Supporting Information

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SI Materials and Methods

In Vitro Mammalian Cell Culture. COS-7 cells grown in DMEM 1885 containing 10% FBS, 2 mM glutamine, 180 units/mL penicillin, and 45 μg/mL streptomycin at 37 °C and 10% CO_2. Approximately 40 h before use, cells were transiently transfected with the pcDNA3.1(+) CCR6 vector by calcium phosphate precipitation. For inositol triphosphate (IP_3) assays, cells were additionally cotransfected with G_{qi4myr}, a large G-protein chimera with the $G\alpha_i$ recognition interface and a $G\alpha_q$ output. The parental cell line of the PathHunter U2OS β-arrestin-2 (A2, DicoveRx) was used for assaying β-arrestin-2 recruitment, and the cells were grown in MEM-α plus GlutaMAX-I (Gibco) supplemented with 10% FBS, 180 units/mL penicillin, 45 μg/mL streptomycin, and 250 μg/mL hygromycin at 37 °C and 5% CO_2. Approximately 40 h before the β-arrestin-2 recruitment assays, the cells were transfected using FuGENE 6.

Molecular Biology. The CCL20 S64C plasmid was generated by site-directed mutagenesis using complementary primers using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Mutant plasmid was confirmed by DNA sequencing. Expression vectors were generated in XL1-blue cells for monoclonal selection and sequence verified.

Protein Purification. CCL20 S64C and CCL20 WT proteins were expressed and purified as previously described (25). Briefly, *E. coli* cells were lysed by French Press and centrifuged for 20 min at 15,000 × *g*. The insoluble fraction was resuspended in a 6 M guanidine buffer and nickel chromatography purified. CCL20 S64C elutions were pooled and refolded dropwise into a 20 mM cysteine, 0.5 mM cystine, and 100 mM Tris (pH 8.0) buffer. CCL20 WT and hBD2 elutions were refolded in a 10 mM cysteine, 0.5 mM cystine, and 100 mM Tris (pH 8.0) buffer. Refolded CCL20 proteins were cleaved with recombinant ULP1 and the SUMO-6XHIS tag and was separated from the recombinant protein by cation exchange chromatography. After exchange chromatography, elutions were HPLC purified and lyophilized. Purity and molecular mass were confirmed by SDS/PAGE and MALDI-TOF spectrometry.

HPLC. Approximately 100 μg of recombinant CCL20 S64C and CCL20 WT proteins were resuspended in H_2O and injected on a Shimadzu HPLC equipped with C18 column (Vydac). A 50–80% gradient of 70% acetonitrile (Sigma) and 0.1% TFA (Sigma) was used to elute proteins.

Mass Spectrometry. Purified CCL20 S64C was diluted to 1 μM in 70% acetonitrile containing 0.1% formic acid. Protein was analyzed by direct infusion using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Analysis was performed with a resolving power set to 140,000. Data were deconvoluted and deisotoped using Xtract and Xcalibur in QualBrowser (Thermo Fisher Scientific).

Reduced/Oxidized SDS/PAGE. Approximately 3 μg of CCL20 S64C or CCL20 WT was resuspended in a SDS buffer in either the absence or presence of β-mercaptoethanol.

NMR Spectroscopy. [U-^{15}N]-CCL20 S64C and CCL20 WT were resuspended in H_2O and diluted to 50 μM in a 25 mM deuterated Mes (pH 6.8), 10% (vol/vol) D_2O, and 0.02% (wt/vol) NaN_3 buffer. ^{1}H–^{15}N heteronuclear single-quantum coherence experiments were performed on a Bruker Avance 500-MHz spectrometer equipped with a TCI cryoprobe at 298 K.

Protein Crystallization. The resulting crystals were flash frozen after passing through a cryoprotection solution of the well solution plus an additional 35% MPD. X-ray diffraction data were gathered for a single crystal using a rotating anode Rigaku Micromax 007 X-ray generator (1.54178 Å) equipped with a Raxis-4++ detector. Diffraction data were collected at 100 K and processed with HKL2000 (55). CCL20 S64C crystals packed in the hexagonal space group ($P6_1$) and diffracted to a resolution of 2.0 Å.

Structure Determination. Molecular replacement was used to evaluate the initial phases using a CCL20 monomer (PDB ID code 1M8A) as the search model. Phenix.AutoMR (56) solved the initial phases and automatically built in the majority of the residues in the dimeric assembly. Models were completed through iterative rounds of manual model building in Coot (57) and refinement with Phenix.refine (56). Geometry of the final structure was validated using Molprobity and Procheck. Ramachandran statistics for the ternary complex were 98.39% and 1.61% for the favored and additionally allowed regions of the Ramachandran plot, respectively. Data collection and refinement statistics for the final model are listed in Table S1. The final model was deposited in the PDB (PDB ID code 5UR7).

Radioligand Displacement Assay. CCR6$^+$ COS-7 cells were seeded in 96-well plates at 35,000 cells per well (in duplicates), and after 24 h the cells were washed into a 50 mM Hepes buffer supplemented with BSA (5 g/L) and chilled to 5 °C. Ligands were added shortly before the ^{125}I-CCL20 tracer (calibrated to result in ∼10% tracer binding), and the cells were incubated at 4 °C for 3 h and washed in a 50 mM Hepes buffer containing BSA (5 g/L) and NaCl (29.22 g/L). The cells were lysed, and gamma radiation of the lysate was measured.

Calcium Flux Assay. Chemokine dilutions were made in buffer and added to the plate after a 20-s baseline reading. Fluorescence at 515 nm was observed using a Flexstation 3 (Molecular Devices) for an additional 80 s. Total fluorescent signal was measured, and four replicates from 3 separate days were measured and are presented as the mean ± SEM. EC_{50} values were calculated through nonlinear fitting to a four-parameter function. For pertussis toxin testing, cells were treated with 200 ng/mL pertussis toxin for 2 h at 37 °C, 5% CO_2. Following the 2-h incubation, treated and nontreated cells were prepared as described above.

IP_3 Accumulation Assay. CCR6$^+$ COS-7 cells transfected with G_{qi4myr} were seeded into 96-well plates at 35,000 cells per well (in duplicates) in growth media supplemented with [^{3}H]myo-inositol (5 μL/mL, 2 μCi/mL) for 24 h. Cells were washed in HBSS buffer and changed to a solution of 10 mM LiCl in HBSS before ligand addition. After 90 min at 37 °C, cells were lysed in 10 mM formic acid, and 35 μL of lysis solution (∼90 vol/vol%) were transferred to white, opaque 96-well plates and mixed with a solution of agitated SPA-Ysi beads (80 μL/well, 12.5 mg/mL; PerkinElmer). After 30 min of shaking, the cells equilibrated for 8 h, and scintillation was measured using a Packard Top Count NXT counter (PerkinElmer).

Chemotaxis Assay. Transfected CCR6$^+$ Jurkat cells were washed in buffer, and 3.6×10^4 cells were added to the upper well and allowed to incubate at 37 °C, 5% CO_2 for 2 h. Migrated cells in the

lower well were quantitated on a BD LSR II flow cytometer. Total cell number is the average of four replicates from 2 separate days and presented as the mean ± SEM.

CCR6 Receptor Internalization. Transfected $CCR6^+$ Jurkat cells were incubated with 70–2,100 nM WT or dimeric CCL20 in complete media at 37 °C for 30 min. Cells were washed in PBS and then incubated with human monoclonal antibody to CCR6 (APC–anti-human; Biolegend), and fluorescence intensity was measured by flow cytometry (Acuri C6; BD Biosciences). Fluorescence values are the average of three replicates from separate days and presented as the mean ± SEM.

Flow Cytometry. FITC–anti-mouse γδ-TCR (clone GL3) monoclonal antibody was purchased from BioLegend. Epidermal sheets (see above) were cut into small pieces with fine scissors and then filtered to recover epidermal cell suspensions as described (8). Live cells were gated initially by forward- and side-scatter parameters, and then analyzed for expression of the γδ-TCR with GDL T cells being defined as those cells with low/intermediate expression of the γδ-TCR (as detected by FITC-labeled GL3). Resident dendritic epidermal T cells differed from GDL T cells by expression of the highest levels of the γδ-TCR. Cells from mouse samples were examined using an Acuri C6 (BD Biosciences). Flow-cytometric data were analyzed using FlowJo, version 10.0.7, software (Tree Star).

Cytokine Injections into Mouse Ears. In a similar manner, we administered an intradermal injection of 20 μL of PBS containing 32 ng (0.1 μM) of recombinant CCL20 S64C mixed with 500 ng of recombinant mouse IL-23, 20 μL PBS containing 16 ng (0.1 μM) of recombinant CCL20 WT mixed with 500 ng of recombinant mouse IL-23. Ear thickness was measured before every injection using a dial thickness gauge (Ozaki).

Quantitative Real-Time PCR (RT-PCR). Extraction of RNA and subsequent analysis of mRNA expression of indicated cytokines from epidermis was performed as described (8). In brief, total RNA of mouse epidermis was prepared using an RNeasy Fibrous Tissue Kit (Qiagen) according to the manufacturer's instructions, and RT-PCR was performed via StepOnePlus Real-Time PCR System (Applied Biosystems).

Histopathological Analysis. Skin specimens were sampled, fixed with 10% formaldehyde, and embedded in paraffin. Those tissues were sectioned at 4-μm thickness, and deparaffinized sections were stained with H&E.

Statistics. All data are expressed as mean ± SEM. Calcium flux, chemotaxis EC_{50} and IC_{50} values, radioligand, IP_3 accumulation, β-arrestin-2 recruitment, and in vivo data were analyzed using GraphPad Prism, version 6 (GraphPad Software). Simple comparisons of means and SEM of data were made by using two-sided Student's *t* test, and multiple comparisons were made by using Tukey's test. A *P* value of less than 0.05 is considered statistically significant.

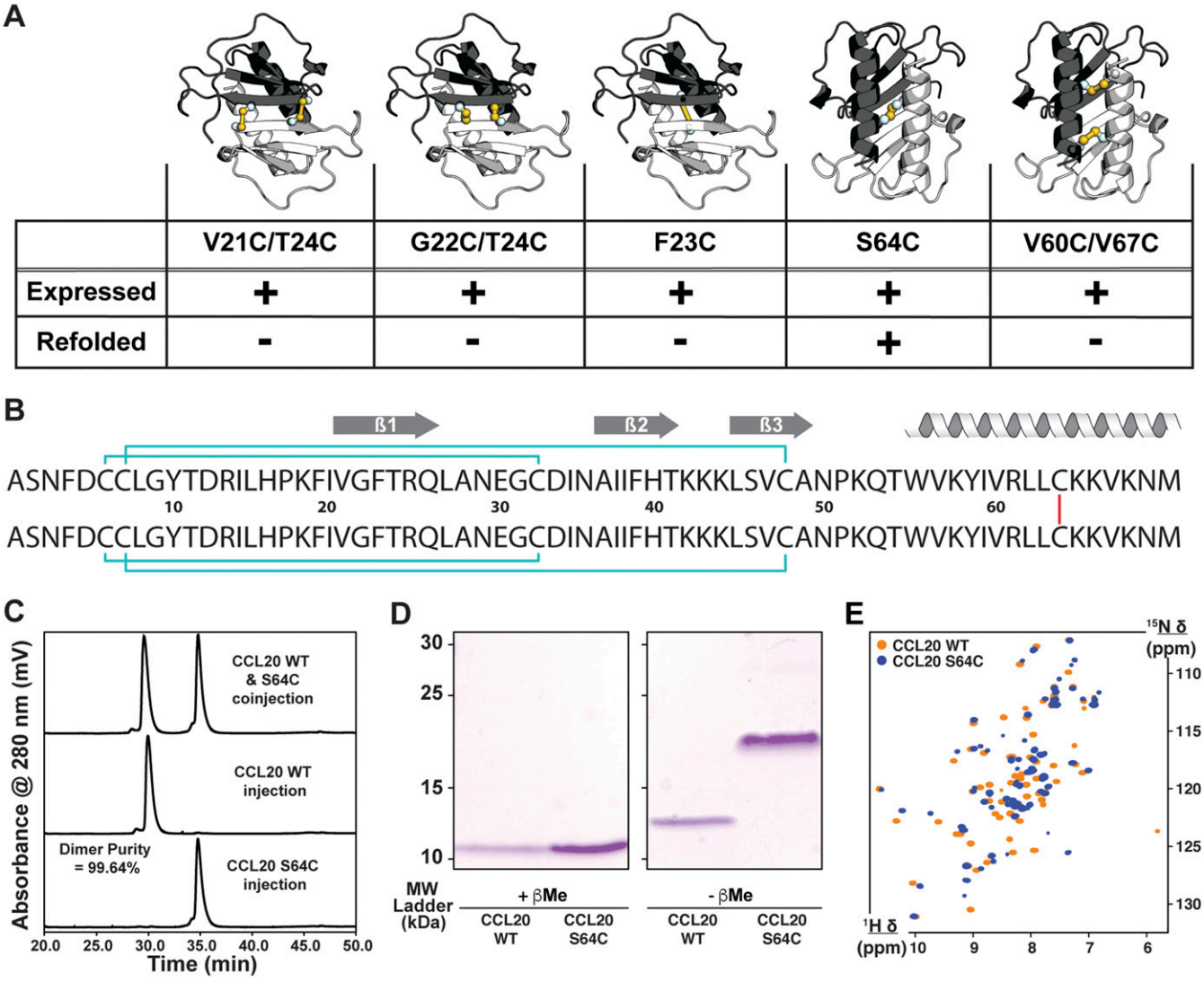

Fig. S1. A disulfide-linked CCL20 is a stably folded dimer in solution. (*A*) Expression testing and refolding results of CCL20 variants and corresponding structural models with predicted disulfide bonds shown in yellow. (*B*) The CCL20 S64C amino acid sequence with native (cyan) and nonnative (red) disulfide bonds shown. (*C*) Reverse-phase HPLC chromatograms of CCL20 WT and S64C. Protein elution peaks are separated by ~5 min. Dimer purity was estimated to be 99.64%. (*D*) SDS/PAGE of CCL20 WT and CCL20 S64C in the presence and absence of a reducing agent, β-mercaptoethanol. (*E*) Heteronuclear single-quantum coherence spectra overlay of CCL20 WT (orange) and CCL20 S64C (blue).

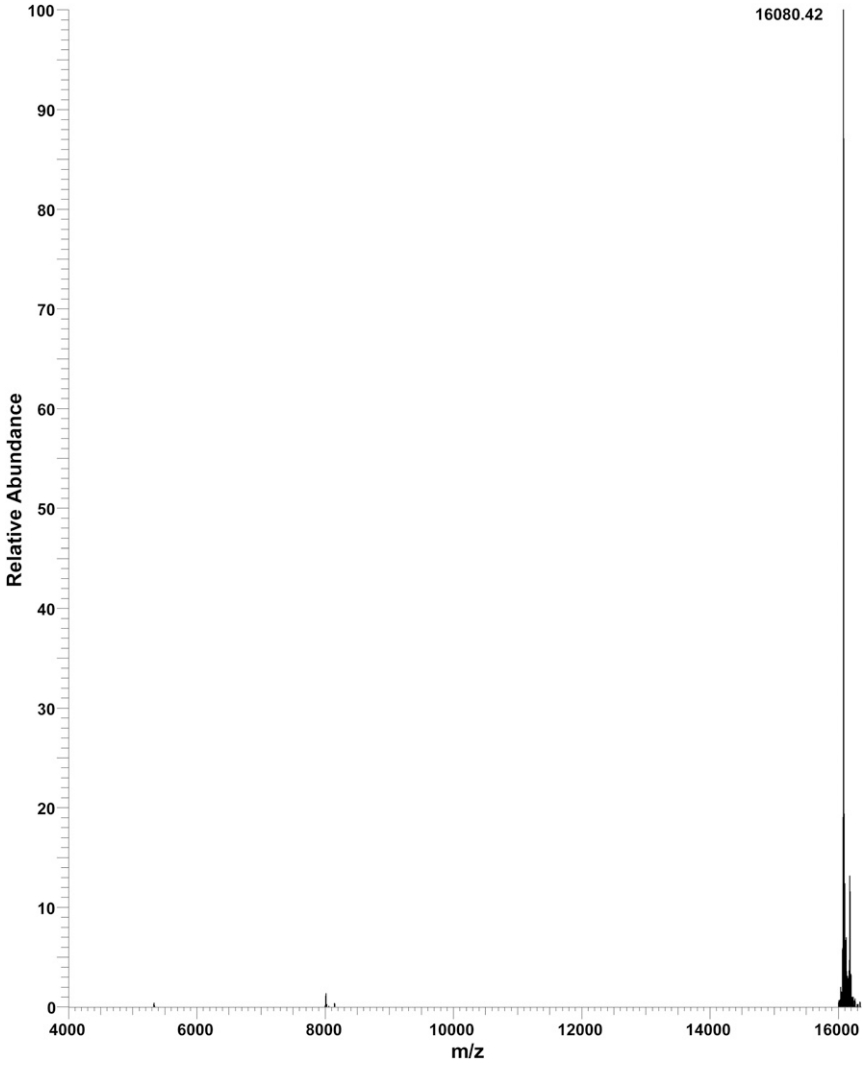

Fig. S2. Mass spectrometry spectra of CCL20 S64C. Spectra of CCL20 S64C shows a molecular mass of 16,080.4 Da, collected on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer.

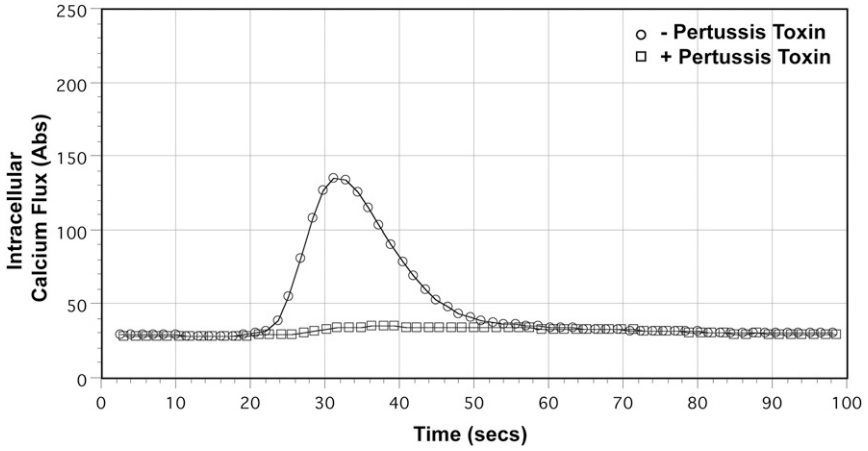

Fig. S3. CCL20 S64C activation of CCR6 is pertussis toxin sensitive. Raw calcium flux trace of $CCR6^{+}$ Jurkat cells exposed to 700 nM CCL20 S64C after a 2-h incubation with 200 ng/mL pertussis toxin.

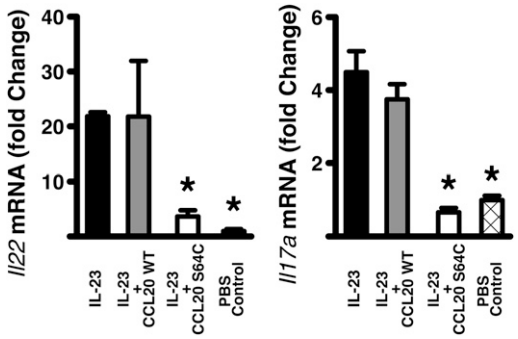

Fig. S4. CCL20 S64C blocks the expression of psoriasis biomarkers. Epidermal expression of *Il17a* and *Il22* mRNA normalized for *Gapdh* mRNA vs. untreated control ear skin ($n = 3$). Plotted values are averaged from two pooled mouse ears. Similar results were obtained in two independent experiments. All experimental data are expressed as mean ± SEM. *$P < 0.05$ vs. IL-23–alone groups for all experiments.

Table S1. Data collection and refinement statistics

	PDB ID code 5UR7
Data collection	
Space group	$P6_1$
Cell dimensions	
a, *b*, *c*, Å	71.679, 71.679, 71.561
α, β, γ, °	90, 90, 120
Resolution, Å	46.905–2.000 (2.072–2.000)[†]
R_{merge}	0.042 (0.206)
R_{meas}	0.044 (0.218)
R_{pim}	0.14 (0.071)
$I/(I)$	57.7 (12.0)
$CC_{1/2}$	(0.985)
CC^*	(0.996)
Completeness, %	99.2 (98)
Redundancy	10.3 (9.5)
Refinement	
Resolution, Å	46.905–2.000
No. reflections	14,051
R_{work}/R_{free}	0.160/0.197
No. atoms	1,208
Protein	1,084
Ligand/ion	20
Water	104
B factors	
Protein	25.200
Ligand/ion	45.520
Water	32.896
Rms deviations	
Bond lengths, Å	0.007
Bond angles, °	0.866

Dataset is from a single crystal, and initial phases were solved by molecular replacement.

[†]Values in parentheses are for highest-resolution shell.