF of patients with RA contains 10–100 times more leucocytes compared with healthy individuals. In patients with chronic RA, >60% of the leucocytes in the SF are neutrophils [37]. Further inflammatory conditions characterized by an abundant neutrophil infiltration and activity include AS [38, 39], ulcerative colitis [40] and a set of autoimmune diseases such as small vessel vasculitis, Behçet’s disease and SLE [41, 42]. There have been attempts to predict clinical response to infliximab based on the presence of synovial lymphocyte aggregates [43]. However, in contrast to this study, a clear correlation between the type of cellular infiltrate and the response to therapy has so far not been reported [43].

The results presented in this article provide a rationale for the clinical development of a fully human analogue of F8-IL4, in combination with DXM, for the treatment of patients with neutrophil-driven inflammatory conditions.

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Supplementary data

Supplementary data are available at Rheumatology online.

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F8-IL4 plus DXM cures CIA by inhibiting neutrophils


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