Membrane-bound form of fractalkine induces IFN-γ production by NK cells

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Natural killer (NK) cells participate in both innate and adaptive immunity, in part by their prompt secretion of cytokines including IFN-γ, a pro-inflammatory cytokine with an important role in Th1 polarization. To assess the involvement of fractalkine in inflammatory processes, we examined the effect of fractalkine on IFN-γ production by NK cells. Although soluble chemokines, including MCP-1 and RANTES as well as fractalkine, had a negligible effect on IFN-γ production, immobilized fractalkine markedly induced IFN-γ production by NK cells in a dose-dependent manner. Pretreatment of NK cells with the phosphatidylinositol 3-kinase (PI 3-K) inhibitor, wortmannin, completely inhibited the production of IFN-γ induced by fractalkine, and pretreatment with the protein tyrosine kinase inhibitor, herbimycin A, partially suppressed the response, suggesting that augmentation of IFN-γ production in response to fractalkine treatment of NK cells involves signaling through PI 3-K and protein tyrosine kinases. Furthermore, co-culture of NK cells with fractalkine-transfected 293E cells markedly enhanced IFN-γ production by NK cells compared with co-culture with control 293E cells. These findings may indicate a paracrine feedback loop system in which endothelial cells may be activated to produce more fractalkine, and also suggest a role for fractalkine expressed on endothelial cells in Th1 polarization through the stimulation of IFN-γ production by NK cells.

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1 Introduction

Natural killer (NK) cells, which express cytolytic activity without prior antigenic stimulation, are thought to mediate immunity against virus infection and neoplastic transformation [1, 2]. NK cells are also believed to contribute to immune regulation through the production of pro-inflammatory cytokines such as IFN-γ and TNF-α, which activate various types of cells including T cells, macrophages and NK cells [3, 4]. IFN-γ also enhances expression of VCAM-1 and ICAM-1 on endothelial cells, resulting in consequent leukocyte trafficking across endothelial surfaces [5, 6]. Furthermore, IFN-γ induces a variety of cell types to produce chemokines, which can regulate immune responses, for example, through Th1 polarization [7–9].

Chemokines play an important role in the recruitment of various types of cells to the inflammatory sites [10, 11]. Chemokines are divided into four subfamilies depending on the arrangement of cysteine residues in the N-terminal region: CXCL, CCL, CL and CX3CL [9, 12]. Fractalkine is the only known CX3CL chemokine and is structurally distinct from other chemokines. Fractalkine exists as a membrane-bound molecule with the chemokine

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Abbreviations: MCP: Monocyte chemotactic protein **RANTES:** Regulated upon activation, normal T cell expressed and secreted **VCAM-1:** Vascular cell adhesion molecule-1 **PI 3-K:** Phosphatidylinositol 3-kinase

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domain at the end of a mucin-like stalk, and is released in soluble form through cleavage at a membrane proximal site [13, 14]. We have previously reported that CX3CR1, a receptor for fractalkine, is expressed on NK cells [15] and that fractalkine increases NK cell activities, such as granule exocytosis and cytotoxicity [16]. Fractalkine is expressed on endothelial cells activated by TNF-α, IFN-γ and IL-1, and rapidly captures cells expressing its receptor, CX3CR1 [13–15]. Since the endothelium plays an important role in the recruitment and emigration of circulating leukocytes into sites of inflammation, fractalkine expressed on inflamed endothelium may function as the vascular gatekeeper for immune responses [17]. In the present study, we report that immobilized and membrane-bound fractalkine markedly induced IFN-γ production by NK cells, data that suggest a role for fractalkine in Th1 cell polarization as well the possible existence of a paracrine loop of endothelial and NK cell activation.

2 Results and discussion

2.1 Effects of fractalkine on interferon-γ production by NK cells

NK cells are important in the early phases of immune responses against microbial infections through production of cytokines, including IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5, IL-10, and IL-15 [18, 19]. However, chemokine-induced cytokine production by NK cells remains to be defined. Since NK cells express fractalkine receptor CX3CR1 and adhere to immobilized full-length fractalkine [16], we examined whether the soluble or immobilized form of fractalkine induces production of IFN-γ by NK cells. NK cells (5×10^5/ml) were stimulated with soluble (10 nM) or immobilized (polybeads pretreated with 10 nM) fractalkine, and supernatants were collected and examined for production of IFN-γ by ELISA. The effect of immobilized fractalkine on IFN-γ production by NK cells was dose-dependent (Fig. 1A). Time-course studies revealed that IFN-γ production was observed after 24-h stimulation with the maximum response achieved at 48 h (Fig. 1B). Although some variability in magnitude was observed among individuals, immobilized fractalkine induced IFN-γ production from NK cells in repeated tests using cells from seven different donors (Fig. 1C).

Several CC chemokines, including macrophage inflammatory protein-1α (MIP-1α), IFN-γ-inducible protein-10 (IP-10), monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, and regulated upon activation, normal T cell expressed and secreted (RANTES), have been reported to activate NK cells, resulting in increased cytosolic free Ca^{2+}, chemotaxis, granule exocytosis and cytotoxicity [20, 21]. Therefore, we examined whether either the soluble or immobilized forms of MCP-1 and RANTES induce IFN-γ production by NK cells. NK cells (5×10^5/ml) were cultured in the presence of soluble forms of fractalkine, MCP-1 and RANTES (each 10 nM) or stimulated with polybeads pretreated with 10 nM of each chemokine at 37°C for 48 h. After incubation, supernatants were collected and IFN-γ production was examined. Although NK cells express CCR2 and CCR3 [22], MCP-1 and RANTES of either soluble or immobilized form did not induce more than marginal enhancement of IFN-γ production. However, immobilized fractalkine, but not MCP-1 nor RANTES, markedly enhanced IFN-γ production by NK cells (Fig. 1D). Fractalkine contains an extended mucin-like stalk, a transmembrane domain and intracellular domain, and fractalkine itself functions as an adhesion molecule to support cell adhesion [15, 23, 24]. In this regard, results of transfection studies in which the fractalkine mucin-like stalk was replaced with a rod-like segment of E-selectin or the chemokine domain of fractalkine was replaced with other soluble chemokines, such as MCP-1 and RANTES, revealed that the mucin-like stalk extends the chemokine domain to support cell adhesion [25, 26]. Thus, immobilized fractalkine-mediated NK cell activation leading to IFN-γ

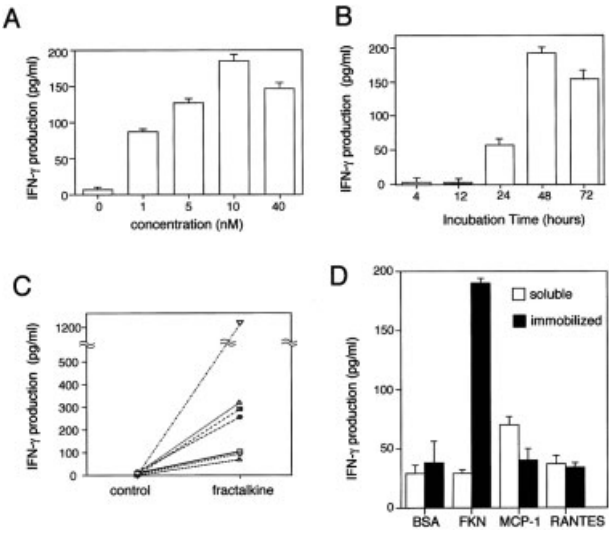

Fig. 1. Effect of immobilized fractalkine on IFN-γ production by NK cells. (A) NK cells were incubated for 24 h in the presence of various concentrations of immobilized fractalkine. (B) NK cells were incubated for the indicated period with fractalkine immobilized on polybeads. (C) NK cells were isolated from seven different healthy donors and cultured in the absence or presence of immobilized fractalkine for 24 h. (D) NK cells were incubated for 24 h with soluble or immobilized fractalkine (FKN), MCP-1 or RANTES. Supernatants were collected and IFN-γ was measured by specific ELISA.

production may be explained by the unique architecture of fractalkine, which may efficiently aggregate signal components associated with CX3CR1, consistent with a model proposed by Rodriguez-Frade et al. [27].

2.2 Effects of inhibitors on fractalkine-mediated IFN-γ production

Pretreatment of NK cells with 10 μg/ml of cycloheximide for 60 min nearly abolished fractalkine-induced IFN-γ production (Fig. 2A), suggesting that fractalkine transduces signals for *de novo* synthesis of IFN-γ. Chemokine receptors identified to date, including CX3CR1, all manifest a seven transmembrane G protein-linked architecture and transduce signals that lead to Ca^{2+} influx, cytoskeletal reorganization, integrin activation, and other functions leading to increased adhesion and migration of the cells [9, 12]. Al-Aoukaty et al. [28] have reported that fractalkine activates Gαi and Gαz types of G proteins. We have also observed that a Gαi protein inhibitor, pertussis toxin, completely blocked granule exocytosis from fractalkine-activated NK cells [16]. In addition to G protein activation, fractalkine has been reported to transduce cell activation signals through extracellular signal-related kinases (ERK1 and ERK2), stress-activated protein kinases (SAPK1/JNK1 and SAPK2/p38), Akt [down stream of phosphatidylinositol 3-kinase (PI 3-K)], src and syk kinases [22, 29, 30]. Therefore, we examined the effects of the protein tyrosine kinase inhibitor, herbimycin A, and PI 3-K inhibitor, wortmannin, on fractalkine-mediated NK cell activation leading to IFN-γ production.

The results of the experiment depicted in Fig. 2B show that herbimycin A significantly inhibits fractalkine-induced IFN-γ production, suggesting that tyrosine kinases are involved, at least in part, in fractalkine-mediated NK cell activation. We also confirmed that wortmannin completely suppressed IFN-γ production. Although further investigations to delineate the signaling pathway of CX3CR1 are required, the proposed model for a signaling pathway through G protein-coupled receptors may explain the different roles of tyrosine kinase and PI 3-K in fractalkine-mediated signal transduction [31].

2.3 IFN-γ production by co-culture of NK cells with fractalkine-expressing cells

Finally, we examined whether membrane-bound fractalkine activates NK cells. To examine this possibility, we transfected fractalkine cDNA into 293E cells, producing transient expression of membrane-bound fractalkine without up-regulation of other adhesion molecules including ICAM-1 and VCAM-1 (data not shown). Freshly isolated NK cells were incubated with confluent monolayers of 293E (control 293E) cells or fractalkine-transfected 293E (FRK-293E) cells for 24 h. After incubation, supernatants were collected and examined for IFN-γ production. As shown in Fig. 3, FRK-293E cells strongly enhanced IFN-γ production by NK cells compared to control 293E cells. This enhancement was partially but significantly inhibited with anti-fractalkine Ab. Both control 293E cells and FRK-293E cells did not produce IFN-γ spontaneously.

There is growing evidence that the chemokine-cytokine network is crucial for inflammatory responses, and the association of chemokines with the Th1 or Th2 phenotypes has been well established [32, 33]. It has been reported that CX3CR1 is preferentially expressed in Th1

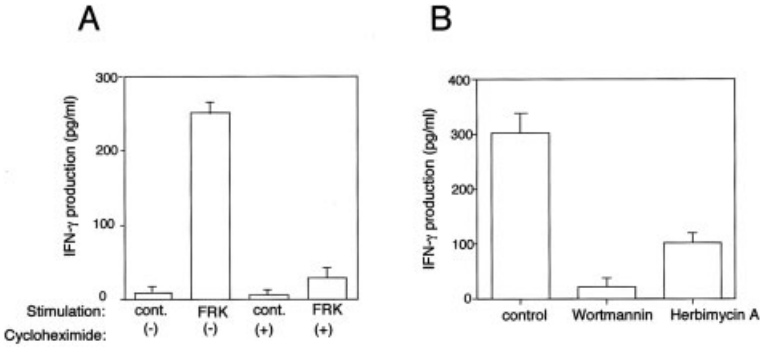

Fig. 2. Effects of inhibitors on IFN-γ production by NK cells. NK cells were pretreated with cycloheximide (A), wortmannin and herbimycin A (B). After pretreatment, NK cells were washed extensively and incubated with immobilized fractalkine (FRK) for 24 h. Then supernatants were collected and IFN-γ was measured by ELISA. The data are presented as means of three independent experiments and error bars indicate SD.

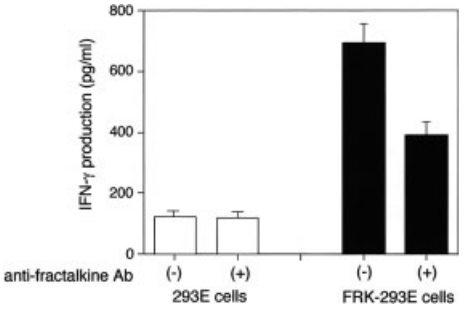

Fig. 3. Effect of membrane-bound fractalkine on IFN-γ production. Freshly isolated NK cells were co-cultured with control 293E cells or fractalkine-transfected (FRK-293E) cells in the absence or presence of anti-fractalkine neutralizing Ab for 24 h, and IFN-γ production was examined. The data are presented as means ± SD from three independent experiments.

compared with Th2 cells, and that Th1 but not Th2 cells respond to fractalkine [34], suggesting that expression of fractalkine may mediate Th1 polarization directly at the inflammation sites. In this regard, it has been reported that fractalkine is expressed on endothelial cells in cases of psoriasis, a Th1-dominated skin disorder, but not in cases of Th2-driven atopic dermatitis [34]. Overall, the Th1/Th2 balance is primarily controlled by two antagonistic cytokines, IFN-γ and IL-4 [8, 35]. Since IFN-γ activates dendritic cells to produce IL-12, which is a strong activator of NK cells and plays a selective role in driving Th1 responses [36, 37], a positive feedback loop between fractalkine and IFN-γ may accelerate Th1-based immune responses each other.

Very recently, we have reported that surface expression of CX3CR1 clearly defines cytotoxic lymphocytes commonly possessing high levels of intracellular perforin and granzymes B, which include NK cells, γδ T cells and terminally differentiated $CD8^+$ T cells [38]. Furthermore, soluble fractalkine preferentially attracts cytotoxic lymphocytes, and membrane-bound fractalkine enhances their migration in response to secondary chemokines, resulting in increased transmigration of cytotoxic lymphocytes regardless of their lineage and mode of target cell recognition [38]. Thus, fractalkine and CX3CR1 are likely to function as a vascular gatekeeper for cytotoxic lymphocytes at the inflammation sites and may be involved in the pathophysiology of vascular and tissue injuries of various clinical diseases [17].

Since IFN-γ is known to activate endothelial cells [39], the increased IFN-γ produced by NK cells may further augment expression of fractalkine on endothelial cells, contributing to a paracrine positive feedback loop. Taken together, a chemokine-to-cytokine-to-chemokine cascade may be involved in various immune responses [40], and our results presented here suggest a role for fractalkine in the Th1-based immune response, anti-viral and anti-tumor immune response, as well as its direct toxicity to endothelium.

3 Materials and methods

3.1 Monoclonal antibodies and reagents

Monoclonal antibody (mAb) against CD3 (OKT3) was purified from ascites as previously described (hybridoma from the American Type Culture Collection, Manassas, VA) [41]. Anti-fractalkine mAb was made by immunizing mice with recombinant human fractalkine expressed in a baculovirus expression system [15]. Anti-CD14 and anti-CD19 mAb were purchased from Immunotech (Marseilles, France). FITC-conjugated goat anti-mouse Ab were purchased from Becton Dickinson (Mountain View, CA). Recombinant human fractalkine, RANTES and MCP-1 were purchased from Genzyme (Cambridge, MA). Polybeads, polystyrene microspheres (2.5% solid-latex, diameter 6 μm) were purchased from Polysciences (Warrington, PA). Wortmannin and herbimycin A were purchased from Wako Chemicals (Osaka, Japan), and cycloheximide from Sigma (St. Louis, MO).

3.2 Cells and NK cell purification

293/EBNA-1 (293E) cells were purchased from Invitrogen (Carlsbad, CA). For transient expression of membrane-bound fractalkine, the expression plasmid pCAGG-Neo-fractalkine-1 [15] was transfected into 293E cells (FRK-293E) by electroporation using GENE-PULSER II (Bio-Rad, Japan). Cells were maintained with G418 contended Dulbecco's modified Eagle medium containing 10% heat-inactivated fetal calf serum and were used for assay after 24-h incubation.

PBMC were isolated from peripheral venous blood from consenting healthy volunteers by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density-gradient centrifugation. NK cells were isolated by negative selection using a mixture of anti-CD3, anti-CD14 and anti-CD19 mAb and immunomagnetic beads (PerSeptive Diagnostics, Cambridge, MA) to deplete T cells, monocytes and B cells, respectively, as described previously [41]. NK cell populations used in all experiments were >85% pure as confirmed by flow cytometric analysis for the presence of CD56 and the absence of CD3 (FACS Caliber, Becton Dickinson) [16].

3.3 Cell stimulation and measurement of cytokines

Freshly isolated NK cells (5×10^5) were cultured with soluble RANTES, MCP-1 and fractalkine for 24 h at 37°C in 96-well plates (IWAKI Glass, Osaka, Japan). For NK cell stimulation with the immobilized chemokines, polybeads were pre-coated with the indicated concentration of RANTES, MCP-1 or fractalkine in PBS, pH 8 at 4°C overnight, then treated with 1% BSA/PBS, pH 7.4 at room temperature for 2 h prior to use. Purified NK cells (5×10^5) were cultured in 96-well plates with chemokine-coated polybeads (cell:beads 1:4) for 24 h at 37°C. For the stimulation with membrane-bound fractalkine, NK cells were co-cultured with control 293E or transfected FRK-293E cells (1×10^5) for 24 h. In some experiments, NK cells were pretreated with wortmannin, herbimycin A (1 μM each for 20 min), or cycloheximide (10 μg/ml for 60 min) at 37°C. After incubation, the plates were centrifuged, and cell-free supernatants were collected and assessed for cytokine production using ELISA kits (BioSource International, CA) according to the manufacturer's protocols. The detectable range of each ELISA kit was 5–1,000 pg/ml and the optical density of individual wells was determined at 450 nm using microplate reader (Bio-Rad).

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