Antibody-based delivery of IL4 to the neovasculature cures mice with arthritis

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Antibody–cytokine fusion proteins (immunocytokines) are innovative biopharmaceutical agents, which are being considered for the therapy of cancer and chronic inflammatory conditions. Immunomodulatory fusion proteins capable of selective localization at the sites of rheumatoid arthritis (RA) are of particular interest, as they may increase the therapeutic index of the cytokine payload. The F8 antibody recognizes the alternatively spliced extra domain A of fibronectin, a marker of angiogenesis, which is strongly overexpressed at sites of arthritis. In this study, we investigated the targeting and therapeutic activity of the immunocytokine F8-IL4 in the mouse model of collagen-induced arthritis. Different combination regimes were tested and evaluated by the analysis of serum and tissue cytokine levels. We show that F8-IL4 selectively localizes to neovascular structures at sites of rheumatoid arthritis in the mouse, leading to high local concentrations of IL4. When used in combination with dexamethasone, F8-IL4 was able to cure mice with established collagen-induced arthritis. Response to treatment was associated with an elevation of IL13 levels and decreased IL6 plasma concentrations. A fully human version of F8-IL4 is currently being developed for clinical investigations.

interleukin 4 | targeted therapy | vascular targeting | armed antibody

Rheumatoid arthritis (RA) is a common, chronic, inflammatory disorder of the joints predominantly affecting young adults and premenopausal women. The disease is characterized by a progressive inflammatory synovitis, manifested by polyarticular joint swelling and tenderness. The synovitis results in erosion of articular cartilage and marginal bone with subsequent joint destruction. This destruction of the bone is thought to be irreversible. There is no known cure for RA (1).

Cytokines play a crucial role in inflammatory processes, and monoclonal antibodies, blocking the interaction of certain proinflammatory cytokines (e.g., TNF and IL6) with their receptor, provide a substantial benefit to a fraction of RA patients (2–4). For example, a 50% reduction in American College of Rheumatology parameters (ACR50) has been documented in 35% and 40% of RA patients treated with adalimumab and tocilizumab, respectively (3, 5, 6). Indeed, cytokine-blocking antibodies and antibody-based fusion proteins represent one of the largest sectors of modern pharmaceutical biotechnology.

As an alternative therapeutic strategy, the antibody-based pharmacodelivery of anti-inflammatory cytokines (such as IL10) has been considered (7, 8). Thanks to advanced technologies (9), high-affinity human antibodies can be raised against virtually any accessible marker of disease and may selectively accumulate at the site of disease, thus facilitating the development of pharmacodelivery strategies. In particular, the F8 antibody, specific to the alternatively spliced extra domain A (EDA) of fibronectin, a marker of angiogenesis, has been shown to strongly react with neovascular structures at sites of chronic inflammation in human specimens and in the mouse (7, 10, 11), whereas the antigen is only found in placenta, endometrium, and some vessels of the ovaries in normal adult tissue (7). The immunocytokine F8-IL10 is currently being investigated in clinical trials in patients with active RA (12).

Interleukin 4 (IL4) is a cytokine involved in the proliferation of immune cells and the polarization of the immune environment toward a T helper type 2 response (13). Recombinant IL4 has previously been investigated in preclinical models of rheumatoid arthritis, showing disease-modifying efficacy (14–18). However, the clinical application of recombinant IL4 was not overly successful, as at the dose tested, the cytokine did not display potent activity (19). The antibody-based targeted delivery of IL4 to sites of arthritis in vivo may result in a therapeutic action, which is dramatically more potent, compared with the nontargeted IL4 cytokine.

Results and Discussion

Recombinant IL4 has previously been investigated in preclinical models of rheumatoid arthritis, showing disease-modifying efficacy and prevention of bone erosion (14, 15, 17). Despite these encouraging results, recombinant IL4 has never been tested in arthritis patients. The antibody cytokine fusion protein F8-IL4 consists of the F8 antibody in noncovalent homodimeric scFv format ["diabody" (20)], fused to murine IL4. The diabody format has previously been shown to allow an efficient accumulation at sites of disease, while being rapidly cleared from circulation (10, 21). We expressed F8-IL4 and antibody KSF-IL4 (an immunocytokine of irrelevant specificity in the mouse, serving as negative control as it recognizes hen egg lysozyme) in CHO cells, yielding homogenous protein preparations after affinity chromatography (Fig. 1 *A*–*C*). A radioiodinated preparation of F8-IL4, incubated with mouse blood, remained in the plasma after a centrifugation step, indicating that the protein is not efficiently trapped by leukocytes (Fig. 1*D*). An i.v. administration of radioiodinated F8-IL4 and

Significance

Disease-homing antibody–cytokine fusion proteins (immunocytokines) are considered as innovative biopharmaceutical agents for the therapy of cancer and chronic inflammatory conditions with the potential to modulate the activity of the immune system at the site of disease. The immunocytokine F8-IL4 was able to selectively localize to arthritic sites in vivo and exhibited a potent single-agent activity in the collagen-induced arthritis model in mice. Surprisingly, the combination treatment of F8-IL4 with dexamethasone cured 100% of treated mice with established arthritis. To our knowledge, this is the first report of durable and complete regressions in mice with established RA. These findings are of clinical significance as the F8 antibody recognizes its cognate antigen, the extra domain A of fibronectin, with comparable affinity in mouse and man.

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The authors declare a conflict of interest (such as defined by PNAS policy). Dario Neri is a cofounder and shareholder of Philogen SpA (Siena, Italy), the company that owns the F8 antibody. Teresa Hemmerle is a consultant for Philochem AG (Otelfingen, Switzerland).

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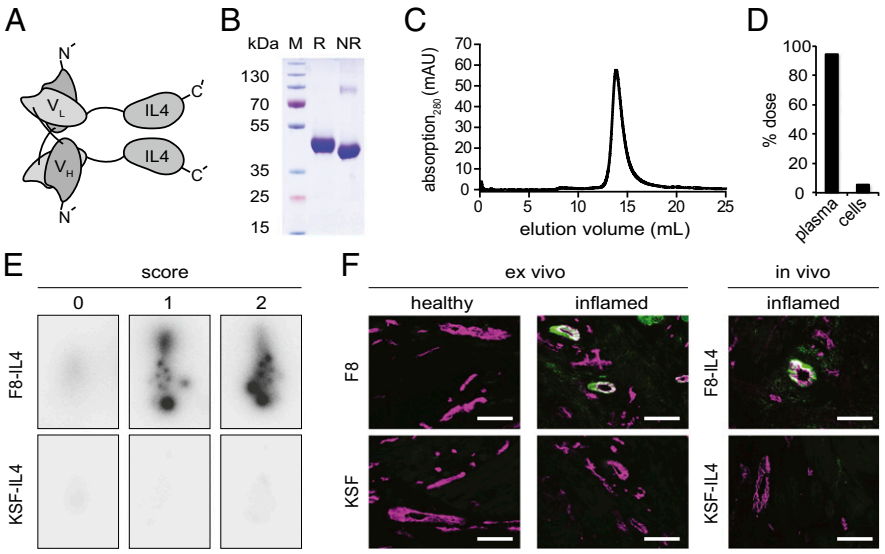

Fig. 1. Cloning, expression, and characterization of F8-IL4. (*A*) Schematic representation of an antibody–cytokine fusion protein based on the F8 antibody in diabody format and IL4 (F8-IL4 and KSF-IL4). (*B*) SDS/PAGE analysis of purified fusion protein (M, molecular-weight marker; R, F8-IL4 under reducing conditions; NR, F8-IL4 under nonreducing conditions). Monomeric fusion proteins are expected to have a molecular weight of 40 kDa. (*C*) Size-exclusion chromatography profile of purified F8-IL4. The peak eluting at a retention volume of 13.2 mL corresponds to the noncovalent homodimeric form of F8-IL4. (*D*) Incubation experiment of radiolabeled protein preparation with murine blood. Radioiodinated F8-IL4 (50 μg/mL) was incubated with fresh mouse blood containing inhibitors of coagulation. After centrifugation and separation of plasma from the cell pellet, radioactivity was counted and expressed as percent of initial dose. (*E*) Investigation of selective accumulation of F8-IL4 in inflamed paws. Arthritic mice were injected with ^{125}I-labeled F8-IL4 or KSF-IL4 (untargeted IL4; specific to hen egg lysozyme; negative control). Uptake of radioiodinated antibodies was analyzed by phosphorimaging 24 h after injection (score 0, no inflammation or swelling; score 1, one inflamed and swollen toe; score 2, two or more inflamed and swollen toes). (*F*) Immunofluorescence analysis of targeting. Healthy and inflamed paw tissues were stained ex vivo with SIP(F8) or SIP(KSF) (green). Additionally, mice with established arthritis were injected with F8-IL4 or KSF-IL4, and antibody accumulation was analyzed by staining for IL4 (green). (Vascular CD31 staining in magenta; Scale bar, 100 μm.)

KSF-IL4 into mice with collagen-induced arthritis revealed that F8-IL4 (but not KSF-IL4) was able to selectively localize at sites of arthritis (e.g., inflamed toes and paws) in the mouse, as revealed by an autoradiographic analysis 24 h after injection (Fig. 1*E*). A microscopic analysis of antigen expression and of the immunocytokine localization in arthritic lesions confirmed that the F8-IL4 fusion protein was able to selectively localize on the subendothelial extracellular matrix of newly formed blood vessels (Fig. 1*F*). The therapeutic activity of F8-IL4 was assessed in mice with collagen-induced arthritis. F8-IL4 showed a dose-dependent disease-modulating effect and superior performance to a murine analog of etanercept, a tumor necrosis factor receptor Fc fusion protein (TNFR-Fc) (22) in a model of severe arthritis (Fig. 2*A*). The therapeutic benefit also resulted in a reduction of body weight loss and correlated with decreased IL6 and increased IL13 serum levels (Fig. 2*A* and Fig. S1). The F8 antibody alone exhibited no inhibition of arthritis (Fig. S2). When comparing F8-IL4 to untargeted IL4 (KSF-IL4), the F8-based immunocytokine exhibited a clear superiority over KSF-IL4 ($P < 0.01$) (Fig. 2*B* and Fig. S3). A comparison between i.v. and s.c. administration of F8-IL4 was also performed, as the s.c. route is more convenient for patients. Both treatments were similarly efficacious in terms of disease score, but the s.c. administration of F8-IL4 resulted in a decreased body weight loss (Fig. 2*C*). Although the combination with murine TNFR-Fc (22) exhibited no superior disease reduction over F8-IL4 as monotherapy (Fig. 2*D* and Fig. S3), the treatment with a combination of F8-IL4 and a previously described IL10-based immunocytokine (L19-IL10) (7, 8) resulted in disease stabilization, which lasted for 28 d (Fig. 3 *A*–*C*). Surprisingly, the combination of F8-IL4 with dexamethasone resulted in a highly potent disease-modulating activity, with a complete disappearance of any sign of arthritis in 100% (9/9) of the study animals (Fig. 3 *A*–*C*). A cytokine analysis in paws of animals at the end of the experiment confirmed that the F8-IL4 plus dexamethasone combination treatment resulted in a complete normalization of cytokine concentrations. The most striking reduction of cytokine levels was observed for IL10, IL13, IL17, IL21, IL22, IL27, and TNF levels (Fig. 3*D* and Figs. S4 and S5). By contrast, only a trend to normalization in anti-collagen antibody levels was observed (Fig. S6).

The striking beneficial effect of the combination treatment may result from a synergistic effect of the glucocorticoid and IL4 (15, 17, 18, 23, 24). Dexamethasone has an anti-inflammatory activity and inhibits leukocyte infiltration at the site of inflammation, interferes with the function of mediators of inflammatory response, suppresses humoral immune responses, and reduces edema. IL4 is known to have anti-inflammatory effects in established disease due to the induction of the differentiation of naive helper T cells (Th0 cells) to Th2 cells and the suppression of Th1 responses.

F8-IL4 treatment did not lead to increased IgE levels, in keeping with the observation that IL4 blockade had no effect on circulating eosinophils and IgE levels in allergy patients (Fig. S7) (25).

To our knowledge, this is the first report of durable and complete regressions in mice with established RA. The findings are likely to be of clinical significance, because dexamethasone is often used to treat RA patients, the F8 antibody reacts with identical affinity against murine and human EDA, and other immunocytokines specific to fibronectin splice isoforms are currently being investigated in clinical trials. The observed correlation between F8-IL4 treatment and cytokine changes in serum and in inflamed joints may facilitate the monitoring of patients and/or the implementation of patient stratification procedures. Although some anti-inflammatory therapeutic proteins have been associated with an elevation of cancer risk in patients, F8-IL4 has been shown to mediate a strong antitumoral activity in immunocompetent mouse models of cancer (26).

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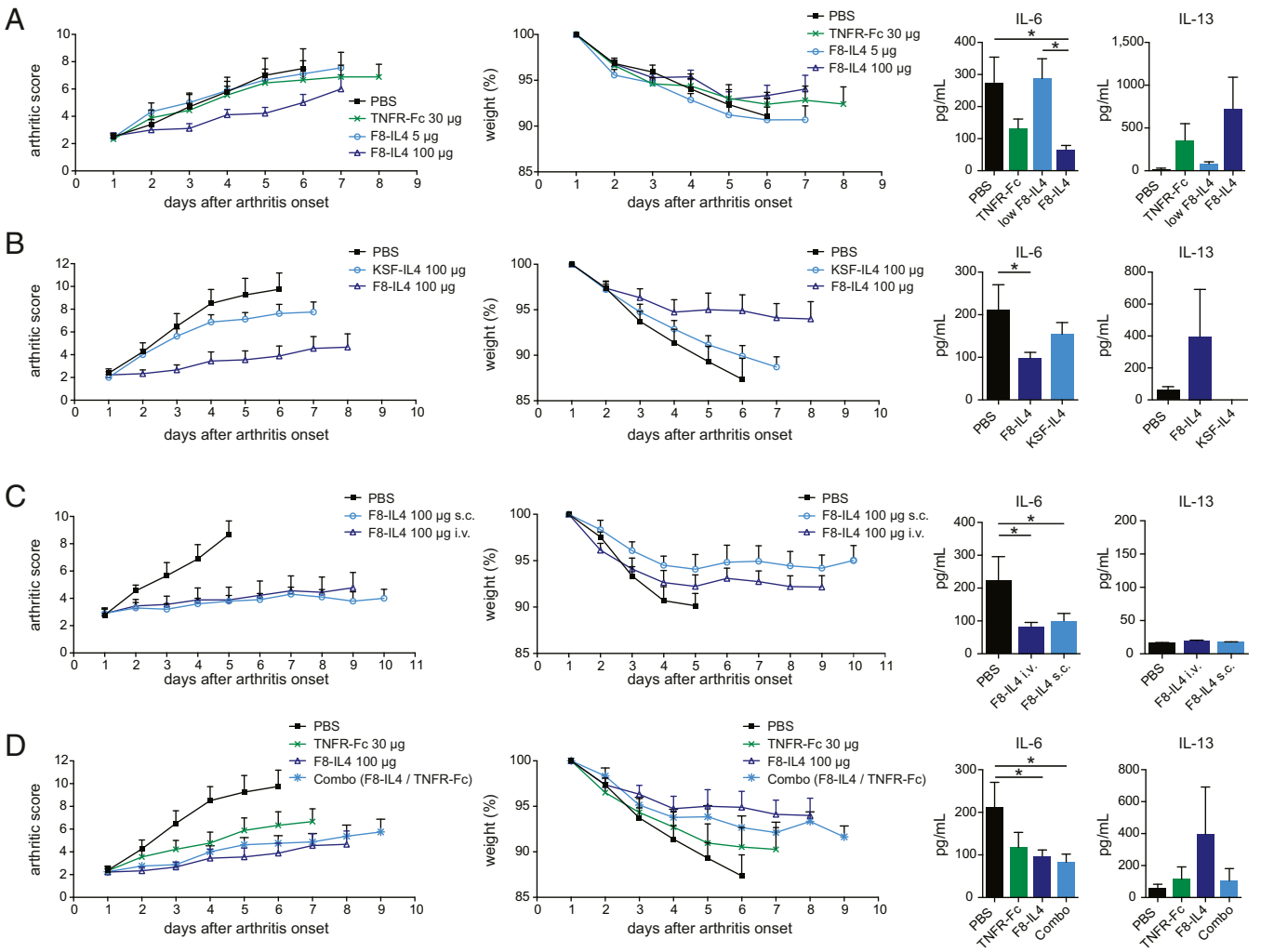

Fig. 2. Therapeutic activity of F8-IL4 in the collagen-induced arthritis model. The therapeutic proteins were administered three times every 72 h (n = 7–9; SEM; *$P < 0.05$). Cytokine levels were determined in serum of terminal blood. (*A*) Dose-finding and proof of principle study with low-dose F8-IL4 (5 µg) and high-dose F8-IL4 (100 µg) compared with vehicle (PBS; negative control) and 30 µg murine TNFR-Fc (positive control). IL6 and IL13 levels were determined in serum. (*B*) Comparison of targeted to untargeted delivery of IL4. F8-IL4 displayed superior therapeutic activity to KSF-IL4 (IL6 and IL13 levels were determined in serum). (*C*) Effect of the route of administration on efficacy of F8-IL4. No significant difference of s.c. to i.v. administration could be observed (IL6 and IL13 levels were determined in serum). (*D*) Combination therapy with murine TNFR-Fc. No additive effect of F8-IL4 with TNF-blockade was observed (IL6 and IL13 levels were determined in serum).

Methods

Cell Lines, Proteins, and Animals. CHO-S cells in suspension (Invitrogen) were cultured in PowerCHO-2CD (Lonza) with 8 mM ultraglutamine (Lonza), HT supplement (Gibco), and antibiotics/antimycotics (Gibco) in shaker incubators. The cloning and production of murine TNFR-Fc (22), F8-IL4, and KSF-IL4 (26), as well as L19-IL10 in diabody format (7, 8), has been previously described. Male DBA/1J and female BALB/c mice were obtained from Janvier.

Collagen-Induced Arthritis Model. For the induction of rheumatoid arthritis in mice, male DBA/1J mice (8 wk old) were immunized by s.c. injection at the base of the tail with an emulsion of bovine type II collagen emulsified in Completes Freund's Adjuvant (CFA) (Hooke Laboratories). Three weeks later, a booster injection of bovine collagen/CFA was given to the mice. After the booster injection, mice were inspected daily, and disease was monitored by applying a clinical score to every paw (0 = normal; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw inflamed and swollen or mild inflammation and swelling of entire paw; 3 = entire paw inflamed and swollen; and 4 = very inflamed and swollen paw). A total maximum score of 16 can be reached per mouse. In addition, swelling of affected paws was measured daily with a caliper under anesthesia (isoflurane). Paw thickness is expressed as the mean of all four paws of each animal. Animals were included into a therapy group when showing signs of joint inflammation with a total score of 1–4. Experiments were performed in agreement with the Swiss regulations and under a project license granted by the cantonal veterinary office Zurich (208/2010).

Blood Incubation Assay. The ability of F8-IL4 to interact with blood cells was determined by a blood cell binding assay using ^{125}I-labeled protein preparations as previously described (22). F8-IL4 was labeled with iodine-125 (Perkin–Elmer) and incubated at a concentration of 50 µg/mL with fresh murine blood from a BALB/c mouse in Microtainer tubes with lithium heparin (BD Bioscience) to prevent coagulation. After 10 min of incubation, tubes were centrifuged for 3 min at 2,000 × g. Plasma was separated from blood cells, and radioactivity was counted using a Cobra gamma counter (Packard). Radioactivity was expressed as percent of the input.

Autoradiography Experiments. The in vivo targeting performance of F8-IL4 and KSF-IL4 was evaluated by autoradiography analysis (7, 8). Radioiodinated protein preparations were injected into the lateral tail vein (100 µg; 7 µCi ^{125}I-F8-IL4, 8 µCi ^{125}I-KSF-IL4). Mice were killed 24 h after injection, and paws were exposed for 16 h to a phosphorimager screen

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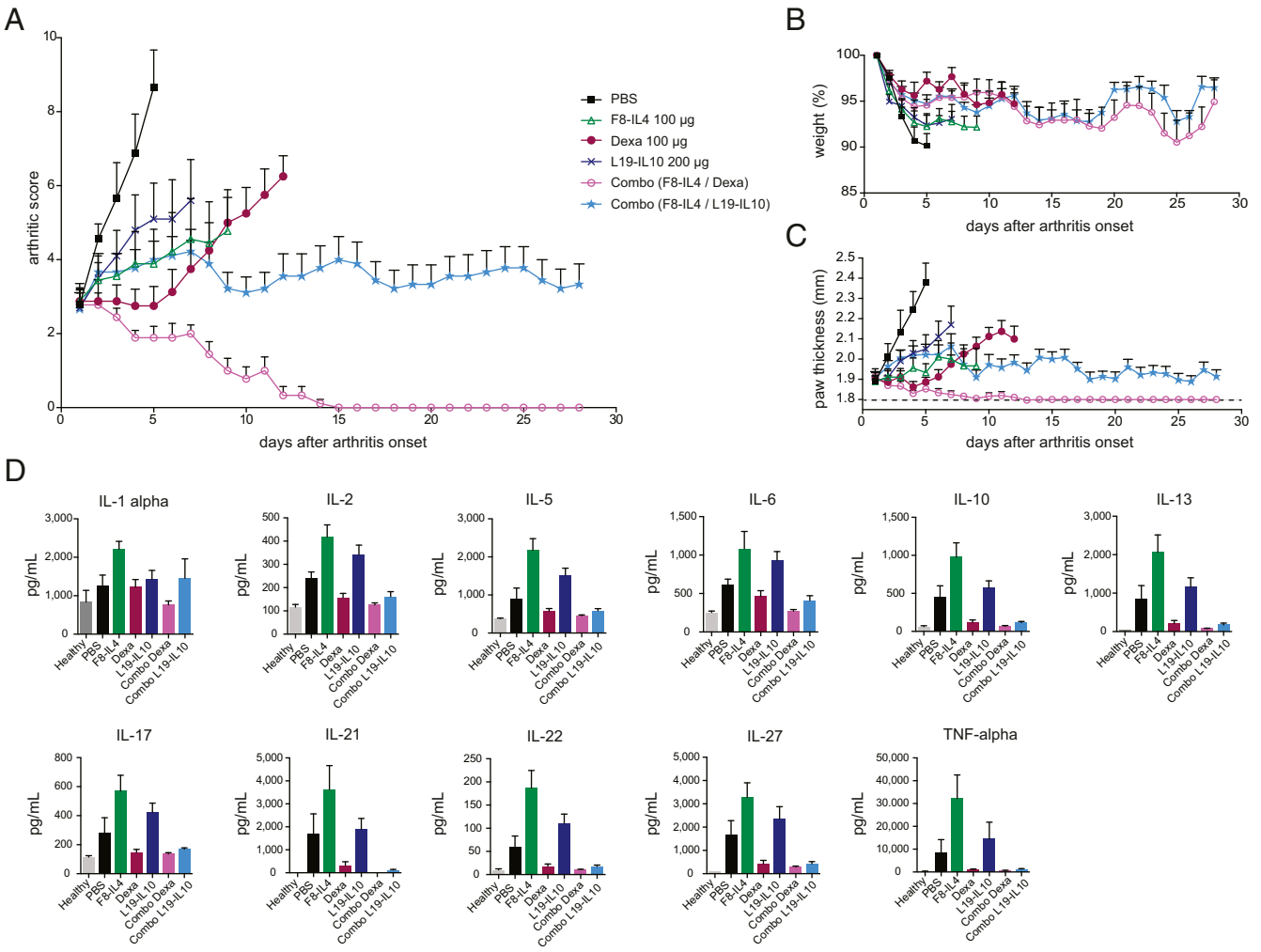

Fig. 3. The combination of F8-IL4 and dexamethasone cures mice with collagen-induced arthritis. Therapeutic activity was investigated by arthritic score, measurement of paw thickness, observation of changes in weight, and analysis of cytokine levels. Therapeutic proteins were administered three times every 72 h, and dexamethasone was administered every 24 h for 9 d (n = 8–10; SEM). (*A*) F8-IL4 in combination with L19-IL10 stabilized arthritis progression over a 4 wk period. Combination of F8-IL4 with dexamethasone led to complete regression of arthritis in all mice. (*B*) Therapeutic regimes were well tolerated. Weight was monitored daily and expressed as percent of weight loss. (*C*) Combination treatment with F8-IL4 and dexamethasone decreased paw swelling to baseline value (dotted line at 1.8 mm, determined with healthy mice). (*D*) Cytokine levels were determined in paw tissue lysates.

(Fujifilm). Accumulated radioactivity was read with a phosphorimager (BAS5000, Fujifilm).

Immunofluorescence Analysis. Frozen sections of healthy and arthritic paws were fixed in ice-cold acetone and stained for EDA expression using biotinylated F8 and KSF antibody in small immune protein (SIP) format. Bound antibody was detected using Streptavidin–Alexa Fluor conjugate (Invitrogen) (green). To detect the in vivo accumulation of immunocytokine, arthritic mice were injected with F8-IL4 or KSF-IL4 (100 µg per mouse, three injections, every 72 h). Sections of swollen paws (score 3) were stained with a rat anti-mouse IL4 antibody (eBioscience) and an anti-rat IgG Alexa Fluor coupled secondary antibody (Invitrogen) (green). Vascular structures were stained using an anti-CD31 antibody (Santa Cruz) and secondary Alexa Fluor coupled antibody (Invitrogen) (magenta). Slides were mounted with fluorescent mounting medium (Dako) and analyzed with an Axioskop2 mot plus microscope (Zeiss).

Dose-Finding Therapy Experiment. Mice were immunized with 50 µg bovine collagen/CFA emulsion for the first immunization and 60 µg for the second immunization. When mice developed a new clinical score of 1–4, they were randomly assigned to a treatment group, and therapy was started (n = 7–8 mice per group). PBS (vehicle), 30 µg murine TNFR-Fc, 5 µg F8-IL4, or 100 µg F8-IL4 were injected into the lateral tail vein on day 1, 4, and 7. Mice were monitored daily for the arthritic clinical score, the thickness of inflamed paws, and weight and killed due to the arthritic score (≥score 2 on more than one paw for more than 4 d) and weight loss (>15%) in accordance with local regulations.

Comparison of Targeted to Untargeted IL4 and Combination Therapy with Murine TNFR-Fc. For a moderate strength in arthritic inflammation, mice were immunized with 50 µg bovine collagen/CFA emulsion for the first immunization and 40 µg for the second immunization and included in a treatment group with a new clinical score of 1–4 (n = 8–9 mice per group). On day 1, 4, and 7, mice were treated i.v. with either PBS (vehicle, buffer control), 30 µg murine TNFR-Fc, 100 µg F8-IL4, 100 µg KSF-IL4, or the combination of F8-IL4 with murine TNFR-Fc (100 µg F8-IL4 with 30 µg murine TNFR-Fc).

Comparison of s.c. to i.v. Administration of F8-IL4 and Combination Therapy with Dexamethasone or the Antibody-Mediated Delivery of IL10. Mice, immunized for moderate arthritis strength (50 and 40 µg), with a new clinical score of 1–4, were included in a treatment group and treated with either i.v. PBS (vehicle control), i.v. 100 µg F8-IL4, s.c. 200 µg L19-IL10, the combination of i.v. F8-IL4 with s.c. L19-IL10, s.c. 100 µg F8-IL4, i.p. 100 µg dexamethasone, or the combination of i.v. F8-IL4 and i.p. dexamethasone (n = 8–10 mice per group). Immunocytokine treatments were administered three times (every 72 h), and dexamethasone was administered daily until day 9.

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Analysis of Cytokine Levels in Serum. Blood was obtained at the end of therapy from each mouse by cardiac puncture and processed to serum. To quantify cytokine levels of treated and control mice, a multiplex bead-based flow cytometry analysis was performed using the mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBioscience). FACS analysis was performed on a BD FACS Canto (BD Bioscience), and data were evaluated with FlowCytomix Pro-3.0 software (eBioscience).

Analysis of Cytokine Levels in Paw Tissue. To compare cytokine levels in paws of treated and control mice, hind paws were taken at the end of the therapy experiment. After detaching the skin, paws were cut into small pieces, and the tissue fragments were suspended in a 50 mM Tris, 150 mM NaCl buffer containing complete protease inhibitor mixture (Roche Diagnostics). For homogenization, a 5-mm stainless steal bead (QIAGEN) was added, and the tissue was homogenized in a QIAGEN Tissue Lyzer (4 × 1 min, 4 °C, 30 Hz). The supernatant was harvested after centrifugation (5 min, 4 °C, 16,000 × g). The protein concentrations of the extracts were determined by a bicinchoninic acid assay (Thermo Fisher Scientific), and samples were normalized according to total protein concentration. For the quantification of cytokine levels, a multiplex bead-based flow cytometry analysis was performed using the mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBioscience). FACS analysis was performed on a BD FACS Canto (BD Bioscience), and data were evaluated with FlowCytomix Pro-3.0 software (eBioscience).

Analysis of Serum IgE Levels. For the determination of IgE levels in mice treated with F8-IL4 compared to levels in healthy mice and mice treated with vehicle control (PBS), serum was analyzed using Mouse IgE Ready-Set-Go ELISA kit (eBioscience) according to the supplier's protocol. Briefly, wells were coated with anti-mouse IgE monoclonal antibody, and bound IgE was detected with biotinylated anti-mouse IgE antibody.

Analysis of Serum Anti-collagen Antibodies. For the determination of anti-bovine collagen type II specific antibody levels in mice treated with F8-IL4 compared to mice treated with PBS levels in healthy mice compared to mice treated with PBS, the combination F8-IL4 with L19-IL10, or the combination of F8-IL4 with dexamethasone, serum was analyzed by ELISA technique as previously described (7). Bovine collagen II solution (5 μg/mL) was coated, and serum samples were tested in triplicates at a 1:1,000 dilution. Bound total IgG, IgG1, and IgG2a were detected by incubation with horseradish peroxidase conjugated goat anti-mouse Fc (for IgG), IgG1, or IgG2a antibodies (Santa Cruz).

Statistical Analysis. Data are expressed as the mean ± SEM. Differences between therapy groups were analyzed using Graphpad Prism's grouped two-way ANOVA multiple comparisons (Bonferroni corrected) analysis (GraphPad Software, Inc.).

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