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The cytokines cardiotrophin-like cytokine/cytokine-like factor-1 (CLC/CLF) and ciliary neurotrophic factor (CNTF) differ in their receptor specificities

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ABSTRACT

Ciliary neurotrophic factor (CNTF) and cardiotrophin-like cytokine (CLC) are two cytokines with neurotrophic and immunomodulatory activities. CNTF is a cytoplasmic factor believed to be released upon cellular damage, while CLC requires interaction with a soluble cytokine receptor, cytokine-like factor 1 (CLF), to be efficiently secreted. Both cytokines activate a receptor complex comprising the cytokine binding CNTF receptor α (CNTFRα) and two signaling chains namely, leukemia inhibitory factor receptor β (LIFRβ) and gp130. Human CNTF can recruit and activate an alternative receptor in which CNTFRα is substituted by IL-6Rα. As both CNTF and CLC have immune-regulatory activities in mice, we compared their ability to recruit mouse receptors comprising both gp130 and LIFRβ signaling chains and either IL-6Rα or IL-11Rα which, unlike CNTFRα, are expressed by immune cells. Our results indicate that 1) mouse CNTF, like its human homologue, can activate cells expressing gp130/LIFRβ with either CNTFRα or IL-6Rα and, 2) CLC/CLF is more restricted in its specificity in that it activates only the tripartite CNTFR. Several gp130 signaling cytokines influence T helper cell differentiation. We therefore investigated the effect of CNTF on CD4 T cell cytokine production. We observed that CNTF increased the number of IFN-γ producing CD4 T cells. As IFN-γ is considered a mediator of the therapeutic effect of IFN-β in multiple sclerosis, induction of IFN-γ by CNTF may contribute to the beneficial immunomodulatory effect of CNTF in mouse multiple sclerosis models. Together, our results indicate that CNTF activates the same tripartite receptors in mouse and human cells and further validate rodent models for pre-clinical investigation of CNTF and CNTF derivatives. Furthermore, CNTF and CLC/CLF differ in their receptor specificities. The receptor α chain involved in the immunomodulatory effects of CLC/CLF remains to be identified.

1. Introduction

CNTF and CLC are IL-6 family cytokines that activate a receptor comprising a glycosylphosphatidylinositol-anchored non-signaling subunit, CNTFRα and two signaling transmembrane chains, LIFRβ and gp130 [1–6]. CNTF, which is expressed by Schwann cells, astrocytes and T cells [7–9], has potent trophic effects on neurons, oligodendrocytes and muscle cells *in vitro* and *in vivo* (reviewed in [8]). This led to its evaluation in clinical trials for the treatment of amyotrophic lateral sclerosis (ALS) [10]. The toxicity of CNTF limited the concentration that could be used in patients and no beneficial effects on ALS was observed [10,11]. Loss of weight was one of the side effects observed in ALS patients, an effect also documented in rodent obesity models [10,12,13]. This led to clinical trials of a CNTF derivative, Axokine, in the treatment of obesity and type II diabetes, with limited benefits [14,15].

CNTF is a protein devoid of a signal peptide and is therefore believed to be a cytoplasmic protein released by damaged cells [16]. Supporting the concept that CNTF is mainly a trauma factor, a mutation inactivating the *CNTF* gene has been observed at a high frequency in healthy populations [17]. This suggests that CNTF has only limited non-redundant roles during development in human. *CNTF* gene deficiency has been associated in some (but not all) studies with an early onset of ALS and multiple sclerosis (MS) [18–21]. CLC differs from CNTF in that it possesses a signal

Abbreviations: ALS, amyotrophic lateral sclerosis; CLC, cardiotrophin-like cytokine; CLF, cytokine-like factor 1; CNTF, ciliary neurotrophic factor; EAE, experimental autoimmune encephalitis; LIF, leukemia inhibitory factor; mAb, monoclonal antibody; MS, multiple sclerosis; R, receptor.

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peptide but requires interaction with the soluble cytokine receptor CLF for efficient secretion [1]. Mutations in the genes encoding CLC and CLF (*CLCF1* and *CRLF1*, respectively) result in cold-induced sweating and Crisponi syndromes, the latter being often lethal early in life. This indicates that CLC/CLF, unlike CNTF, plays an important role during development [22–26]. The similarity between the cold-induced sweating syndromes observed in patients with mutation in *CLCF1* and *CRLF1* strongly support the notion that the association between CLC and CLF observed *in vitro* [1] plays a physiological, non-redundant role *in vivo* [25,27]. Patients with mutations in LIFRβ, one of the CNTFR signaling chains, suffer from Stuve-Wiedemann/Schwartz-Jampel Type 2 syndrome (SWS/SJS2 [28]. The broad overlap between the clinical manifestations of Crisponi syndrome and SWS/SJS2 imply that CLC/CLF is a key CNTFR ligand during development [23,24,28]. Interestingly, CLC/CLF can also regulate kidney development and CLC has been identified as a potential permeability factor in the serum of patients with focal segmental glomeruloscerosis [29,30].

While these results point toward roles of CNTF and CLC/CLF on cells expressing CNTFR, both cytokines are believed to have immunoregulatory functions: CNTF is produced by T cells [9] and activates an alternative tripartite receptor expressed by immune cells comprising IL-6Rα, LIFRβ and gp130 [31]. CLC and CLF are expressed in the immune system and both cDNAs were cloned from T cells [32–34]. Injection of high doses of CLC or overexpression of CLC in mice results in B cell expansion, despite the lack of CNTF receptor expression by these cells [33,35]. This suggests that CLC/CLF can recruit, besides CNTFR, an alternative as yet unidentified receptor. Since mice have been used to study the immunomodulatory effects of CLC/CLF, [33,35], we compared the effects of mouse CNTF and CLC/CLF on transfectants co-expressing the signaling chains of the CNTF receptor (gp130 and LIFRβ) and the membrane anchored α chains specific for the members of the IL-6 family, CNTFRα, IL-6Rα and IL-11Rα. Our results indicate that, like its human homologue [31], mouse CNTF can activate a receptor comprising IL-6Rα, gp130 and LIFRβ but that this property is not shared by CLC/CLF. As both LIF (which activates the same signaling chains as CNTF) and IL-6 influence mouse T helper (Th) cell differentiation [36–38], we investigated the effects of CNTF on CD4 T cell cytokine expression *in vitro*. We observed that CNTF regulates the production of IFN-γ by T cells, a property which could contribute to the immunomodulatory effects of this cytokine in the experimental autoimmune encephalitis (EAE) model of MS [39].

2. Materials and methods

2.1. Generation of stable Ba/F3 transfectants

The cDNA coding for mouse LIFR-β (RIKEN clone F630311N01) and gp130 (IMAGE ID 6834623), were obtained from the FANTOM Consortium, The Institute of Physical and Chemical Research (RIKEN, Saitama, Japan) Genome Exploration Research Group (provided by K.K. DNAFORM, Ibaraki, Japan) and Open Biosystems (Open Biosystems Products, Huntsville, AL), respectively. The two cDNA were re-cloned in the multigenic expression vector pMG (InvivoGen, Cedarlane, Burlington, ON). Linearized plasmids were transfected by electroporation in Ba/F3 cells [40]. Stable transfectants were selected using hygromycin (1 mg/ml). Clones were expanded using mouse IL-3 (2 ng/ml; Peprotech, Rocky Hill, NJ) and selected for proliferation in response to mouse LIF (10 ng/ml StemCell Technologies Inc. Vancouver, BC). The mouse IL-6Rα, IL-11Rα and CNTFRα cDNA (IMAGE IDs 0130735, 3585497 and 6389656, respectively; Open Biosystems) were re-cloned in the expression vector pSFFV-neo [41]. A Ba/F3 clone expressing LIFRβ and gp130 was re-transfected by electroporation with the α chain cDNAs. Transfectants were selected using G418 (1 mg/ml). Clones were further selected for proliferation in response to mouse IL-6 (10 ng/ml R&D Systems, Minneapolis, MN), IL-11 (10 ng/ml R&D Systems) or CNTF (10 ng/ml, produced as described previously [42]).

2.2. Proliferation assays

Ba/F3 transfectants (5×10^3 cells/well in 96 well plates) were incubated in triplicates with the indicated dilutions of recombinant proteins for 72 h in RPMI-1644 medium supplemented with 5% FBS. The anti-IL-6Rα mAb D7715A7 was obtained from BD Biosciences (Mississauga, ON). When used, the purified 2B10 mAb [43] or its isotype IgG1 control were used at a final concentration of 5 μg/ml on Ba/F3 transfectants incubated with 10 ng/ml of the tested cytokines. Proliferation was measured using an Alamar blue fluorometric assay as described previously [40].

2.3. Measurement of STAT1 and STAT3 activation by flow cytometry

Ba/F3 transfectants were serum- and cytokine-starved for 4 h. Cells were activated for 15 min at 37 °C using mouse LIF (50 ng/ml), IL-6 (50 ng/ml), CLC/CLF (50 ng/ml; produced as described previously [1,40]) or CNTF (50 ng/ml). CLC/CLF and CNTF were produced as described previously [1,40,42]. The concentration of the recombinant mouse CNTF leading to half maximum proliferation of Ba/F3 expressing LIFRβ, gp130 and CNTFRα was 190 pg/ml). Cells were fixed and stained as described previously [40] and fluorescence was analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR).

2.4. CD4 T cell differentiation assays

C57BL/6 spleen and lymph node CD4 T cells were enriched by depleting CD8 and NK cells or purified by magnetic negative bead-selection following manufacturer's instructions (StemCell Technologies Inc.). CD8 and NK cell-depleted or purified CD4 T cells were plated in 96-well round-bottom plates at a density of 5×10^6 cells per ml. Cells were stimulated using plate-coated anti-CD3 and soluble anti-CD28 (both at 1 μg/ml; BD Biosciences). Cultures were supplemented with anti-IFN-γ and anti-IL-4 (both at 10 μg/ml; BD Biosciences). When added, CNTF, and IL-27 (R&D Systems) were used at 100 ng/ml each while IL-6 (R&D Systems) and TGFβ were used at 20 ng/ml and 2 ng/ml, respectively. CD4 T cells were supplemented with fresh medium and reagents on day 3. After 96 h of culture, cells were restimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 h. During the last 2 h, cytokine secretion was blocked with brefeldin A (5 μg/ml). Cells were collected and stained with FITC-labeled anti-CD4 (BD Biosciences) for 30 min on ice. Cells were washed and fixed with formaldehyde (2%) for 30 min at room temperature. Cells were permeabilized with saponin (0.5%) and stained with either allophycocyanin (APC)-labeled anti-IFN-γ (BD Biosciences) or phycoerythrin (PE)-labeled anti-IL-17 (eBiosciences, Cedarlane, Burlington, ON) for 1 h at room temperature. Fluorescence was measured using a FACSCalibur flow cytometer and data were analyzed using the FlowJo software.

3. Results

3.1. CNTF and CLC/CLF differ in their capacity to induce the proliferation of Ba/F3 cells expressing IL-6Rα, LIFRβ and gp130

The IL-3-dependent cell line Ba/F3 can be rendered responsive to either CNTF or CLC/CLF by transfection with the appropriate cytokine receptor cDNA [1,31]. To determine whether mouse IL-6Rα or