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Rational Design of High Affinity Interaction Between CC Chemokine Binding Protein vCCI and CCL17/TARC

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ABSTRACT: The poxvirus-derived protein vCCI (viral CC chemokine inhibitor) binds almost all members of the CC chemokine family with nanomolar affinity, inhibiting their pro-inflammatory actions. Understanding the affinity and specificity of vCCI could lead to new anti-inflammatory therapeutics. CCL17, also known as TARC, is unusual among CC chemokines by having only micromolar binding to vCCI. We have used sequence analysis and molecular simulations to determine the cause of this weak binding, which identified several locations in CCL17 where mutations seemed likely to improve binding to vCCI. Based on the aforementioned analysis, we expressed and tested multiple mutants of CCL17. We found two single point mutants V44K and Q45R that increased binding affinity to vCCI by 2−3-fold and, in combination, further improved affinity by 7-fold. The CCL17 triple mutant G17R/V44K/Q45R yielded a K_d of 0.25 ± 0.13 μM, a 68-fold improvement in affinity compared to the complex with wild-type CCL17. A quadruple mutant G17R/V44K/Q45R/R57W showed high affinity (0.59 ± 0.09 μM) compared to the wild type but lower affinity than the triple mutant. This work demonstrates that sequence comparisons and molecular simulations can predict chemokine mutations that increase the level of binding to vCCI, an important first step in developing engineered chemokine inhibitors useful for anti-inflammatory therapy.

One of the most effective inhibitors of chemokines is poxviral protein vCCI (also called 35K). This protein tightly binds and inhibits many members of the CC chemokine subfamily, which are involved in inflammation and are so named because they have a conserved pair of cysteines near their N-terminus. As such, vCCI has been suggested as an anti-inflammatory protein that may be useful against a wide variety of immune-related pathologies, including asthma, traumatic brain injury, and arthritis.[1]

All chemokines share the same general fold, and CC chemokines have a high degree of sequence similarity. For instance, chemokine CCL17 (also called TARC) is a small protein that binds the receptor CCR4 on the surface of certain immune cells, causing activation and chemotaxis. CCL17 shares 28% amino acid sequence identity and 57% similarity to CCL2 and 35% identity and 61% similarity to CCL11, which both tightly bind vCCI. Despite these similarities, vCCI was reported to have limited binding affinity for CCL17.[2] We confirmed this in a recent study, showing a vCCI:CCL17 binding K_d of 14 μM.[3] This is several orders of magnitude weaker than vCCI binding to similar CC chemokines such as CCL2, CCL4, and CCL11, which bind vCCI at low nanomolar (nM) or even sub-nM levels.[4] In this work, we use a combination of molecular dynamics simulations and sequence comparisons to engineer CCL17 mutants with improved binding to vCCI and succeed in improving the binding of CCL17 by 68-fold. This significant gain of affinity demonstrates the promise of combined computational and experimental biochemistry to determine the key amino acids mediating protein interactions.

The vCCI-chemokine system provides an ideal test for engineering protein binding, because the chemokines all share the same fold, while exhibiting a range of affinities for vCCI. Hence, it should be possible to identify the pairwise interprotein contacts that strengthen or weaken binding and mutate amino acids on either the chemokine or vCCI to modulate the binding affinity. Additionally, there is an experimentally determined structure of the vCCI:CCL4 complex[5] which can be used as a template for molecular dynamics simulations of vCCI binding to other chemokines.[6,7] This work is conceived as a proof-of-concept study toward eventually redesigning vCCI itself in a complementary fashion to obtain affinity and specificity for CCL17 or other CC chemokines. Mutant vCCIs that are specific for certain sets of chemokines could be useful therapeutically, allowing for precise inhibition of those particular chemokines that cause damage while allowing protective inflammatory chemokine signaling to persist.

We began by performing a sequence alignment of CCL17 with five other chemokines with a high affinity for vCCI (Figure 1). This showed that CCL17 differs in several key residues from the other chemokines. Specifically, CCL17: (1) has a glycine instead of a positively charged residue at position

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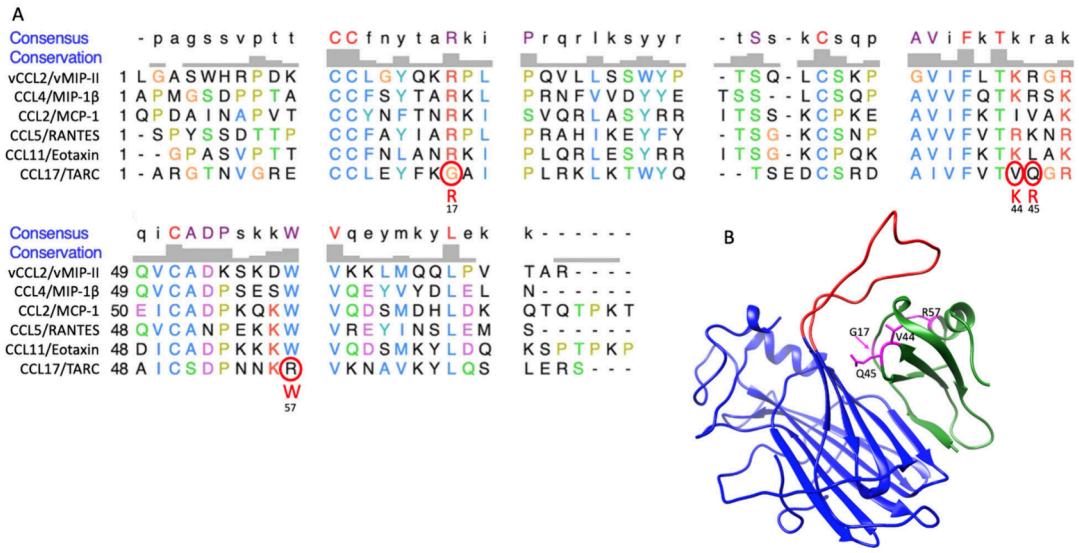

Figure 1. (A) Sequence comparison of several CC chemokines that bind vCCI with high affinity, along with CCL17, which binds vCCI several orders of magnitude more weakly. (B) Model of the vCCI:CCL17 interaction, as determined by molecular dynamics. vCCI is shown in blue with the acidic loop denoted in red. CCL17 is shown in green. Amino acids in CCL17 hypothesized to be important in the vCCI:CCL17 interaction are shown in pink.

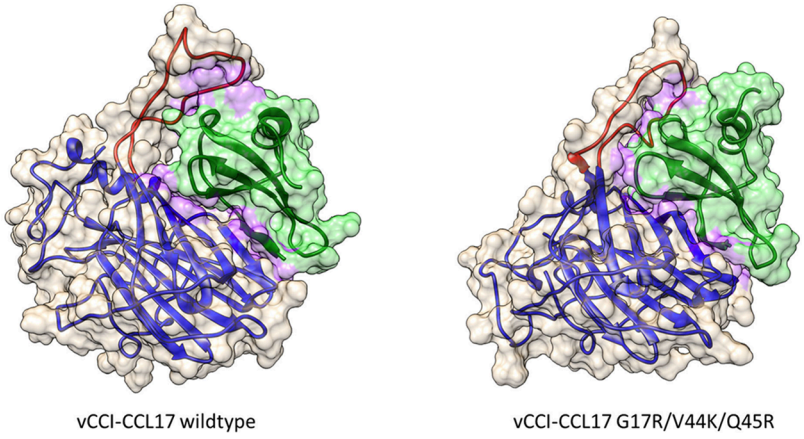

Figure 2. Molecular dynamics model of vCCI WT in complex with the CCL17 WT (left) and CCL17 triple mutant (right). vCCI is shown in blue, with the acidic loop denoted in red. CCL17 is shown in green. The purple surface shading shows close contacts between vCCI and the chemokine.

17; (2) lacks a positively charged residue at positions 44 and/or 45 present in most other chemokines; and (3) has an arginine instead of the highly conserved tryptophan at position 57. These positions are marked on the vCCI-bound CCL17 model shown on the right in Figure 1 and were suggested by molecular simulations (see below). The sites at 17, 44, and 45 are in locations that previous experimental structures and molecular simulations have shown can contact vCCI residues. Based on these observations, to create a mutant CCL17 that should have improved binding to vCCI, we identified the following three mutations: G17R, V44K, and Q45R. It is less clear what role the conserved W57 may play in the other chemokines' binding affinity to vCCI. In the experimental vCCI:CCL4 structure the chemokine W57 is not located near the vCCI interface, and, if it were able to contact vCCI, the R57 in CCL17 might be expected to contribute to stronger binding to vCCI like the other positively charged residues on the chemokines.

After the sequence analysis, we used a structural analysis of the vCCI-CCL17 complex to validate mutations likely to increase binding. There are established methods for identifying interacting residues including rigid docking and mapping complementary regions of surface potential on each protein, but given the flexibility of the vCCI binding pocket and the large number of possible interactions between the many negatively charged residues on the vCCI and positive charges on the chemokines, we instead opted for the more laborious technique of using molecular dynamics (MD) simulations of the bound complexes to identify persistent residue−residue interactions over an extended simulation time. We simulated wild-type CCL17 bound to vCCI and also the triple mutant G17R/V44K/Q45R for a full 1 microsecond (μs) of molecular dynamics. The starting structures were built using as a template the experimentally determined structure of vCCI bound to a human CC chemokine (MIP-1β).[5] (Details of the MD methodology are in the Supporting Information.) Figure 2 (and Supporting Information Figure 1) shows the bound conformations after 1 μs of MD, with vCCI:CCL17(wild type) on the left and vCCI:CCL17(triple mutant) on the right. The Supporting Information figure shows the bound structures

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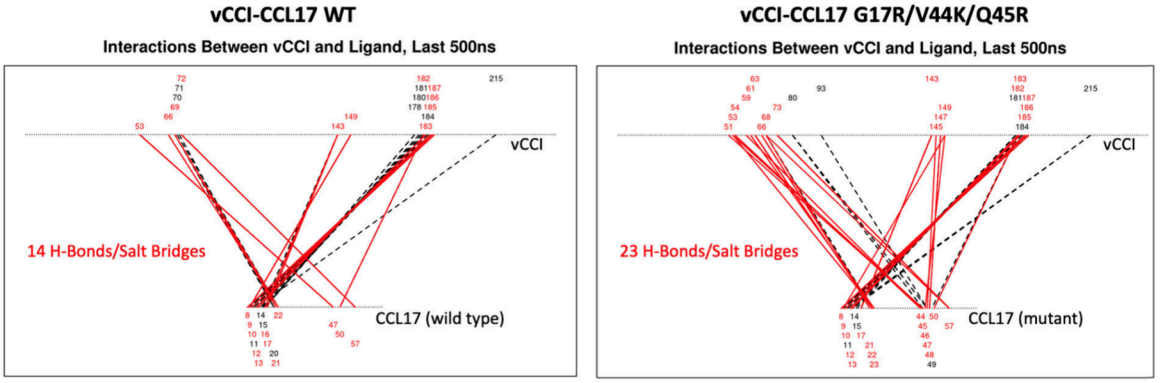

Figure 3. Contacts between the amino acids in the vCCI and CCL17, as determined from PDBePISA analysis of the molecular dynamics trajectories. The horizontal dotted lines represent the vCCI and CCL17 residues. The red lines indicate hydrogen bonds or salt bridges, and the black dashed lines show other close contacts. All contacts present in at least 1/3 of the sampled frames are shown, and the amino acids involved are listed by number (See Supporting Information for more details).

"opened" to reveal the binding interface, and the purple surface shading shows where there are close contacts between the vCCI and chemokine. The most obvious difference in the interface between the CCL17 wild type and triple mutant is in the contacts with the large vCCI loop (residues 51−74) which has more complete contact with the mutant CCL17 than the wild type. Quantitatively, the interface surface areas calculated using PDBePISA are 1419.6 Å^2 and 1210.9 Å^2 for the CCL17 triple mutant and wild type, respectively, suggesting stronger interaction between the CCL17 mutant and the vCCI. PDBePISA also calculates an empirical approximation of the binding ΔG, which yields a binding free energy for the triple mutant 1.1 kcal/mol stronger than for the wild type, which corresponds to a K_d 7-fold smaller for the triple mutant than the wild type.

We analyzed the specific amino acid interactions between the vCCI and chemokines using PDBePISA. This allowed us to count the number of hydrogen bonds (including salt bridges) and also allowed us to identify close interprotein contacts that are not hydrogen bonds. We analyzed the last 500 ns of the 1 μs trajectories, sampling every 20 ns, and only noted interactions that were present in at least 1/3 of the sampled frames. Figure 3 plots these contacts between the amino acids in the vCCI (represented as the top line of dots in each graph) and the chemokine (represented as the bottom line of dots). The red lines show the persistent hydrogen bonds (including salt bridges), and the dashed black lines represent the close contacts that do not involve a hydrogen bond. The numbers above and below the axes indicate the residues involved in each interaction, color coded by the type of interaction (red for a hydrogen bond, black for close contact). These results show that each of the CCL17's mutated residues is involved in persistent hydrogen bonds with vCCI. The CCL17 R17 hydrogen bonds to vCCI E143, the CCL17 K44 hydrogen bonds to vCCI D59 (in the vCCI loop), and the CCL17 R45 hydrogen bonds to vCCI E53 (also in the vCCI loop). Therefore, these sites in CCL17 appear to be promising sites to experimentally mutate to improve binding to vCCI. Overall, the triple mutant CCL17 was found to have 23 persistent hydrogen bonds to vCCI and the wild type had only 14, indicating a stronger binding affinity for the mutant.

This simulation also sheds light on the possible effect of the CCL17 R57W mutation on the binding. In the simulation of the triple mutant CCL17, the R57 is able to form hydrogen bonds to vCCI intermittently during the 1 μs simulation. For example, Supporting Information Figure 2 shows the R57 in the CCL17 triple mutant forming a hydrogen bond with D73 in the vCCI at the 800 ns point in the simulation. Additionally, the contact maps shown in Figure 3 show that R57 in both the wild type and mutant CCL17 has persistent hydrogen bonds with acidic residues in vCCI. If CCL17 R57 were replaced by a tryptophan, we would expect the interactions with the vCCI acidic residues to be weakened. Therefore, the molecular modeling predicts that the CCL17 R57W mutation will not increase binding to vCCI, despite the tryptophan being highly conserved at that location in tight-binding chemokines.

We experimentally tested the effects of the G17R, V44K, Q45R, and R57W mutations by synthesizing and assaying the mutations individually, in pairs, and as triple (G17R/V44K/Q45R) and quadruple (G17R/V44K/Q45R/R57W) mutants. The gene sequence for each CCL17 variant was made by PCR or ordered from a commercial source, and these mutants were expressed in BL21(DE3), solubilized in 6 M guanidinium chloride followed by Ni^{2+} affinity chromatography, then refolded, and finally purified by reversed-phase chromatography. The mutants were verified to be folded by NMR (Supporting Information Figure 3). Similarly, wild-type vCCI from rabbitpox was expressed in BL21(DE3), refolded, and purified with ion exchange chromatography as previously described (for details see Supporting Information).[7]

To ascertain the vCCI:CCL17 complex stoichiometry, Size Exclusion Chromatography with Multiangle Light Scattering (SEC/MALS) was carried out to demonstrate that vCCI:CCL17 does indeed form a 1:1 complex at high concentrations (10 mg/mL) as observed for other chemokines.[5] To determine the affinity for each CCL17 variant for vCCI, competition fluorescence anisotropy was used. In this technique, a 1:1 complex of vCCI with tight-binding ligand vMIP-II having a fluorophore (vMIP-fluor) was first formed, and anisotropy was measured. Then a CCL17 variant was added to the complex at increasing concentrations and equilibrated. As the CCL17 mutant binds to vCCI, displacing the vMIP-fluor, the anisotropy signal of the vMIP-fluor decreases because an increasing amount of it is in the unbound, i.e., low-anisotropy, form. Using the known K_d (0.06 nM) for vCCI:vMIP-II,[7] the curve of anisotropy versus concentration of CCL17 can be fit to obtain a K_d for the CCL17 variant.[3]

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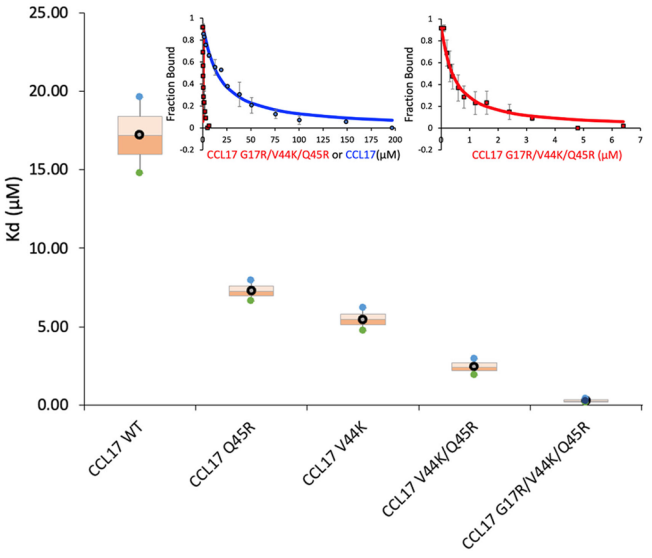

Figure 4. Main chart: K_d values were measured for five different CCL17 mutants bound to vCCI. Fluorescence anisotropy of CCL17 (left, blue) or triple mutant CCL17 G17R/V44K/Q45R (right, red) in competition with a preformed complex of vCCI:vMIP-II-fluor. The left inset figure also shows the triple mutant in red to demonstrate binding on the same scale as the wild-type protein.

As shown in Figure 4 and Supporting Information Figure 4, the designed changes in CCL17 do indeed increase affinity for vCCI. Single mutations show a decrease in K_d of 2−3 fold relative to the wild-type CCL17 (K_d of 17.0 ± 3.4 μM): CCL17 Q45R has a K_d of 7.3 ± 0.9 μM, and similar variant CCL17 V44K has a K_d of 5.5 ± 1.0 μM. The effect is more pronounced for the double mutant CCL17 V44K/Q45R, which has a K_d of 2.4 ± 0.8 μM. Further, when multiple areas of contact are mutated in CCL17 to provide a strong ionic interaction with vCCI, significant improvement is observed, with triple mutant CCL17 G17R/V44K/Q45R showing a K_d of 0.25 ± 0.13 μM, a 68-fold increase in affinity compared to wild-type CCL17 binding to vCCI (Figure 4).

In addition to this triple mutant (G17R/V44K/Q45R) which we accurately predicted would increase the level of CCL17 binding to vCCI, we also created a quadruple mutant (G17R/V44K/Q45R/R57W) to include the highly conserved tryptophan. This change was not predicted by structural modeling to enhance affinity, and indeed, the quadruple mutant does not improve the binding to vCCI beyond that of the triple mutant, giving a K_d of 0.59 ± 0.09 μM.

To put these results in the context of earlier vCCI:chemokine mutation studies by us and others, more than 20 years ago alanine scanning mutagenesis was applied to the chemokine CCL2 to determine which residues were important to binding vCCI. These determined that most single point mutations were either neutral or reduced binding to vCCI, with the exception of K49A, which increased binding to vCCI by 2−3-fold.[8,9] Our earlier work expanded on this, using multiple chemokines, further demonstrating the key role of positively charged chemokine residues in vCCI binding. For example, in a study of CCL11 we showed that the replacement of positively charged R and K residues with A typically led to a weaker binding with binding constants up to 4.8 times, 18 times, or 134 times larger for single, double, or triple mutants, respectively.[4] Replacement of a single basic residue, R, with an acidic residue, E, in CCL11 led to a more dramatic 34-fold increase in the binding constant. Only one CCL11 mutation, K47A, led to improved binding, with an 8-fold decrease in K_d, similar to the earlier CCL2 K49A result mentioned above. These and other mutation studies were important in identifying specific residues required for tight binding of chemokines to vCCI, but they did not lead to mutant chemokines for which significantly increased binding was measured, which was the goal of the present study.

The vCCI:chemokine interaction is important both for the impressive ability of vCCI to tightly bind many different chemokines and also because this binding interaction has great therapeutic potential. An important goal of the study of this interaction is to discern the reasons why very few CC chemokines such as CCL17 do not bind vCCI well. Most mutagenesis studies involve changing amino acids with the hypothesis that the changes will show the importance of these sites by *decreasing* binding to an important binding partner. However, in the current work, hypotheses were formed about how to *improve* the binding to vCCI through mutagenesis of CCL17. We used a combination of molecular modeling and biochemical methods to design variants of the chemokine CCL17 to increase the number of favorable interactions between the two proteins, resulting in an overall change in the binding constant from 17.0 ± 3.4 μM to 0.25 ± 0.13 μM, a remarkable 68-fold increase in binding affinity.

This work illustrates how modifications to the chemokines can lead to stronger vCCI:chemokine interaction, but the complementary approach of designing mutant chemokine inhibitors with modified specificity is an essential next step toward developing therapeutics. An important proof-of-concept of this approach was recently published for evasins, a family of chemokine-binding proteins distinct from the vCCIs. Along with other work by this group,[10,11] Bhusal et al. showed mutation in the evasin EVA-P974 showed increase in binding to CCL17.[12] Our group is currently working on the design and testing of mutant vCCI tailored for strong binding to specific chemokines, including CCL17, as well as other chemokines important to a variety of inflammatory conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.4c00298.

Experimental methods, structures of vCCI and CCL17, results of MD simulation, NMR spectra, Fluorescence anisotropy competition binding curves, additional references (PDF)

Accession Codes

Rabbitpox vCCI: O10647 Human CCL17: Q92583 vMIP-II: Q98157

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CCL17, CC chemokine ligand 17, also called TARC; vCCI, (viral CC chemokine inhibitor); WT, wild type

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