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Antiinflammatory properties of a peptide derived from interleukin-4

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ABSTRACT

Interleukin-4 (IL-4) is a potent antiinflammatory cytokine. However its use in the clinic is hampered by side effects. We here describe the identification of a novel synthetic peptide, termed Ph8, derived from α-helix C of IL-4, which interacts with IL-4 receptor α (IL-4Rα). Employing various cultured genetically engineered cell lines and primary lymphocytes, surface plasmon resonance, qPCR, ELISA and immunoblotting techniques we found that Ph8 bound IL-4Rα and mimicked the anti-inflammatory effects of IL-4 by inhibiting TNF-α production by macrophages *in vitro*. It induced phosphorylation of STAT6 65 kD but inhibited phosphorylation of STAT6 110 kD induced by IL-4 in a B-cell line that expressed the type I receptor. It also inhibited the IL-4-stimulated expression of a STAT6-inducible reporter gene in cells that expressed the type II receptor. Ph8 inhibited the proliferation of Th1/2 cells and downregulated the production of IFN-γ in stimulated Th1 cells. Moreover, Ph8 did not induce any shift in Th1/Th2 profile. This is a favorable effect and it is indicating that Ph8 could block general T cell activation and inflammatory responses without further inducing the side effects generally associated with IL-4 signaling. These data collectively show that Ph8 is only a partial agonist of IL-4 mimicking its desirable properties. In agreement, Ph8 treatment of rats with collagen-induced arthritis, a Th1- and antibody- mediated disease of joint, delayed the manifestation of chronic inflammation and reduced acute inflammation in carrageenan-induced edema. Our findings indicate that Ph8 is a promising potential drug candidate for the treatment of inflammatory diseases.

1. Introduction

Interleukin-4 (IL-4) is one of the antiinflammatory cytokines secreted by Th2 cells. Deficits in IL-4 aggravate severe inflammation in collagen-induced arthritis (CIA), a widely used animal model of rheumatoid arthritis. Overexpression of IL-4 or exogenous administration of IL-4 significantly delayed the onset and reduced the severity of experimental autoimmune encephalomyelitis (EAE) and CIA and attenuated the pathogenesis of Alzheimer's disease in transgenic mice [1–6]. IL-4 knockout mice have been found to be more sensitive in models of transient focal cerebral ischemia [7]. IL-4 has been shown to enhance the antileukemic immune response [8]. It can also induce apoptosis of breast cancer cells [9]. The IL-4 receptor has been used as target for antitumor cytotoxin therapy [10,11]. Clinical trial of IL-4 in psoriasis has demonstrated its effectiveness to correct imbalances in immune function [12]. One of the mechanisms responsible for the antiinflammatory effects of IL-4 is attributable to the suppression of tumor necrosis factor α (TNF-α) production by macrophages [13], involving signal transducer and activator of transcription 6 (STAT6) activation-induced TNF mRNA destabilization [14]. The antiinflammatory potential of IL-4 makes its receptor a target for therapeutic intervention.

IL-4 can mediate immunological responses via both type I and type II receptor complexes (IL-4R-I and IL-4R-II, respectively). IL-4 binds to and signals through the two combinations of three receptor chains, IL-4Rα, IL-13Rα1, and the common γ-chain (γc). IL-4R-I, a heterodimer of IL-4Rα and γc, is the main receptor on hematopoietic cells, and it induces the activation of the Janus

Abbreviations: IL, interleukin; IL-R, IL-receptor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; STAT, signal transducer and activator of transcription; NF-κB, nuclear factor-κB; JAK, Janus kinase; HEK, human embryonic kidney; MOG_{35-55}, myelin oligodendrocyte glycoprotein 35–55; SEAP, secreted embryonic alkaline phosphatase; APCs, antigen-presenting cells; DLNs, draining lymph nodes; BrdU, bromodeoxyuridine; CIA, collagen-induced arthritis; CFA, complete Freund's adjuvant; CII, collagen type II; MTX, methotrexate; Dx, dexamethasone 21-disodium phosphate.

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kinase 3 (JAK3)-STAT6 pathway. IL-4R-II, a heterodimer of IL-4Rα and IL-13Rα, can bind both IL-4 and IL-13 and is the primary receptor complex utilized by nonhematopoietic cells [15,16]. Type I and II IL-4 receptors exert distinct effects on immune response. While IL-4R-I is more active in regulating Th2 development, IL-4R-II is not found on T-cells and is more active in regulating cells that mediate airway hypersensitivity and mucus secretion [17,18].

The present study identifies a novel synthetic peptide, termed Ph8 that is a partial agonist of IL-4Rα. *In vitro,* it mimicked the anti-inflammatory effects of IL-4 by inhibiting TNF-α secretion by macrophages and modulating the proliferation and survival of Th cells and production of cytokines. *In vivo*, Ph8 reduced edema acutely induced by carrageenan and delayed the manifestations of chronic inflammation in the CIA rat model.

2. Materials and methods

2.1. Peptides

The Ph8 peptide (AQFHRHKQLIRFLKRA), biotinylated Ph8 (both dimers composed of two monomers coupled to a lysine backbone), and myelin oligodendrocyte glycoprotein 35–55 (MOG_{35-55}) were purchased from Schafer-N (Copenhagen, Denmark).

2.2. Cell cultures

AMJ2-C8, an immortalized murine alveolar macrophage cell line from the American type culture collection (ATCC) and L929, a murine fibroblast cell line (European Collection of Cell Cultures, Salisbury, United Kingdom), were maintained as described previously [19,20]. Human embryonic kidney (HEK)-Blue cells (HEK-Blue IL-1β, HEK-Blue IL-6, and HEK-Blue IL-4/IL-13; InvivoGen, San Diego, CA, USA) are HEK293 cells that express an inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene that is quantitatively detected using the QUANTI-Blue SEAP colorimetric detection medium. The cells were maintained as described by the manufacturer.

Ramos B-lymphocyte cells (Invitrogen, Life Technologies, Nærum, Denmark) were maintained as described by the manufacturer.

Th2 cells were prepared from the spleens of OT2-C57BL/6 mice (Taconic, Ry, Denmark). $CD4^+$ T-cells were isolated with Miltenyi Biotech CD4 (L3T4) microbeads on an autoMACS (Fisher Scientific, Slangerup, Denmark). The negative fraction was irradiated with 30 Gy and used as antigen-presenting cells (APCs). T-cells (2×10^5) were cultured with 2×10^6 APCs and stimulated with 10 μg/ml ovalbumin peptide ($OVA_{323-339}$, AnaSpec, Fremont, CA, USA), 10 μg/ml anti-interferon-γ (IFN-γ) antibody (BD Pharmingen), 10 μg/ml anti-IL-12 antibody (BD Pharmingen), 5 ng/ml IL-2 (obtained from IL-2-transfected X63 cell line supernatants), and the indicated amounts of recombinant IL-4 (PeproTech Nordic) or Ph8. OT2 cells (8×10^3) were incubated with or without 5 μg/ml ConA (GE Healthcare) and varying concentrations of Ph8 peptide for 48 h in triplicate. Th1 cells were obtained from draining lymph nodes (DLNs) of immunized mice and prepared and cultured with or without MOG_{35-55}, ConA for 48 h prior to bromodeoxyuridine (BrdU) labeling as described previously [15].

2.3. Surface plasmon resonance analysis

Binding experiments were performed on a Biacore 2000 instrument (Biacore AB) at 25 °C using phosphate-buffered saline (PBS; pH 7.4) as running buffer. Biotinylated Ph8 was immobilized on a streptavidin-coated SA-sensor chip. The human receptors IL-4Rα, IL-6R, and IL-1R (R&D Systems) were injected at a flow rate of 10 μl/min. The data were analyzed by nonlinear curve-fitting using BIAevaluation 4.1 software.

2.4. Macrophage activation

AMJ2-C8 cells (2.5×10^5 cells/well) were incubated with Ph8, 100 μM hydrocortisone, or 25 ng/ml IL-1R antagonist (Sigma–Aldrich) for 24 h at 37 °C before 0.01 μg/ml IFN-γ (Sigma–Aldrich), 0.1 ng/ml IL-1, or 0.1 ng/ml IL-6 (R&D Systems) was added to the cultures. L929 cells (0.2×10^2 cells/ml) were incubated for 24 h at 37 °C. Conditioned medium from macrophages was collected and added to the cultures of L929 cells together with actinomycin D (0.6 μg/well; Sigma–Aldrich). After 24 h of incubation at 37 °C, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; Promega, Madison, WI, USA) was added to each well and incubated at 37 °C in the dark for 40 min, and the optical density was measured at 490 nm using an enzyme-linked immunosorbent (ELISA) reader (Wallac Multilabel counter 1420, PerkinElmer, Skovlunde, Denmark).

2.5. Signaling assay in HEK-Blue cells

HEK-Blue IL-1β and HEK-Blue IL-6 cells (5×10^4 cells/well) were treated with Ph8 together with 100 ng/ml IL-1 or 100 ng/ml IL-6, respectively. After 24 h of incubation at 37 °C, 150 μl of the cell supernatants was added to each well together with 50 μl QUANTI-Blue (InvivoGen) and incubated at 37 °C for 40 min and then measured in an ELISA reader at 600 nm to determine the expression levels of reporter genes activated by nuclear factor-κB (NF-κB) and AP1 (HEK-Blue IL-1β) or STAT3 (HEK-Blue IL-6). The detection of the STAT6-inducible SEAP reporter gene was performed in HEK-Blue IL-4/IL-13 cells (3.5×10^5 cells/well) as described above.

2.6. Determination of STAT6 phosphorylation

Ramos B-lymphocyte cells (2×10^7) were starved overnight in medium without serum. Pretreatment with 40 μM Kaempferol (Sigma–Aldrich) was performed 1 h before stimulation with IL-4 (R&D Systems) or Ph8 for 15 min. The cells were lysed by 500 μl RIPA lysis buffer that contained 1% (v/v) nonidet P-40 (Sigma–Aldrich), 0.2% (v/v) sodium dodecyl sulfate (SDS; Sigma–Aldrich), complete protease inhibitors (1:50; Roche), and phosphatase inhibitors (Calbiochem inhibitor cocktail III; 1:100) in PBS. 60 μg protein from each lysate was separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore). Immunoblotting was performed using a polyclonal antibody against phosphorylated Tyr 641 of STAT6 (Santa Cruz Biotechnology) and IRDye 680LT-conjugated goat anti-rabbit IgG (diluted 1:10000; Li-COR, Biosciences) in 5% (w/v) nonfat dry milk. The immune complexes were developed by Li-COR and visualized and quantified using Odyssey 3.0 V image analysis software (Bioscience). For the analysis of total STAT6 expression, 60 μg protein from each lysate was separated by SDS–PAGE and analyzed by Western blotting using a rabbit polyclonal antibody against STAT6 (Santa Cruz Biotechnology). The amount of actin was visualized using rabbit antibody against actin (Sigma–Aldrich).

2.7. Immunization of mice to induce Th1-mediated immune response

C57BL/6 mice, aged 10–20 weeks, were subcutaneously immunized with 250 μg MOG_{35-55} (Schafer-N, Copenhagen, Denmark) and complete Freund's adjuvant (CFA) that contained 500 μg *Mycobacterium tuberculosis* H37Ra (Difco). Spleens and DLNs were collected 27 or 10 days after immunization, respectively ($n = 3$).