Characterizing structure-function relationships in the CCR6/CCL20 chemokine receptor-ligand pair

Background

G-protein coupled receptors (GPCRs) are transmembrane proteins crucial for generating intracellular responses from extracellular signals, including hormones and neurotransmitters, as well as other signaling molecules. Around 35% of all FDA-approved drugs target GPCRs and GPCR-related proteins. This makes GPCRs one of the main targets for drug discovery and highlights the importance of understanding GPCR structure-function relationships for therapeutic applications.

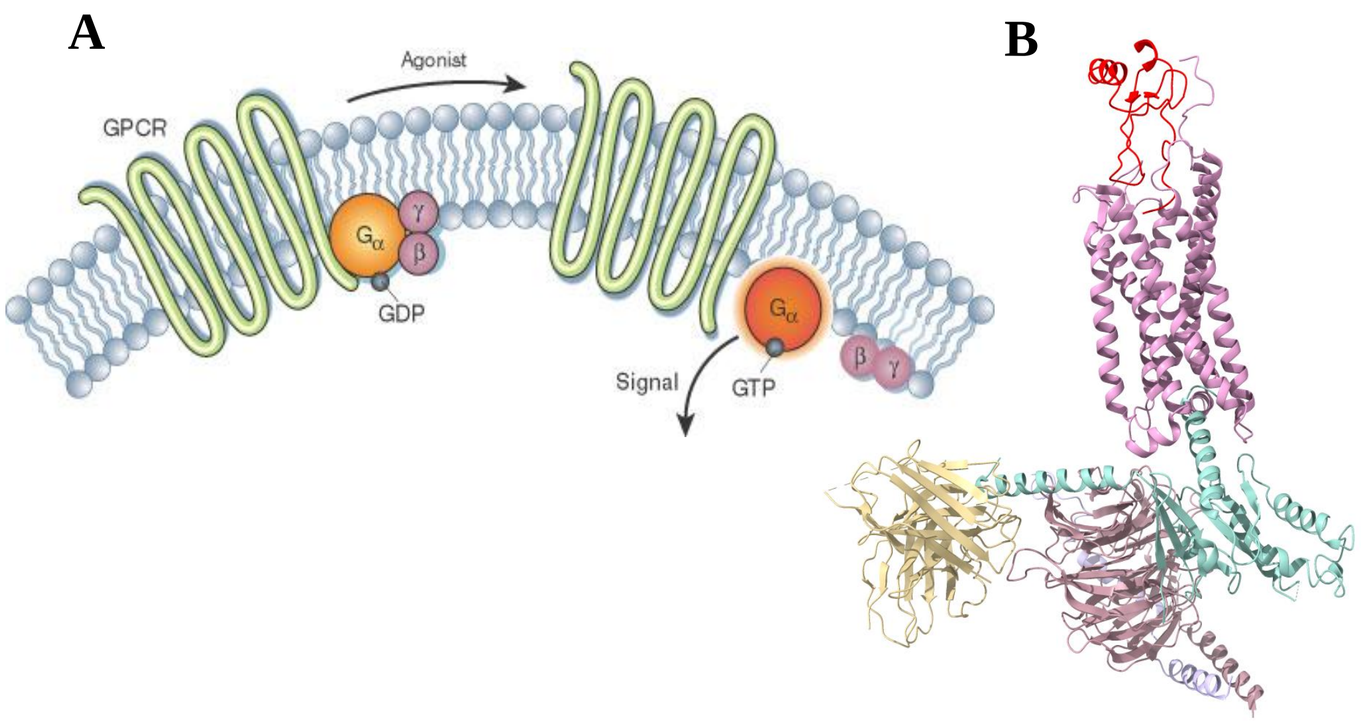


Fig 1A. The GPCR contains an extracellular domain, seven transmembrane helices, as well as an intracellular domain. The intracellular portion can interact with a heterotrimeric G-protein complex. When an agonist binds to the GPCR at the extracellular binding pocket, the heterotrimeric G-protein complex dissociates into the alpha, beta, and gamma subunits, triggering downstream signaling and prompting a cellular response. Li, J. *et al.* The Molecule Pages database. *Nature* 420, 716-717 (2002).
Fig 1B. CCR6-CCL20 structure (PDB ID: 6WWZ). The CCR6 receptor (pink) is bound to the CCL20 ligand (red). The heterotrimeric G-protein complex (green, tan, purple) is bound to the receptor on the intracellular side. An scFv16 antibody (yellow) helps stabilize the complex in active formation.

CCR6 is a type of GPCR that mediates directed cell migration in response to its ligand, CCL20. My goal is to study the importance of specific CCR6 amino acid residues through site specific mutagenesis and functional characterization of the resulting mutants. I measured protein expression and intracellular G-protein coupling and saw how different mutations affected those aspects. Through this, I gained more insight into the functional importance of each binding site. More broadly, this knowledge can be utilized to modulate cell migration patterns in the body and develop novel therapeutics.

Design Rationale

- Selected CCR6 residues that form strong hydrogen bonds and salt bridges with their ligand complements
- Used charge-swap mutagenesis to swap out positively charged amino acid residues for negatively charged amino acids, and vice versa
- Designed multiple single, double, and combo mutational variants

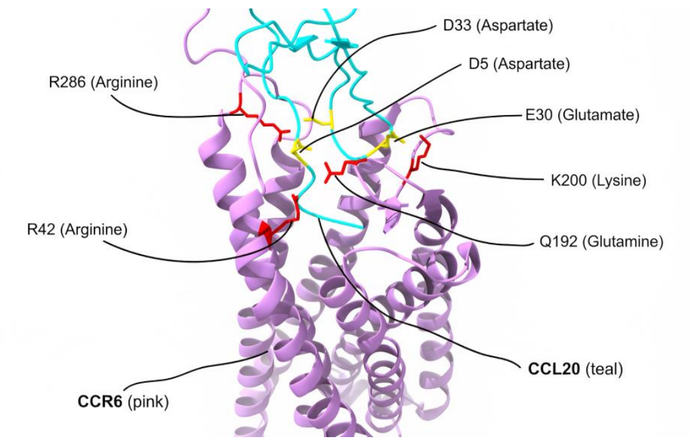
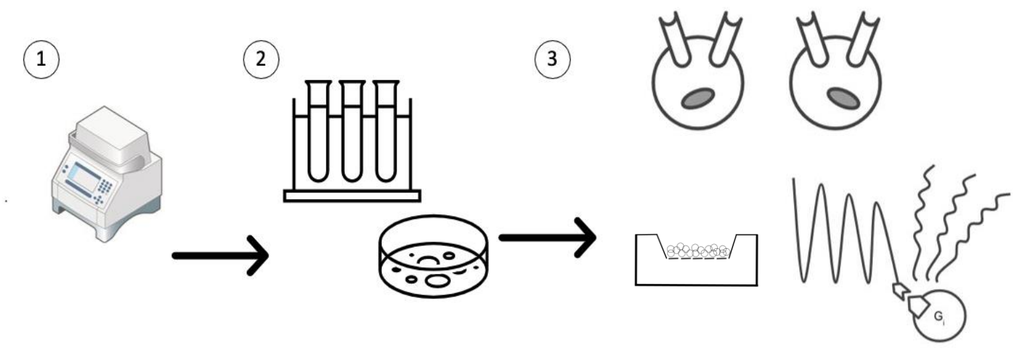


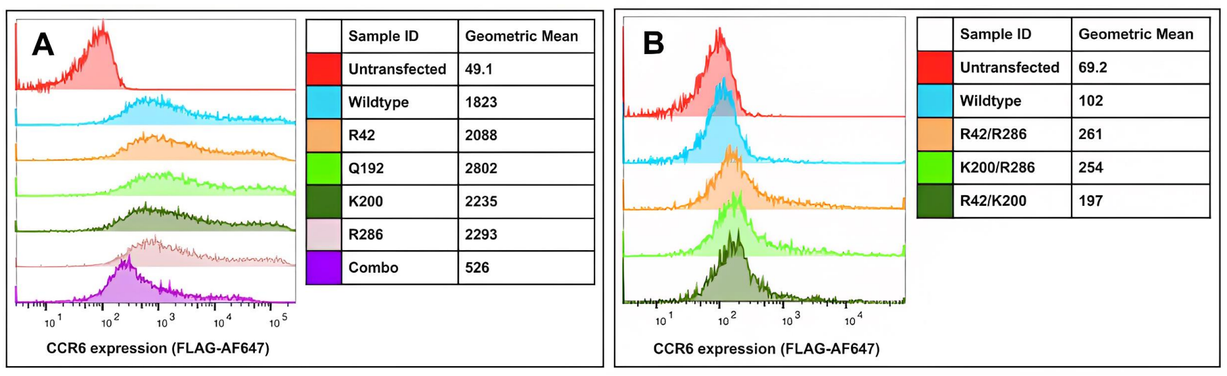
Figure 2. **Charge-swap mutagenesis performed on select CCR6 residues.** Sites R42 (red) and Q192 (red) interact with site D5 (yellow) on the CCL20 ligand (teal). R42 forms a salt-bridge with D5, a strong bond between two oppositely charged moieties, while Q192 forms a hydrogen bond. CCR6 residues K200 and R286 (red) interact with E30 and D33 (yellow), respectively, and are also found near the receptor surface. Disruptions to the four CCR6 (pink) residues may interfere with receptor-ligand formation and receptor activation. Image produced with ChimeraX (PDB ID: 6WWZ).

Methodology



Results

CCR6 mutants express robustly in HEK-293 cells



Sample ID	Geometric Mean
Untransfected	49.1
Wildtype	1823
R42	2088
Q192	2802
K200	2235
R286	2293
Combo	526

Sample ID	Geometric Mean
Untransfected	69.2
Wildtype	102
R42/R286	261
K200/R286	254
R42/K200	197

Fig 3. **CCR6 receptor expression in HEK-293 cells, as measured via flow cytometry.**
A. Single mutants, containing altered side chain charges at R42 (orange), Q192 (neon green), K200 (dark green), and R286 (tan) expressed at levels comparable with wildtype (blue). The combo mutant contains all four mutations at R42/Q192/K200/R286 (violet). Insignificant levels of fluorescence were observed in untransfected cells (red).
B. Double mutants, with charge-swap mutations at R42/R286 (orange), K200/R286 (neon green), R42/K200 (dark green), as well as wildtype (blue), expressed poorly, as compared with untransfected cells (red).

CCR6 mutants weaken receptor activation in response to CCL20

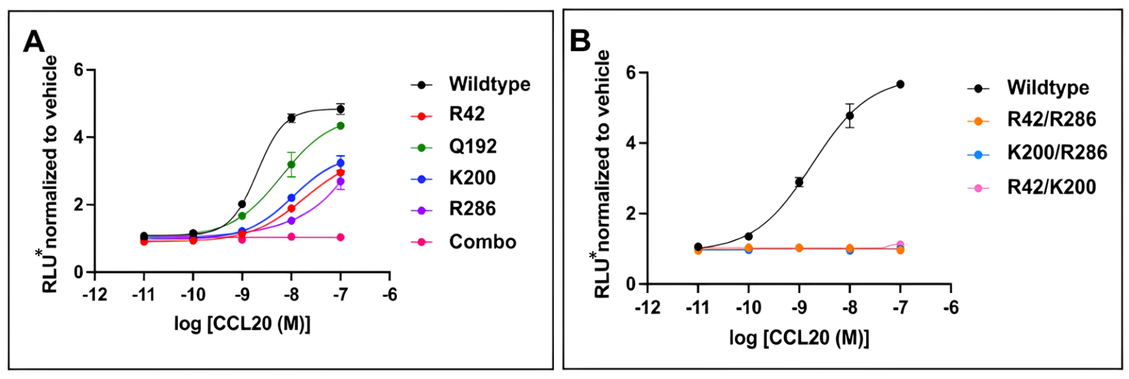
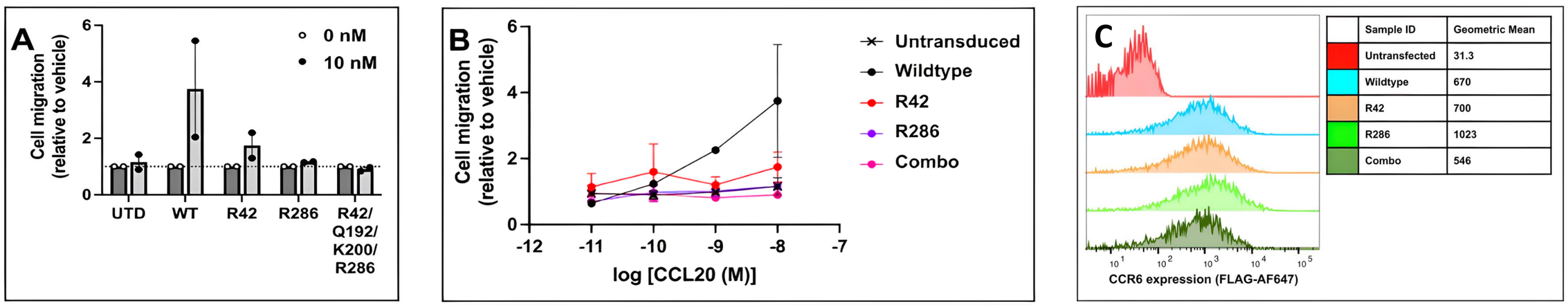


Fig 4. **G protein coupling as measured by NanoBiT assay.** When a ligand binds to the receptor, two protein units fused to the receptor and G protein form the functional luciferase enzyme, which catalyzes substrate added into the cell. The assay measures the amount of luminescence coming from the cells, with higher luminescence indicating higher receptor activation.
A. Mutation Q192 (green) slightly disrupted G_i protein coupling, while mutations at K200 (blue), R42 (red), and R286 (purple) exhibited substantially stronger disruption. The combo mutant fully ablated receptor activation.
B. Mutations at R42/R286 (green), K200/R286 (purple), and R42/K200 (orange) also fully eroded GPCR activation.
*RLU: Relative Luminescence Units.

CCR6 mutants successfully diminish cell migration in response to CCL20



Sample ID	Geometric Mean
Untransfected	31.3
Wildtype	670
R42	700
R286	1023
Combo	546

Fig 5A. Comparison of cell migration at 10 nM CCL20 versus baseline. Jurkat cells expressing the wildtype cell line exhibited substantially higher cell migration towards 10 nM of CCL20. Cells expressing the receptors with mutations at R286 and the combo did not show increased cell migration in the presence of 10 nM of CCL20. Cells with receptor mutations at R42 displayed lower cell migration at 10 nM compared to wildtype, although a lack of statistical significance suggests that this data point may be a result of background noise. The number of migrated cells was normalized to the background level (no CCL20) to account for ligand-independent passive migration.
Fig 5B. Cell migration of CCR6 mutant-expressing Jurkat cells in the absence and presence of CCL20. Jurkat cells expressing the wildtype receptor showed a moderately linear increase in cell migration. R42 fluctuated at different points; higher cell migration occurred at lower concentrations and decreased at 1 nM CCL20. The untransduced, R286, and combo cell lines all maintained relatively consistent cell migration throughout all concentrations.
Fig 5C. CCR6 expression on Jurkat cells. All receptors exhibited positive expression on Jurkat cells. Consistent with previous flow cytometry data, single mutation receptors and wildtype receptors expressed at similar levels. Expression levels for different receptors were comparable.

Conclusions

- Identified residues critical for CCR6 binding/activation
- Single mutants had insignificant effects on protein expression
- The combo mutant strongly disturbed G-protein coupling but also disrupted protein folding, leading to lower receptor expression
- Double mutants strongly disrupted G-protein coupling
- Developed a framework for studying structure-function relationships in a broad class of GPCRs
  - Design mutations --> measure protein expression --> G-protein coupling assay --> functional assay

Disclaimer: All images and graphics were self-produced unless noted otherwise.

Future Directions

- Use mutant receptors for in-vivo modulation of immune cell migration
  - Develop high-affinity super-agonists to enhance trafficking of immune cells to deep-tissue tumors such as glioblastomas or pancreatic cancers
  - Design competitive inhibitors to reduce CCR6-mediated immune cell migration to hyper-inflammatory areas caused by autoimmunities such as Crohn's disease, rheumatoid arthritis, and encephalomyelitis

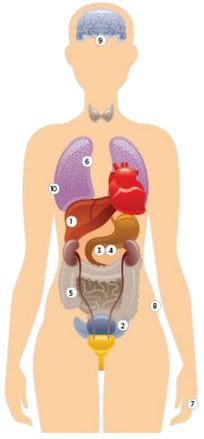


Figure 8. Common inflammatory diseases in the human body.
1. Fatty liver disease
2. Endometriosis
3. Type 2 diabetes mellitus
4. Type 1 diabetes mellitus
5. Inflammatory bowel disease (IBD)
6. Asthma
7. Rheumatoid arthritis
8. Obesity
9. Alzheimer's and Parkinson's' disease
10. Cancer

Institute for Biomolecular Science. *Common inflammatory diseases.* University of Queensland, (2018).