Supplementary Information

Development and Characterization of an Aptamer Binding Ligand of Fractalkine Using Domain Targeted SELEX

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Schematic of Fractalkine



Fig. S1 Graphic representation of fractalkine (not to scale). The chemokine domain is attached to a mucin-like stalk. A single pass transmembrane domain anchors fractalkine to the cell.

Materials and Methods

Materials

Recombinant human fractalkine containing the chemokine domain, mucin-like stalk and a poly-histidine tag (FKN; Catalog Number 365-FR-025) and an anti-fractalkine chemokine domain antibody (FKN Ab; Catalog Number AF365) were obtained from R&D Systems (Minneapolis, MN). The anti-polyhistidine tag antibody was obtained from Millipore (His Ab; Billerica, MA, Catalog Number AB3517). The aptamer library and all primers were obtained through Integrated DNA Technology, Inc. (Coralville, IA, USA). Ni-NTA Magnetic Agarose Beads (Catalog Number 36111) and Streptavidin MagneSphere Paramagnetic Particles (Catalog Number Z5481) were obtained from Qiagen (Valencia, CA) and Promega (Madison, WI) respectively. All PCR reagents, Hot-Start Taq DNA Polymerase (Catalog Number CB4040-1), 10x PCR buffer (Catalog Number CB3702-7) and dNTP master mix (Catalog Number CB4421-4) were obtained from Denville Scientific (South Plainfield, NJ). The TOPO-TA cloning kit with one-shot chemically competent TOP10 E. *coli* cells (Product Number K4500-01) was obtained from Invitrogen (Carlsbad, CA). Chemokines CCL8 and CXCL16 were obtained from PeptroTech (Rocky Hill, NJ).

Selection of Aptamers Against Fractalkine

The aptamer library, consisting of a 40-mer random region with flanking forward and reverse priming regions, WP-18 and WP-20 respectively, are shown below. Primer WP-20 contains a biotin tag on the 5' end to facilitate strand separation.

Aptamer Library: 5'-GTGCAGTCAAAGACGTCC-N40-GACCATGAAGTGCGATTGCC-3' Primer WP-18: 5'-GTGCAGTCAAAGACGTCC - 3'

Primer Biotin WP-20: Biotin-5'-GGCAATCGCACTTCATGGTC -3'

The fractalkine was immobilized through a polyhistidine-Ni²⁺ interaction. Approximately 50 μ L of Ni-NTA magnetic agarose beads were combined with 1 μ g of fractalkine and incubating for 1 hour at room temperature on a rotisserie shaker. The beads were washed three times to remove un-immobilized fractalkine.

For the initial round of SELEX, 50 pmol of fractalkine was incubated with 500 pmol of aptamer library for 120 minutes on a rotisserie shaker at room temperature in selection buffer (phosphate buffered saline (PBS) with 10 mM imidazole and 0.005% (v/v) Tween-20, pH 7.4). The aptamer library was heat denatured and snap cooled in ice water to eliminate hybridization. Following incubation, the beads were quickly washed three times with selection buffer followed by three 5 minute washes. The aptamer-fractalkine complex was eluted by 2 additions of 50 μ L of 250 mM imidazole in PBS. The aptamer pool was amplified using polymerase chain reaction (PCR) using 1 μ M of primers WP-18 and WP-20, 800 μ M total dNTP and 1.25 units of Taq per 50 μ L reaction. Touchdown PCR was used to amplify the aptamer pool according to the

following program: 5 minutes at 95°C, 10 cycles of 5 minutes at 95°C, 15 sec at 94°C, 15 sec at 72°C*, and 15 sec at 72°C with the * temperature decreased by 1°C per cycle, followed by 15 sec at 94°C, 15 sec at 62°C, and 15 sec at 72°C with an extension step of 1 minute at 72°C. The number of cycles used was adjusted depending on the SELEX round to prevent byproduct formation. The anti-sense strand was removed by denaturing the aptamer at 95°C for 5 minutes followed by snap cooling in ice water and incubation for 5 minutes on ice with Streptavidin MagneSphere Paramagnetic Particles. In subsequent rounds, the aptamer pool was reduced from 500 to 100 pmol while the fractalkine was reduced from 6 pmol to 1 pmol depending on the round to increase the stringency of selection. All binding occurred for 30 minutes at room temperature in selection buffer. SELEX was stopped after the 12th round when the PCR cycles needed for amplification of the aptamer leveled off between rounds 10 to 12 (Figure S2).



Fig. S2 The number of PCR cycles needed for aptamer pool amplification per round. The two chemokine selection steps are denoted by 2c and 6c. The chemokine domain selection steps required fewer cycles because rather can collecting the beads, only the supernatant was amplified which contained significantly more sequences.

Domain Targeted SELEX: Chemokine Domain Selection Steps

Selection of chemokine domain aptamers was performed after rounds 2 and 5. Fractalkine was denatured by heating to 95°C for 10 minutes followed by snap cooling. The fractalkine was bound to Ni-NTA beads for 1 hour. The aptamer was added and incubated in selection buffer for 60 and 30 minutes at room temperature for counter selections following rounds 2 and 6 respectively. Unbound aptamer was collected and amplified by PCR.

EMSA Analysis of Aptamer Pool

An electrophoretic mobility shift assay (EMSA) demonstrated binding of the aptamer pool to fractalkine. Asymmetric PCR was used to create biotinylated aptamer with the following reagent concentrations: DNA Polymerase (1.25 units per 50 μ L reaction), 10x PCR buffer, dNTP (total dNTP concentration of 500 μ M), Biotin-WP-18 (1 μ M), WP-20 (0.01 μ M), water (added to 50 μ L) and SELEX round 12 aptamer template (0.1 pmol).

Varying concentrations of 5' biotinylated round 12 aptamer pool was incubated with 1 pmol of fractalkine for 30 minutes at room temperature in selection buffer. A similar procedure was used for the supershift assay. An anti-polyhistidine tag antibody, fractalkine and aptamer were incubated for 30 minutes at room temperature. For the chemokine blocking experiment, 15 pmol of anti-chemokine antibody was pre-incubated with fractalkine for 15 minutes prior to aptamer addition. After binding, the samples were run at 80V on a non-denaturing 6% polyacrylamide gel in 0.5x TBE buffer (45 mM Tris-Base, 45 mM Boric Acid, 1 mM EDTA). The samples were transferred to a nylon membrane (Pall Biodyne B) at 380 mA for 30 minutes and crosslinked by UV exposure. The membrane was developed using a LightShift

Chemiluminescent EMSA Kit (Thermo Scientific; Product Number 20148) according to manufacturer's instructions.

Figure 1 from the main text is recreated in Figure S3-A to show the entire gel which shortened for space considerations. An electrophoretic mobility shift assay confirmed that the aptamer pool bound fractalkine in a dose dependent manner (Figure S3-B). In Figure S3-C an increasing concentration of an anti-polyhistidine tag antibody (His Ab) bound the polyhistidine tag of the recombinant fractalkine causing a supershift of the fractalkine-aptamer band. Increasing antibody concentrations intensified the supershift while decreasing the fractalkine-aptamer does not bind the antibody.

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Fig. S3 (A) Full image of Figure 1 from the main text. EMSA analysis of the 12th SELEX round. Lane 1: Aptamer with no FKN. Lane 2: Aptamer with FKN. Lane 3: Addition of the His Ab causes a supershift. Lane 4: The aptamer does not bind the His Ab. Lane 5: The aptamer does not bind BSA. Lane 6: An anti-chemokine domain antibody blocks aptamer binding. All lanes have 5 nM aptamer concentration. (B) Increasing concentration of SELEX aptamer pool binding to

fractalkine (FKN) (C) Supershift assay with increasing concentrations of an anti-polyhistidine antibody (His Ab) showing an increased supershift corresponding to the His Ab-fractalkine-aptamer complex. The aptamer concentration was 5 nM with 1 pmol of FKN used in each lane.

Cloning and Sequencing of Aptamer Pool

The aptamer pool was cloned into a TA vector (Invitrogen pCR2.1 Topo TA cloning kit) and heat shocked into chemically competent *E. coli* (One Shot TOP10 Chemically Competent *E. coli*; Invitrogen) according to the manufacturer's instructions. The transformed E. coli were plated onto LB agar plates with 50 µg/ml kanamycin and 40 mg/mL of X-gal and successfully transformed colonies were selected through blue/white screening. The sequencing region was amplified by colony PCR and amplicon size verified by agarose gel electrophoresis. ExoSAP-IT (Affymetrix, Inc.; Product Number 78201) was used to remove the excess primers. The clones were sequenced of the M13 Forward primer on an ABI 3730xl using ABI BigDye version 3.1 terminator chemistry by the University of Minnesota BioMedical Genomic Center sequencing facility. Aptamer candidates were aligned using MEGA 5 software.

FKN-S2 Aptamer Homologous Competitive Binding Assay

FKN-S2 was labeled with γ -³²P ATP using a T4 polynucleotide kinase (PNK) (Roche Applied Science) according to the manufacturer's instructions. Briefly, 10 units of PNK were added to 25 pmol of aptamer in the supplied PNK buffer. The reaction proceeded for 45 minutes at 37°C and was stopped by the addition of 10 µL of a 0.5 M EDTA solution. Unincorporated γ -³²P ATP was removed using a Sephadex G-50 spin column.

The aptamer dissociation constant was measured using a homologous competitive binding assay. The aptamer concentration was quantified by UV absorbance measured using a

Thermoscientific Nanodrop spectrophotometer and an extinction coefficient obtained from Integrated DNA Technology, Inc based on sequence composition.

All binding experiments occurred in binding buffer composed of PBS with 0.5 µg/mL poly dA:dT and 50 µg/mL bovine serum albumin (BSA), pH 7.4. Unlabeled full length FKN-S2 aptamer was diluted in binding buffer and a constant concentration of radiolabeled full length FKN-S2 aptamer added to each dilution. Full length fractalkine or an equivalent concentration of BSA was diluted in binding buffer to 50 pM. Aptamer and fractalkine/BSA was incubated for 1 hour at room temperature. Following incubation, samples were filtered through a nitrocellulose membrane using a bio-rad dot blot apparatus. Each well was washed 3 times with 200 µL of PBS to remove unbound aptamer. The membrane was dried and exposed to a storage phosphor screen overnight and imaged (Packard Cyclone). Binding was quantified using ImageJ and the Dot Blot

Analyzer plug-in. The data was fit to the following equation $AB = AB_{MAX} \left(\frac{[A]}{[A] + [X] + K_d} \right) - b$

where AB is the measured signal, AB_{Max} is the maximum signal, [X] is the concentration of unlabeled competitor, [A] is the hot aptamer concentration, b is the background binding, and K_d is the dissociation constant. Data was fit using non-linear regression analysis with the program Origin 8.

Truncated FKN-S2 Aptamer Competitive Binding Assay

Unlabeled aptamer was serially diluted from 10,000 nM to 0.1 nM in binding buffer (PBS with 0.1 μ g/mL Poly (dA:dT) and 50 μ g/mL BSA, pH 7.4). 1 nM of unlabeled FKN-S2 and 5,000 cpm/ μ L (counts per minute/ μ L) of radio-labeled full length FKN-S2 were heat denatured and snap cooled before addition to either fractalkine or a BSA control at a concentration of 0.1

nM and incubated for 1 hour at room temperature. The solutions were filtered through a nitrocellulose membrane and exposed to a storage phosphor film overnight and imaged. The binding was quantified using ImageJ and the Dot Blot Analyzer plug-in. Data was fit using the

non-linear regression program Origin 8 to the equation $AB = AB_{MAX} \left(1 - \frac{[X]}{[X] + IC_{50}} \right)$ where AB

is the measured signal, AB_{Max} is the maximum signal, [X] is the concentration of unlabeled competitor and IC₅₀ is the competitor concentration at which the signal is reduced by 50%.

Anti-fractalkine Antibody Blocking Assay



Fig. S4 Anti-fractalkine antibody blocking of binding of the scrambled FKN-S2 aptamer to fractalkine. No binding was seen for the scrambled aptamer. 100 nM of antibody that is specific for the chemokine domain of fractalkine (FKN Ab) was allowed to bind for 30 minutes prior to aptamer addition. Results show the mean \pm standard error from 4 independent experiments (n=4). Two tailed t-test with unequal variances was used to determine significance, $\dagger p > 0.01$.

FKN-S2 Binding to Heat-Denatured Fractalkine and Chemokines CCL8 and CXCL16

A saturation binding experiment was performed examining the binding of FKN-S2 to heat-denatured fractalkine and chemokines CCL8 and CXCL16. CCL8 represents the CC family of chemokines while CXCL16 was chosen because it is structurally similar to fractalkine. CXCL16 contains a chemokine domain atop a mucin like stock which is anchored to the cell with a transmembrane domain. The membrane bound protein is cleaved to produce a soluble version of the protein. Membrane bound CXCL16 can also act as an adhesion molecule to certain T cell types. These similarities to fractalkine made it an excellent candidate to test FKN-S2 binding.

FKN-S2 was labeled with γ -³²P ATP using a T4 polynucleotide kinase as described in the *FKN-S2 Aptamer Homologous Competitive Binding Assay* section. Unlabeled FKN-S2 aptamer

and 10⁶ counts per minute of radiolabeled FKN-S2 aptamer were mixed in binding buffer (PBS with 50 µg/mL BSA and 2.5 µg/mL Poly (dA:dT); pH 7.4) and the aptamer was denatured at 95°C for 5 minutes followed by snap cooling in ice water for 5 minutes. The aptamer was then serial diluted in binding buffer and the dilutions added to full length fractalkine, heat-denatured fractalkine and chemokines CCL8 (PeproTech Catalog Number 300-15) and CXCL16 (PeproTech Catalog Number 300-55). All proteins were at a concentration of 0.2 nM. Fractalkine was denatured by heating to 95°C for 10 minutes followed by incubation on ice until use. The aptamer and protein were incubated for 1 hour at room temperature. Bound aptamer was collected by filtering the samples thorough at nitrocellulose membrane and signal captured with a phosphor film and imaged. The background was measured from wells containing an identical concentration of BSA without the protein. Binding was quantified using ImageJ. Results are shown in Figure S5. FKN-S2 did not bind to the chemokines CCL8 or CXCL16, however, some binding was seen to heat-denatured fractalkine. Heat-denaturing reduced binding by 2.5 fold at saturation. The binding that was seen may be due to incomplete denaturing or refolding of fractalkine during the experiment.



Fig. S5 FKN-S2 binding to fractalkine, heat-denatured fractalkine, chemokine CCL8 and chemokine CXCL16. The aptamer and the protein (0.2 nM) were incubated for 1 hour at room temperature. Results show the mean \pm standard error from five independent experiments (n = 5).