# TNF-α and IL-4 regulate expression of fractalkine ($CX_3CL1$) as a membrane-anchored proadhesive protein and soluble chemotactic peptide on human fibroblasts

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**Abstract The $CX_3C$ chemokine, fractalkine (FKN, $CX_3CL1$), has multiple functions and exists as two distinct forms, a membrane-anchored protein and a soluble chemotactic peptide that cleaves from the cell surface FKN. In this study, we first demonstrated the expression of FKN in tumor necrosis factor (TNF)-α- and interleukin (IL)-4-stimulated human fibroblasts. The induction of FKN was observed for both forms. We also demonstrated monocyte chemotactic activity in the culture supernatant from the fibroblasts stimulated with these cytokines. These results suggest that TNF-α- and IL-4-stimulated fibroblasts may play an important role in accumulation of monocytes at inflammatory sites.**
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*Key words:* Fractalkine; $CX_3CL1$; Tumor necrosis factor-α; Interleukin-4; Fibroblast

## 1. Introduction

The trafficking of leukocytes out of circulation and into sites of inflammation requires multiple steps including selectin-dependent rolling and chemokine-induced arrest mediated by integrins [1,2]. More than 50 chemokines have been identified to date, and these are subdivided into four subfamilies on the basis of the number and spacing of the first two cysteine residues in a conserved cysteine structural motif: C, CC, CXC and $CX_3C$ [3]. Different chemokine classes tend to exhibit different ranges of leukocyte specificity. The sole member of the $CX_3C$ subfamily, fractalkine (FKN, $CX_3CL1$), has two structural features that make it unique in the chemokine family: a $CX_3C$ motif and an extended carboxy-terminus that anchors it to the cell surface [4,5]. It is expressed on the surface of endothelial cells, epithelial cells, dendritic cells and neurons and is up-regulated by pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α [6–9]. Furthermore, FKN has multiple functions and exists as two distinct forms, a membrane-anchored proadhesive protein (mFKN) and a soluble chemotactic peptide (sFKN) that is released from the cell surface through proteolytic cleavage at membrane-proximal regions by TNF-α-converting enzyme (TACE), a member of the ADAM (disintegrin and metalloprotease) family, and ADAM10 [4].

Both the adhesion and chemotactic properties of FKN are mediated via a specific G protein-coupled, seven-transmembrane domain receptor, V28, now termed $CX_3CR1$ [10–12]. Surface expression of $CX_3CR1$ has been demonstrated on NK cells, γδ T cells, $CD8^+$ T cells, and $CD4^+$ T cells equipped with cytotoxic granules, monocytes, and mast cells [10,13–16]. $CX_3CR1$ is capable of mediating both leukocyte migration and adhesion. Firm adhesion is not inhibited by pertussis toxin under static and physiological flow conditions in monocytes, T cells and NK cells [13]. Unlike other chemokine/G protein-coupled receptor interactions that require signal transduction and integrin activation for firm cell adhesion, the adhesive interaction between FKN and $CX_3CR1$ is independent of signal transduction and integrin function [13,14]. However, it has recently been demonstrated that FKN can induce adhesion by up-regulating integrin avidity in a pertussis toxin-sensitive manner [17].

Nasal polyposis is a chronic inflammatory disease of the nasal mucosa sometimes affecting patients with allergy. The polyp stroma is highly edematous, with a varying density of inflammatory cells and fibroblasts [18]. In the airway, fibroblasts, which also include myofibroblasts, produce collagen and have been shown to produce specific cytokines that may attract inflammatory cells to the airway and perpetuate the inflammatory and fibrotic processes [19,20].

The purpose of this study was to examine the expression of FKN in human fibroblasts established from human nasal polyp biopsy specimens because of the putative importance of TNF-α and IL-4 in controlling allergic inflammation. We have also analyzed the induction of FKN in lung- and skin-derived fibroblasts.

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## 2. Materials and methods

### 2.1. Reagents

Recombinant TNF-α, interferon (IFN)-γ, IL-4 and IL-13 were purchased from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies (mAb) against FKN (1F3-1 and 3A5-2) were prepared in our laboratory (Kan Research Institute). Polyclonal antibody against FKN was purchased from R&D Systems. Polyclonal antibody against $CX_3CR1$ was purchased from Torrey Pines Biolabs (Houston, TX, USA).

### 2.2. Cell cultures

Human nasal fibroblasts were established from nasal polyp biopsy specimens. Briefly, single cells isolated from nasal polyp specimens were washed with phosphate-buffered saline (PBS), and then cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS; JRH Bioscience, Lenexa, KS, USA), 100 U/ml penicillin–100 μg/ml streptomycin (Gibco BRL, Grand Island, NY, USA) and 3 μg/ml amphotericin B (Sigma, St. Louis, MO, USA). The cells were incubated at 37°C in a humidified incubator containing 5% $CO_2$ in air. The cells were used between passages 3 and 10 for analysis of FKN induction.

A human fetal lung fibroblast line, MRC-5-30, was purchased from Health Science Research Resources Bank (Osaka, Japan) and cultured in Eagle's minimal essential medium (Gibco BRL) with 10% FCS, penicillin–streptomycin and amphotericin B. The cells were used between passages 31 and 34.

Normal human dermal fibroblasts (adult skin) were obtained from Clonetics (Walkersville, MD, USA) and cultured in Fibroblast Basal Medium (Clonetics) with 2% fetal bovine serum, 1 μg/ml human recombinant fibroblast growth factor, 5 μg/ml insulin, 50 μg/ml gentamicin and 50 μg/ml amphotericin B (Clonetics).

### 2.3. Cytokine stimulation of fibroblasts

For cytokine stimulation, fibroblasts were cultured in 6-well or 48-well culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). They were grown to approximately 80% confluence, and then the culture medium was replaced with Dulbecco's modified Eagle's medium/Ham's F12 medium (Gibco BRL) without FCS in the presence of various cytokines.

### 2.4. Relative quantitation of mRNA by reverse transcription polymerase chain reaction (RT-PCR)

Nasal fibroblasts were cultured with TNF-α (50 ng/ml) and IL-4 (10 ng/ml) alone or in combination for 12–48 h. Total RNA was extracted with an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA syntheses were performed using a Ready-To-Go kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The PCR conditions were optimized empirically using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and GeneAmp PCR system 9600 (Applied Biosystems). Forward and reverse oligo primers for FKN (5′-ATG GCT CCG ATA TCT CTG TCG T-3′ for sense and 5′-AAA AGC TCC GTG CCC ACA-3′ for antisense) and GAPDH (5′-GTC TTC ACC ACC ATG GAG AAG GCT-3′ for sense and 5′-CAT GCC AGT GAG CTT CCC GTT CA-3′ for antisense) were designed based on the published sequences of each mRNA. The amplified fragments of FKN and GAPDH were 557 bp and 393 bp, respectively, as expected. DNA polymerase was activated by preheating (1 min at 94°C). Then amplification was performed for 37 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and elongation (2 min at 72°C) followed by final elongation (10 min at 72°C). PCR products were electrophoresed through a 2% agarose gel and visualized with ethidium bromide. For semi-quantitative RT-PCR, the relative band intensities were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

### 2.5. Flow cytometric analysis

Nasal fibroblasts were cultured with TNF-α (50 ng/ml) and IL-4 (10 ng/ml) alone or in combination for 72 h and were detached with a non-enzymatic cell dissociation solution (Gibco BRL). The cells were then incubated with anti-human FKN (1F3-1) mAb in the presence of human IgG (50 mg/ml) for 30 min at 4°C. Fluorescein isothiocyanate-labeled goat anti-mouse Ig Ab (Becton Dickinson, San Jose, CA, USA) was then applied for 20 min at 4°C. As a control, cells were incubated under similar conditions with mouse IgG1 (clone MOPC 21; Sigma). The cells were fixed with 4% formaldehyde in PBS and were analyzed on a FACScan (Becton Dickinson). The fluorescence intensity was determined for 10 000 cells per sample using the CELLQuest software (Becton Dickinson).

### 2.6. Enzyme-linked immunosorbent assay (ELISA) for FKN

For quantification of sFKN, cell culture supernatants were collected and stored frozen at −80°C until use. In some experiments, the cell culture supernatant was concentrated with Ultrafree-MC centrifugal filter units (Millipore, Bedford, MA, USA). To determine total FKN in nasal fibroblasts, cells were solubilized with ice-cold lysis buffer containing 0.2% NP-40 and protease inhibitor cocktail tablets (Roche, Mannheim, Germany), centrifuged, and the soluble extract was prepared. FKN concentrations were analyzed by sandwich-type ELISA. Briefly, a 96-well plate (Nunc, Roskilde, Denmark) was coated with 5 μg/ml of anti-human FKN mAb (3A5-2) overnight at 4°C. The plate was washed and then blocked with blocking solution (Boehringer, Mannheim, Germany) for 1 h at room temperature. Samples or recombinant human FKN (R&D Systems) as a standard were added to the wells, and the plate was incubated at 4°C overnight. After washing with 0.1% Brij-PBS, 0.25 ng/ml of biotinylated polyclonal anti-FKN Ab (R&D Systems) was added, and the plate was incubated for 3 h at room temperature. After washing, 100 μl of streptavidin-horseradish peroxidase (Gibco BRL) was added, and the plate was incubated for 30 min at room temperature. The wells were developed with the TMB microwell substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped with 1 M $H_3PO_4$, and the absorbance was read at 490 nm with a microtiter ELISA reader (MR5000, Dynatech Laboratories, Chantilly, VA, USA). Data were analyzed with the Biolinx 2.10 program (Dynatech Laboratories), and the concentration of FKN was calculated by linear regression analysis from the standard curves.

### 2.7. Chemotaxis assay

The biological activity of FKN in the culture supernatant of cytokine-stimulated fibroblasts was assessed by a chemotaxis assay using human monocytes. The monocytes were separated by the cold aggregation method [21].

Briefly, mononuclear cells were isolated from the peripheral blood of normal healthy volunteers using lymphocyte separation medium (ICN Biomedicals, Aurora, OH, USA). The mononuclear cells at $1\times10^7$ cell/ml in RPMI 1640 containing 10% FCS were then agitated gently for 1 h at 4°C and aggregated monocytes were collected. The purity of the monocytes was determined by flow cytometry using anti-human CD14 Ab and was more than 80%. For the chemotaxis assay, 48-well plates assembled with Boyden chambers (5-μm pore size polycarbonate membranes) were used. Briefly, 29 μl of cell culture supernatants of the fibroblasts stimulated with TNF-α plus IL-4 for 96 h were placed in triplicate in the lower chamber. After assembly of the Boyden chambers, 50-μl suspensions of human monocytes (50 000 cells) in RPMI 1640 containing 0.1% bovine serum albumin (BSA) were placed in the upper chamber. The chambers were then incubated in a humidified $CO_2$ incubator at 37°C for 30 min. After the incubation, the filters were recovered, non-migrating cells were removed from the upper surface by washing with PBS, and then the cells that had migrated across the membrane were fixed, and stained by Diff-Quick. In some experiments, human monocytes were preincubated with 10 μg/ml of purified rabbit anti-human $CX_3CR1$ Ab or rabbit IgG as a control, and the chemotactic activity was measured as described above. Migrated monocytes were counted in three random high-power fields by light microscopy, and specific chemotactic activity was expressed as the net percentage after subtraction of spontaneous migration in fresh culture medium containing TNF-α and IL-4.

### 2.8. Statistical analysis

Data are presented as the mean ± S.E.M. Statistical significance was determined by the paired Student's *t*-test and $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Detection of FKN mRNA by RT-PCR

We first analyzed the mRNA levels of FKN in human nasal

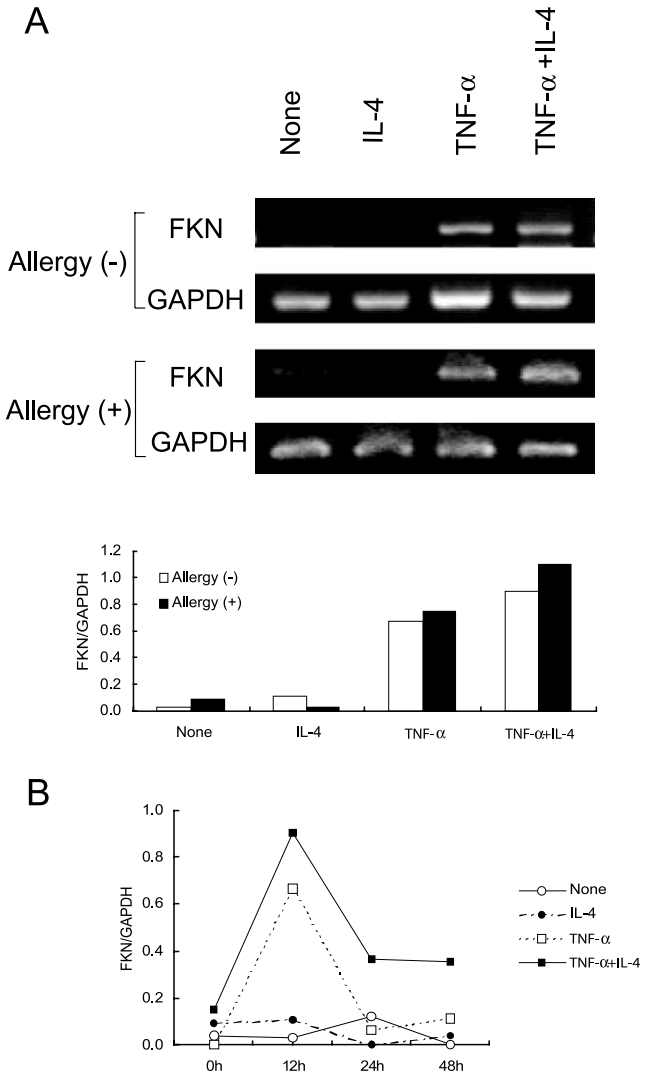


Fig. 1. Expression of FKN mRNA in human nasal fibroblasts. A: Cells from patients with or without allergy were incubated with TNF-α (50 ng/ml) and/or IL-4 (10 ng/ml) for 12 h. FKN mRNA was detected by RT-PCR. For semi-quantitative analysis, RT-PCR products of FKN transcripts were analyzed with an Agilent 2100 Bioanalyzer. The vertical axis indicates the band intensities of FKN corrected for GAPDH. Each RT-PCR analysis is representative of at least three different experiments. B: The time kinetics of FKN expression was determined up to 48 h by RT-PCR.

fibroblasts from patients with or without allergy in the presence of TNF-α and/or IL-4 by RT-PCR (Fig. 1A). The FKN transcript in these fibroblasts was up-regulated in the presence of TNF-α. In contrast, expression of FKN mRNA was not detected in the cells exposed to IL-4 alone. We observed similar induction of FKN mRNA in nasal fibroblasts derived both from patients with and without allergy. We also determined the time kinetics of the expression of FKN mRNA after stimulation with these cytokines. The FKN mRNA was significantly up-regulated by TNF-α in a time-dependent fashion, and reached peak levels within the first 12 h and decreased by 24 h (Fig. 1B). In addition, the combined stimulation with TNF-α and IL-4 showed continuous enhancement of the FKN mRNA level.

### 3.2. Detection of mFKN on the cell surface

We next examined the expression of mFKN protein on the cell surface of human nasal fibroblasts in the presence of cytokines by flow cytometric analysis using a FKN-specific mAb (1F3-1). The mFKN expression on the surface of human nasal fibroblasts was up-regulated by TNF-α for 72 h (Fig. 2). In addition, IL-4 enhanced the expression of mFKN that was induced by TNF-α.

### 3.3. Detection of FKN protein

We performed quantitative analysis of the induction of the two forms of FKN proteins, i.e. sFKN in the cell culture supernatant and mFKN on the cell surface. Therefore, an ELISA for human FKN was developed using a mAb and a polyclonal Ab, as described in Section 2. We determined the concentration of sFKN in the supernatant from the fibroblasts in the presence of cytokines at various time points (Fig. 3A,B). We detected the induction of sFKN in the supernatant from TNF-α-stimulated fibroblasts although IL-4 did not affect the induction. The combined stimulation with TNF-α and IL-4 dramatically showed synergistic enhancement of sFKN protein at 72–96 h (Fig. 3A). In contrast, IL-13 inhibited the induction of sFKN in the supernatant from IFN-γ-stimulated fibroblasts (Fig. 3B). We also determined the dose dependence of TNF-α and IL-4 in the induction of sFKN from nasal fibroblasts cultured with these cytokines (Fig. 3C). The induction of sFKN was observed synergistically with IL-4 in a dose-dependent fashion.

We also tested the induction of sFKN from human fibroblasts derived from different tissues (Fig. 3D). The sFKN protein was detected not only from nasal fibroblasts but also from lung-derived (MRC-5-30) and skin-derived (NHDF7555) fibroblasts in the presence of the cytokines.

To evaluate whether production of sFKN accompanied the expression of intracellular and membrane-anchored FKN, we determined the total FKN in the cells. The amounts of intracellular FKN, including mFKN, in nasal fibroblasts was significantly up-regulated in the presence of TNF-α in a time-dependent fashion, and reached peak levels within the first 72 h of stimulation (Fig. 4). IL-4 markedly enhanced the TNF-α-induced FKN in whole cells.

### 3.4. Chemotactic activity of sFKN derived from fibroblasts

Finally, we tested the chemotactic activity of sFKN derived from the cytokine-stimulated nasal fibroblasts. Human monocytes express a receptor for FKN, $CX_3CR1$, as described above [10]. Therefore, we assessed the chemotactic activity using human monocytes from peripheral blood (Fig. 5). Monocyte chemotaxis was observed in the supernatant derived from the TNF-α- and IL-4-stimulated fibroblasts. Also, 56.2% of the chemotactic activity was suppressed by anti-$CX_3CR1$ Ab (10 μg/ml).

## 4. Discussion

In this study, we provide the first evidence that the expression of FKN is up-regulated by TNF-α in human fibroblasts. In this regard, previous studies determined that FKN was primarily expressed by neurons [6,22,23] and endothelial cells [8,10]. As far as fibroblasts are concerned, FKN expression was reported only for synovial tissue from patients with rheumatoid arthritis [24]. FKN was initially described as being

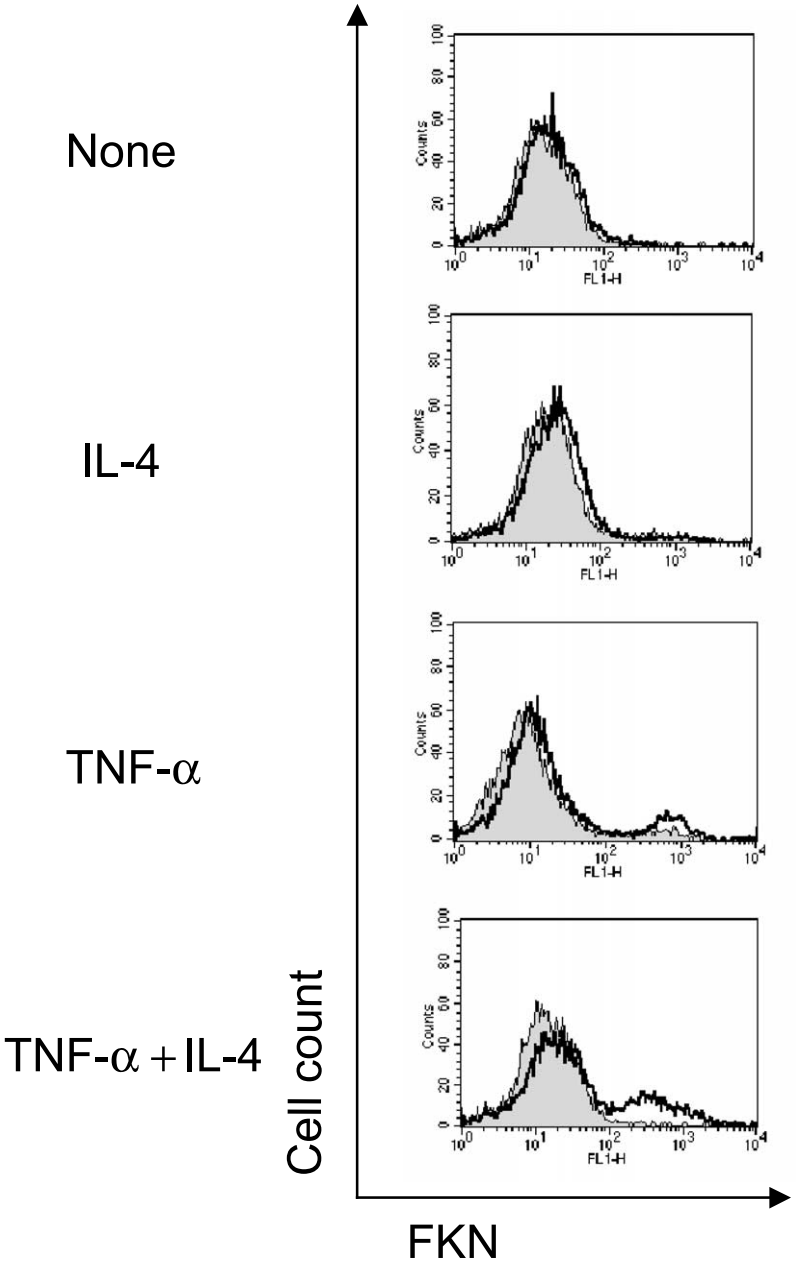


Fig. 2. Expression of mFKN on the cell surface of human nasal fibroblasts. mFKN was detected by flow cytometry. Cells were incubated with TNF-α (50 ng/ml) and/or IL-4 (10 ng/ml) for 72 h. Each histogram is representative of at least three different experiments.

expressed on IL-1β- and TNF-α-activated endothelial cells and having a wide mRNA distribution in human [4] and murine tissues [5]. In this experiment, it is interesting that TNF-α induced FKN in human fibroblasts, which was synergistically up-regulated by IL-4. Recently, Fraticelli et al. reported that IFN-γ and TNF-α induced FKN expression on vascular endothelial cells. In contrast, IL-4 and IL-13 suppressed this induction in the endothelial cells [25]. Our results support the induction of FKN in IFN-γ-stimulated fibroblasts in their finding. However, IL-4 synergistically enhanced TNF-α-induced FKN in human fibroblasts. A similar IL-4 synergistic effect was reported for TNF-α-induced eotaxin in human dermal fibroblasts [26]. Furthermore, a recent study demonstrated that both IL-4 and TNF-α induce eotaxin expression at the level of transcription via a STAT6-mediated pathway [27]. Presumably this different effect of IL-4 is caused by the difference of characteristics between vascular endothelial cells and fibroblasts.

On the cell surface, the two peaks of FKN expression demonstrate a biphasic population upon stimulation, where only a subpopulation up-regulate FKN. This in itself is an extremely interesting observation and may demonstrate the heterogeneity of fibroblast populations, and their complex nature in regulating immune function. One possibility is that FKN production may be a product of this subpopulation only. Future work should include isolation and characterization of this population.

The expression of FKN mRNA reached a peak within the first 12 h. sFKN in the cell culture supernatant increased rapidly during 72–96 h, although the expression of intracellu-

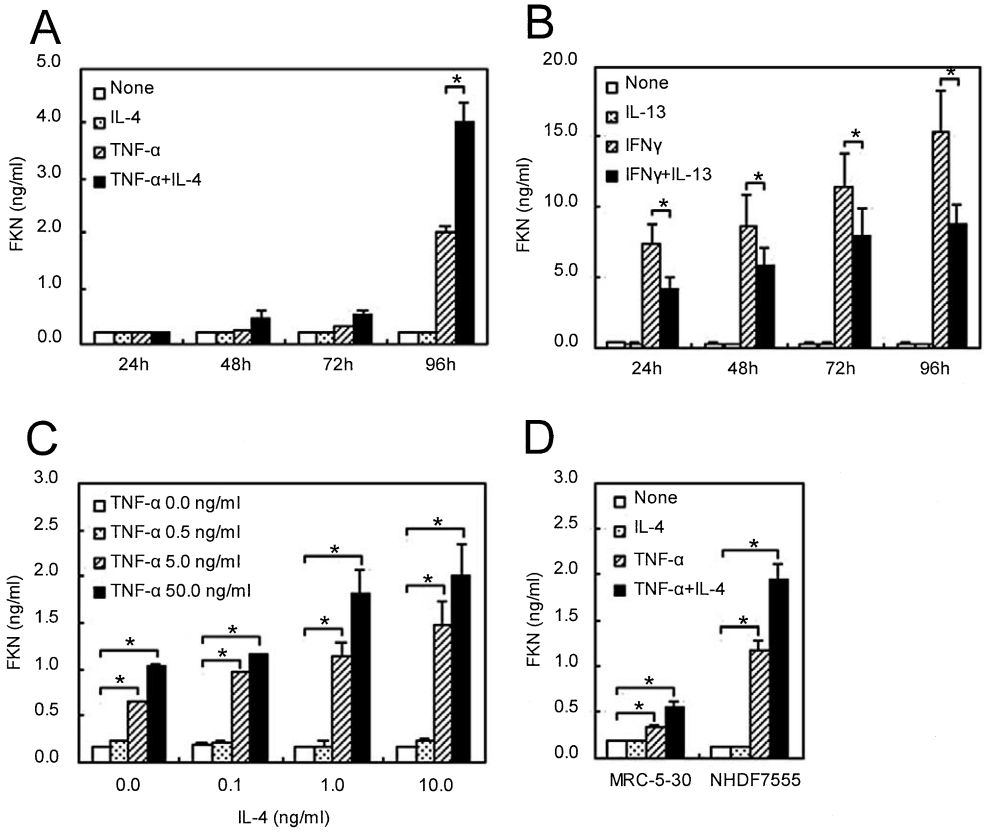


Fig. 3. Induction of sFKN in the culture supernatant from human nasal fibroblasts. A: Cells were incubated with TNF-α (50 ng/ml) and/or IL-4 (10 ng/ml) for 24–96 h. sFKN in the culture supernatant was detected by ELISA. $*P < 0.05$. B: Cells were incubated with IFN-γ (10 ng/ml) and/or IL-13 (10 ng/ml) for 24–96 h. sFKN in the culture supernatant was detected by ELISA. $*P < 0.05$. C: Dose dependence of TNF-α (0.5–50 ng/ml) and IL-4 (0.1–10 ng/ml) in the induction of sFKN. Cells were cultured with TNF-α and/or IL-4 for 72 h. $*P < 0.05$. D: Induction of sFKN in the culture supernatant from human fetal lung-derived fibroblasts (MRC-5-30) and human skin-derived fibroblasts (NHDF7555). These fibroblasts were incubated with TNF-α (50 ng/ml) and/or IL-4 (10 ng/ml) for 72 h. $*P < 0.05$.

lar FKN and mFKN peaked within the first 72 h and gradually decreased in the following 24 h. These results indicate that the extracellular domain of FKN undergoes proteolytic cleavage and is released from the cell surface into the surrounding fluid phase mainly during 72–96 h after stimulation. FKN can be shed after extracellular proteolysis at a mem-

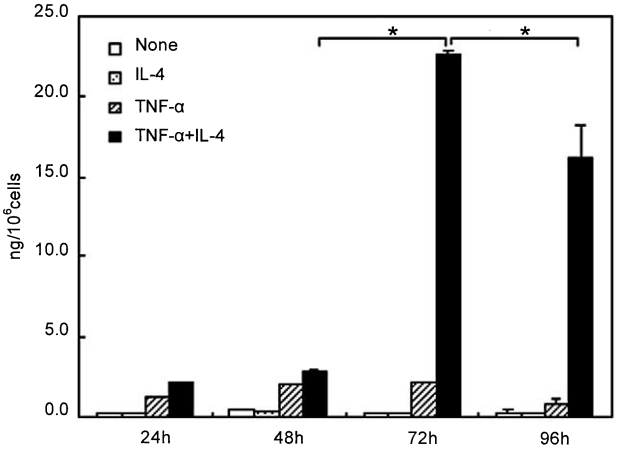


Fig. 4. Induction of FKN protein in the cell lysate of human nasal fibroblasts. Cells were incubated with TNF-α (50 ng/ml) and/or IL-4 (10 ng/ml) for 24–96 h. FKN in the cell lysate was detected by ELISA. $*P < 0.05$.

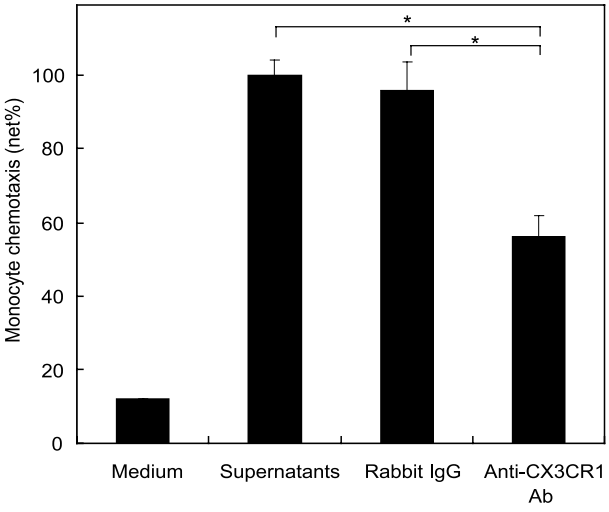


Fig. 5. Monocyte chemotaxis was observed in the supernatant derived from the TNF-α- and IL-4-stimulated fibroblasts with Boyden chambers. Monocytes (50 000 cells in RPMI 1640 containing 0.1% BSA) were seeded in each well and allowed to migrate at 37°C for 30 min. In inhibition assays the cells were incubated with 10 μg/ml of purified rabbit anti-human $CX_3CR1$ Ab or rabbit IgG as a control before seeding in each well. Cells were counted in three random high-power fields by light microscopy, and the chemotactic activity was expressed as the net percentage. $*P < 0.05$.

brane-proximal cleavage site [4]. Regarding the shedding mechanism, a plasma membrane enzyme responsible for the shedding of TNF-α, i.e. TACE, was also shown to cleave transforming growth factor-α, TNF-α receptor types I and II [28,29], the receptors for IL-6, colony-stimulating factor-1 [30,31] and L-selectin [32], and IL-1 type II decoy receptor [33]. Indeed, Garton et al. demonstrated that sFKN can be generated by TACE-dependent cleavage and ectodomain shedding [34].

We also demonstrated monocyte chemotactic activity in the culture supernatant of the fibroblasts stimulated with TNF-α and IL-4. Anti-$CX_3CR1$ neutralizing Ab inhibited approximately 50% of this activity. This result indicates that about half of the monocyte chemotactic activity in the supernatant is sFKN. The above results lead us to hypothesize that the fibroblasts stimulated by these cytokines may accumulate immunocompetent cells expressing $CX_3CR1$ at inflammatory sites.

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