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QTY code-designed water-soluble Fc-fusion cytokine receptors bind to their respective ligands

Shilei Hao[1], David Jin[2], Shuguang Zhang[1*] and Rui Qing[1,3*]

[1]Media Lab, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

[2]Avalon GlobeCare Corp., New Jersey, USA

[3]Koch Institute, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA.

Email: Ruiqing@mit.edu, Shuguang@mit.edu,

Tel.: +1-617-258-7514

*To whom correspondence should be addressed.

ABSTRACT

Cytokine release syndrome (CRS), or "cytokine storm", is the leading side effect during CAR-T therapy that is potentially life-threatening. It also plays a critical role in viral infections such as COVID-19. Therefore, efficient removal of excessive cytokines is essential for treatment. We previously reported a novel protein modification tool called the QTY code, through which hydrophobic amino acids Leu, Ile, Val and Phe are replaced by Gln (Q), Thr (T) and Tyr (Y). Thus the functional detergent-free equivalents of membrane proteins can be designed. Here we report the application of the QTY code on six variants of cytokine receptors, including interleukin receptors IL4αR and IL10αR, chemokine receptors CCR9 and CXCR2, as well as interferon receptors IFNγR1 and IFNλR1. QTY-variant cytokine receptors exhibit physiological properties similar to those of native receptors without the presence of hydrophobic segments. The receptors were fused to the Fc region of IgG protein to form an antibody-like structure. These QTY code-designed Fc fusion receptors were expressed in *E. coli* and purified. The resulting water-soluble fusion receptors bind to their respective ligands with K_d values affinity similar to isolated native receptors. Our cytokine receptor-Fc fusion proteins potentially serve as an antibody-like decoy to dampen the excessive cytokine levels associated with CRS and COVID-19 infection.

Keyword: cytokine release syndrome, protein design, water-soluble membrane protein, antibody-like fusion protein

INTRODUCTION

Chimeric antigen receptor (CAR) T-cell therapy is a novel type of cellular immunotherapy in which a patient's T cells are engineered *in vitro* to target and eliminate cancer cells *in vivo*. In CAR-T treatment, the T cells from a patient's blood are extracted by apheresis. The gene for a specific receptor (CAR) which binds to a certain tumor target is delivered to the T cells by viral vector or non-viral transposon methods (Ittershagen et al., 2019; Jain & Davila, 2018; Srivastava & Riddell, 2015). At present, two anti-CD19 CAR-T products have been approved by the US FDA for the treatment of B-cell acute lymphoblastic leukemia and non-Hodgkin lymphoma; CAR-T therapy for other cancer types are undergoing vigorous clinical studies. CAR-T therapy holds great promise for treating hematologic malignancies, and recent clinical evidence has indicated that similar approaches can also be used to treat solid tumors (Baybutt et al., 2019).

However, there are several potentially fatal side effects during CAR-T treatment including: cytokine release syndrome (CRS), neurologic events, neutropenia and anemia (Xu & Tang, 2014). Among all the side effects, CRS is significant and can be life-threatening. Cytokines are essential immune mediators. Yet, a large and rapid release of cytokines into the blood from immune cells can induce a "cytokine storm", or CRS, which is associated with systemic symptoms of various severity. Most patients with CRS develop a mild flu-like reaction such as fever, fatigue, headache and rash. However, the reaction may progress to an uncontrolled, systemic inflammatory response with extreme pyrexia and become life-threatening (Shimabukuro-Vornhagen et al., 2018).

CRS is not manifested only as a side effect of cellular immunotherapy. It can also be triggered by viral infections such as influenza and hepatitis virus (de Jong et al., 2006; Savarin & Bergmann, 2018; Tisoncik et al., 2012). The current COVID-19 (Coronavirus Disease 2019) triggers CRS in many stages of its pathological course that causes lung fibrosis, acute respiratory distress syndrome, and eventually leads to multi-organ failure (Huang et al., 2020; Xu et al., 2020). Other conditions, including graft-versus-host disease, sepsis, Ebola, avian influenza, smallpox, and systemic inflammatory response syndrome, also involve extensive release of undesired cytokines (Drazen, 2000). To alleviate the symptoms and treat the disease, it is important to remove the excessive cytokines efficiently and rapidly.

The chemokine receptor CCR9, together with its ligand CCL25, contribute to intestinal homing of T cells. This signaling pathway promotes invasion, metastasis, anti-apoptosis and drug-resistance in many types of cancer (Hu et al., 2011; Tu et al., 2016). Specifically, CCR9 is aberrantly expressed in acute and chronic T cell leukemias that contribute to the aggressiveness of the diseases (Somovilla-Crespo et al., 2018). Targeting CCR9 (such as anti-CCR9 monoclonal antibodies with high specificity, affinity and stability) is a rational therapeutic strategy for these diseases.

We have previously devised a novel tool called "QTY code" which regulates the water solubility of redesigned membrane proteins through pairwise substitution of hydrophobic amino acids by hydrophilic ones (Zhang et al., 2018). Hydrophobic amino acids Leu, Val, Ile and Phe are exchanged for hydrophilic Gln, Thr and Tyr in the transmembrane regions of a receptor, based on the structural and electron density maps similarity in their side chains. It has been shown that this protein design approach enables the solubilization of many types of chemokine receptors with tunable functionality (Qing et al., 2019). The QTY code also provides flexibility in studying the physiological and functional properties of transmembrane receptors as well as promoting their utilization, without the requirements of time consuming and expensive detergent screening or use of nanodisks.

We here report the QTY code design of 6 types of cytokine receptors including 2 variants of chemokine receptors CCR9 and CXCR2, 2 variants of interleukin receptors IL4R and IL10R, as well as 2 variants of interferon receptors IFNγR1 and IFNλR1. These QTY code-designed receptors show ligand-binding properties similar to their native receptor counterparts without the presence of hydrophobic patches. The receptors were fused with Fc domain of mouse IgG2a protein to form an antibody-like structure. These Fc-fusion receptors can be expressed and purified in an *E. coli* system with sufficient yield (~mg/L) in LB media. We also showed that these QTY receptors are capable of binding to their respective ligands with affinity close to isolated native receptors on solution-based assays. These QTY code designs of functional, water-soluble Fc-fusion cytokine receptors can potentially be used clinically as decoy therapy to rapidly remove excessive cytokines in the setting of hyperactive immune reactions during CRS including current COVID-19 severely infected patients.

RESULTS

Design of Fc-fusion QTY variant cytokine receptors

Six types of cytokine receptors were selected and redesigned by QTY code. Two variants of chemokine receptors, belonging to the 7-transmembrane (7-TM) G protein-coupled receptor (GPCR) family, and four types single-transmembrane interleukin and interferon receptors were chosen. The L, I, V, F amino acid residues in the transmembrane region of the corresponding receptors were replaced by Q, T and Y accordingly.

Similar to our previous reports for chemokine receptors, sequences of QTY code-designed CCR9 and CXCR2 were aligned with native receptors to compare substitutions of amino acids (Fig.1a and 1b). QTY substitutions were applied to all corresponding residues but only to the 7-TM region. Amino acid changes were highlighted in red color denoting an exchange. Residues in extracellular domain (EC, colored black) or intracellular domain (IC, colored yellow) were untouched. Molecular weight of the QTY variant receptors are slightly increased due to the higher molecular mass of Q, T and Y as compared to L, I, V and F. Despite a total difference of 26.0% (46.4% in 7-TM) in the primary sequence for $CCR9^{QTY}$, and 25.5% (58.9% in 7-TM) for $CXCR2^{QTY}$, the changes in the isoelectric point (pI) of the redesigned

proteins were only 0.05 and 0.06 units, respectively. This is attributed to the non-ionic nature of Q, T and Y as these amino acids do not introduce significant changes in the net charge of a protein. Rather, the substituted amino acids form numerous intra-helical and inter-helical hydrogen bonds that contribute to the structural integrity as well as those to the surrounding water molecules that enhance the overall solubility of a protein (Qing et al., 2019).

Both interferon receptors and interleukin receptors have a single-pass in the transmembrane domain. The ligand binding domain is typically comprised of multiple stranded β-sheets that form two connected anti-parallel β-barrels. The β-barrels are connected to the transmembrane α-helix which is responsible for signal transduction, presumably playing a role in ligand interaction (Richter et al., 2017). In order to best mimic a native receptor, we included the transmembrane domain in our design with a few amino acids in the cytoplasmic region to serve as a short linker so as to optimize the binding and structure of the QTY code modified receptors.

Similar to chemokine receptors, the QTY code was only applied to the transmembrane domains of these receptors, as shown in Fig. 1c, 1d, 1e, 1f. Amino acid exchanges colored in red are selected to eliminate the hydrophobic patches in the designed receptors. Both extracellular domains and intracellular linkers are untouched. Due to the relative weight of TM region, the changes in molecular weight of interferon and interleukin receptors were minimal. pI changes are 0.00, 0.02, 0.18 and 0.01 for IL4Rα^{QTY}, IL10Rα^{QTY}, IFNγR1^{QTY} and IFNλR1^{QTY}, respectively. The larger pI change in IFNγR1^{QTY} was probably due to its larger deviation toward a charge neutral point as compared to other receptors as shown in Fig. 1 (5.10 for IFNγR1^{QTY} compared to 6.15 for IL4Rα^{QTY}, 8.68 for IL10Rα^{QTY} and 8.41 for IFNλR1^{QTY}).

We specifically designed the QTY receptor variants to fuse with the Fc region of IgG protein in order to acquire an antibody-like structure. The primary benefit of Fc fusion is to significantly enhance the half-life of the fused protein in human plasma. It can also improve the safety profile of the fused proteins due to reduced immunogenicity whereas synergistic therapeutic effects from both fusion parts is achievable (Levin et al., 2015). On the other hand, the Fc-fusion is naturally a homodimer through covalent bond. They can be easily tuned to form higher order multimeric states with enhanced stability and efficacy (Czajkowsky et al., 2012). Additionally, Fc fusion further enhances the solubility of QTY-designed cytokine receptors, and serves as an affinity tag for protein purification, as well as for utilization in affinity-based vehicles (beads) for drug delivery. A spacer was introduced to optimize the conformation of QTY- designed receptors in the heavy chain. We used the Fc region of mouse IgG2a in the specific design as it is the functional equivalent of human IgG1. Mouse IgG is chosen over human IgG due to the consideration of implementing mouse cytokine storm model in subsequent experiments, beyond the scope of the current study. The Fc region can be easily exchanged in future designs. Fig. 2 shows a schematic illustration of these cytokine receptor-Fc complex for the 6 QTY receptor variants. The structural illustrations of corresponding cytokine receptors were obtained through PDB (Protein Data Bank) where applicable (Miknis et al., 2010; Moraga et al., 2015; Oswald et al., 2016; Thiel et al., 2000; Yoon et al., 2005) or from a homology model

(CXCR2) (Kwon, 2010). The molecular weight and pI for each of the cytokine receptor-Fc proteins were also denoted in Fig. 1.

Bioinformatics analysis

QTY variant protein sequences were analyzed using a web-based tool TMHMM Server v2.0 to predict the existence of hydrophobic transmembrane segments. The server is based on a Hidden Markov Model (HMM) that takes into account actual biological architectures of a transmembrane helix whereas likelihood of presence is calculated (Sonnhammer et al., 1998).

In Fig. 3, the hydrophobicity of a protein is plotted versus the protein sequences. The X-axis shows the number of amino acids in sequences from N-terminus to C-terminus. Both native sequences (top row) and QTY variant receptor sequences (bottom row) were analyzed and aligned. It is apparent that native CCR9 (Fig. 3a) and CXCR2 (Fig. 3b) exhibits 7 distinctive high- probability hydrophobic segments, corresponding to the 7-TM domains. The segments disappear in QTY designed counterparts. In interleukin and interferon receptors, there is only a single high probability hydrophobic segment near the C-terminus end of each receptor which is also eliminated though QTY modification. The hydrophobicity of both extracellular and intracellular components is unchanged.

E. coli expression and gel-electrophoresis of QTY variant receptors

The corresponding genes with *E. coli* specific codons were synthesized and expressed in sufficient quantities. The throughput for each receptor differed but was all in the mg/L range in LB media. All Fc fusion receptors were expressed into inclusion bodies. They were purified by a) affinity purification, and b) gel filtration in denatured state and then folded into functional state for subsequent analysis. Both arginine and DTT were beneficial for solubilizing the proteins so either or both of them were included in the storage buffer or for ligand binding tests.

The gel-electrophoresis results for purified Fc-fusion QTY variant receptors are shown in Fig. 4. All interleukin and interferon receptors exhibited monomer bands that corresponded well with their respective molecular weight. For the two chemokine receptors, there are several bands above the monomer bands. It is plausible that these bands can be attributed to a dimeric or higher order of multimeric receptors.

Ligand-binding measurement in buffer

The affinity of QTY modified cytokine receptors fused with Fc of IgG for their respective native ligands was measured using microscale thermophoresis (MST). Changes in thermophoretic movement for labeled proteins upon ligand-binding were recorded and plotted as a function of ligand concentration. Both QTY code-designed Fc-fusion interleukin and interferon receptors showed no non-specific adhesion or aggregation during the measurement. The binding data were obtained in early T-Jump period, where rapid changes in fluorophore properties induced by fast temperature change was recorded (Jerabek-Willemsen et al., 2014) because two 7-TM chemokine receptors exhibited minor aggregation during prolonged incubation. For better

visualization, the data were replotted as bound fraction vs. concentration with a scale of 0 to 1, as shown in Fig. 5. The plot was then used to calculate the dissociation constant (K_d) value for receptor-ligand interaction using the K_d model, as presented in the Materials and Methods section. The fitted curves for K_d calculation are also presented in graphs for illustration.

These QTY Fc-fusion receptors exhibit affinity for their respective ligands typically in a range of up to tens of nM (Table 1). The affinities are lower compared to the native receptors without Fc-fusion. The binding affinity of $CXCR2^{QTY}$-Fc with IL8 (60.3±21.0nM) is much lower compared to cell-based assay, e.g. monomeric IL8 binding with native CXCR2 (0.5±0.3nM) but closer to IL8 in dimeric state (8.5±2.0nM) (Rajarathnam et al., 2006). Caccuri et al. also reported a similar dissociation constant (70nM) (Caccuri et al., 2012) that is similar to our affinity measurement of $CXCR2^{QTY}$-Fc with IL8. For affinities of interleukin and interferon receptors, previously-reported studies primarily used human neutrophil cell-based assays with isotope ^{125}I-labeled ligand that is significantly more sensitive than using the purified receptors measured by biophysical instrument; thus they may not be directly comparable. The affinity K_d derived from MST displays similar values compared to previous SPR measurement on purified proteins with the exception of $IL4R^{QTY}$-Fc. The method that was used to determine the K_d in literature is also included in Table 1.

Different types of cytokines are aberrantly expressed during CRS in various pathological conditions. Together with many other types of cytokines, levels of the interleukin IL-8 and IL-10 are elevated over 10 times and 50 times to ~101.7pg/ml and ~100.8 pg/ml, respectively, in the peripheral blood in a non-fatal infection of influenza A (H5N1) (de Jong et al., 2006). Infection with *Francisella tularensis* can lead to an accumulation of excessive IFN-γ, IL-10 and IL-8 to respective levels of ~700pg/ml, ~1ng/ml and ~4ng/ml in the lungs (Sharma et al., 2011). A more recent study on COVID-19 indicate a high-level expression of IL-6 in the blood with an average of 7ng/ml and 12ng/ml detected in discharged and expired patients, respectively. (Ruan et al., 2020). By carefully choosing the vehicle and delivery mechanism such levels of cytokines are surely treatable by our designed Fc fused QTY cytokine receptors. Further data and information from ongoing research with *in vivo* and *in vitro* cytokine release assays as well as mouse animal model studies will be presented in separate reports.

DISCUSSION

In this study, the successful design of QTY code-modified variant chemokine receptors $CCR9^{QTY}$ and $CXCR2^{QTY}$ further expands the plausible applicability of such a protein design algorithm on 7-TM GPCRs. Combined with our prior work (Zhang et al., 2018, Qing et al., 2019), our laboratory has successfully designed and engineered 8 variants of soluble GPCRs while retaining their physiological and functional properties, including 7 variants of chemokine receptors and 1 variant of olfactory receptor. The general applicability of the QTY code for GPCRs may further promote the study of these previously difficult targets in a functionally equivalent form. It is likely that the QTY code may be generally applied to other types of multi-pass membrane proteins and difficult-to-express proteins. Those studies are ongoing.

Although truncated soluble interleukin and interferon receptors also exist *in vivo*, primarily by cleavage between the extracellular and transmembrane segments, the QTY code is still meaningful for designing these single transmembrane receptors for additional studies. While for some receptors the transmembrane α-helix is appears to play a role in ligand interaction (Richter et al., 2017), inclusion of the QTY-designed water-soluble transmembrane helical segment in the entire receptors may contribute to the overall understanding of receptor functional mechanism and signal transduction. On the other hand, not all native-form soluble interleukin and interferon receptors can be readily synthesized and purified in a high-throughput low cost *E. coli* system. In addition, integral multi-transmembrane cytokine receptors such as CCR9 and CXCR2 do not have correspondingly truncated soluble segments.

Our Fc-fusion water-soluble receptor may be able to rapidly soak up excessive cytokines during a cytokine storm unleashed during CAR-T treatment and COVID-19. When the QTY-designed water-soluble Fc-receptors bind to excessive cytokines, they may inhibit excessive cytokine interaction with target cells, thereby reducing the organ damage and toxicity. There are over 20 Fc-fusion proteins commercially available and several of these have been developed as therapeutics (Czajkowsky et al., 2012). Although there have been many Fc-fusion proteins developed for various applications, they are water-soluble proteins in the native state (Czajkowsky et al., 2012; Mekhaiel et al., 2011). Our QTY code designed Fc-fusion receptors, especially chemokine receptors CCR9 and CXCR2, provide a novel platform for further design of other types of fusion membrane receptors for therapeutic and diagnostic applications .

The QTY code design and synthesis of functionally equivalent transmembrane receptor proteins have implications beyond biological and clinical use. Highly specific membrane receptors towards their respective ligands, QTY code modified transmembrane proteins can also serve as ideal candidates for molecular sensing. Complex electrical arrays functionalized with a variety types of water-soluble membrane proteins can potentially mimic cell response *in vitro* and be fabricated into a pseudo cell with electrical readouts.

MATERIALS AND METHODS

Genes identification and QTY modification

Sequences of the selected proteins were obtained from Uniprot: https://www.uniprot.org/. The respective extracellular, transmembrane and cytoplasmic domains were identified. The QTY code was only applied to the transmembrane helical domain to solubilize the proteins.

Bioinformatics analysis

Protein properties were calculated based on their primary sequences via the open access web-based tool ExPASy: https://web.expasy.org/protparam/. The existence of hydrophobic segments within the transmembrane region in native and QTY variant protein sequences was determined via the open access web-based tool TMHMM Server v.2.0: http://www.cbs.dtu.dk/services/TMHMM-2.0/.

E. coli expression system and protein purification

Genes of QTY modified cytokine receptor proteins were cloned into the Fc region of mouse IgG2a as the functional equivalent of human IgG1. The full sequences were codon-optimized for *E. coli* expression and obtained from Genscript. The genes were cloned into pET20b expression vector with Carbenicillin resistance. The plasmids were reconstituted and transformed into *E. coli* BL21(DE3) strain. Transformants were selected on LB medium plates with 100μg/ml Carbenicillin. *E. coli* cultures were grown at 37°C until the OD_{600} reached 0.4-0.8, after which IPTG (isopropyl-D-thiogalactoside) was added to a final concentration of 1mM followed by 4-hour expression. Cells were lysed by sonication in B-PER™ protein extraction agent (Thermos-Fisher) and centrifuged (23,000×g, 40min, 4°C) to collect the inclusion body. The biomass was then subsequently washed twice in buffer 1 (50mM Tris.HCl pH7.4, 50mM NaCl, 10mM CaCl2, 0.1%v/v Trition X100, 2M Urea, 0.2μm filtered), once in buffer 2 (50mM Tris.HCl pH7.4, 1M NaCl, 10mM CaCl2, 0.1%v/v Trition X100, 2M Urea, 0.2μm filtered) and again in buffer 1. Pellets from each washing step were collected by centrifugation (23,000×g, 25min, 4°C).

Washed inclusion bodies were fully solubilized in denaturation buffer (6M guanidine hydrochloride, 1×PBS, 10mM DTT, 0.2μm filtered) at room temperature for 1.5 hour with magnetic stirring. The solution was centrifuged at 23,000×g for 40 min at 4°C. The supernatant with proteins was then purified by Qiagen Ni-NTA beads (His-tag) followed by size exclusion chromatography using an ÄKTA Purifier system and a GE healthcare Superdex 200 gel-filtration column. Purified protein was collected and dialyzed twice against renaturation buffer (50mM Tris.HCl pH 9.0, 3mM reduced glutathione, 1mM oxidized glutathione, 5mM ethylenediaminetetraacetic acid, and 0.5M L-arginine). Following an overnight refolding process, the re-natured protein solution was dialyzed into storage buffer of 50mM Tris.HCl pH 9.0 with various arginine content.

Microscale Thermophoresis

MST is an optical method detecting changes in thermophoretic movement and temperature related intensity change (TRIC) of the protein-attached fluorophore upon ligand binding. Active labelled proteins contribute to the thermophoresis signal upon ligand binding. Inactive proteins influence the data as background but not the signals and only data from binding proteins are used to derive the K_d value. Herein ligand binding experiments were carried out with 5nM NT647-labeled protein in 1 X PBS, 10mM DTT buffer with different concentration of arginine, against a gradient of respective ligands on a Monolith NT.115 pico instrument at 25°C. Synthesized receptors were labeled with Monolith NT™ 2nd generation protein labeling kit RED – NHS (NanoTemper Technologies) so as to obtain unique fluorescent signals. MST time traces were recorded and analyzed to obtain the highest possible signal-to-noise levels and amplitudes, >5 Fnorm units. Multiple rounds of buffer optimization were conducted for $CXCR2^{QTY}$-Fc and $CCR9^{QTY}$-Fc receptors. The data in optimized buffer was reported. The buffer condition was then adopted directly by Fc fused QTY interleukin and interferon variants. The recorded fluorescence was plotted against the concentration of ligand, and curve fitting was performed using the K_d fit

formula derived from the law of mass action. For clarity, binding graphs of each independent experiment were normalized to the fraction bound (0 = unbound, 1 = bound). MST experiments were performed in the Center for Macromolecular Interactions at Harvard Medical School.

K_d fitting model:

K_d model is the standard fitting model based on law of mass action.

Curve fit formula:

$$F(c_T) = F_u + (F_b - F_u) * \frac{c_{AT}}{c_A}$$

$$\frac{c_{AT}}{c_A} = fraction\ bound = \frac{1}{2c_A} * (c_T + c_A + K_D - \sqrt{(c_T + c_A + K_D)^2 - 4c_T c_A})$$

F_u: fluorescence in unbound state

F_b: fluorescence in bound state

K_D: dissociation constant, to be determined

c_{AT}: concentration of formed complex

c_A: constant concentration of molecule A (fluorescent), known

c_T: concentration of molecule T in serial dilution

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Table 1. **Ligand-binding affinity of Fc fused QTY cytokine receptors**

	Native (K_d, nM)	QTY variant (K_d, nM)
$CCR9^{QTY}$-Fc vs CCL25	~8 (Eberhardson et al., 2017)	37.2±15.7
$CXCR2^{QTY}$-Fc vs IL8	0.5±0.3 (Monomer) 8.5±2.0 (Dimer) (Rajarathnam et al., 2006)	60.3±21.0
$IL4R\alpha^{QTY}$-Fc vs IL4	~1 nM (SPR) (LaPorte et al., 2008)	20.9±8.3
$IL10R\alpha^{QTY}$-Fc vs IL10	0.05-0.2 (Cell) (Tan et al., 1993)	1.6±0.9
$IFN\gamma R1^{QTY}$-Fc vs IFNγ	1.7 (Cell) (Celada et al., 1985) 30.8 (SPR) (Mikulecky et al., 2013)	6.2±3.0
$IFN\lambda R1^{QTY}$-Fc vs IL29	—	11.5±2.5

1. The methods used for are included after the reported values.
2. The references for reported K_d values are also cited.

Figures and Figure Legends

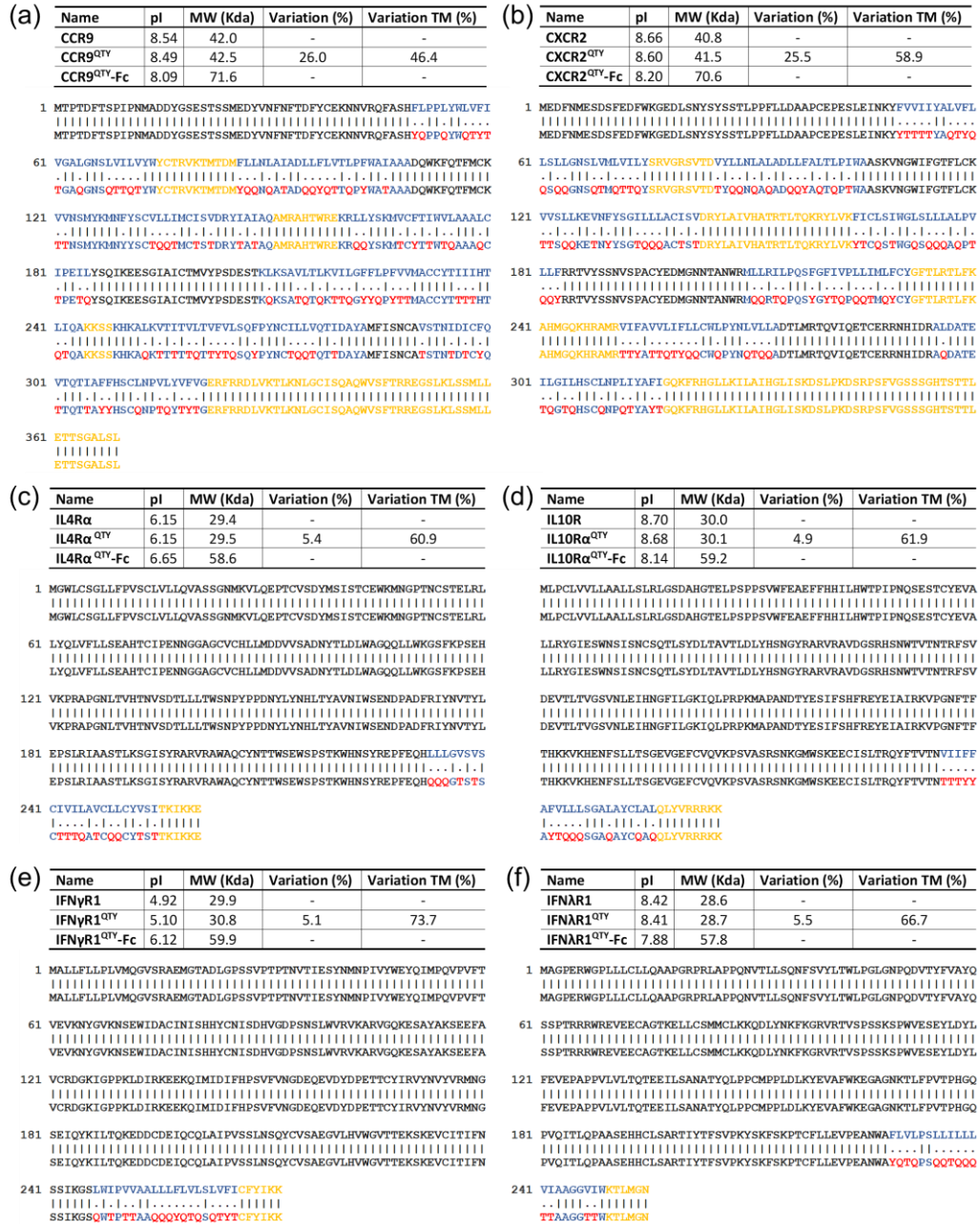

Figure 1. Protein sequence alignment between natural (Top) and QTY redesigned cytokine receptors. (a) CCR9 vs CCR9^{QTY}; (b) CXCR2 vs CXCR2^{QTY}; (c) IL4Rα vs IL4Rα^{QTY}; (d) IL10Rα vs IL10Rα^{QTY}; (e) IFNγR1 vs IFNγR1^{QTY}; (f) IFNλR1 vs IFNλR1^{QTY}. The substitutions of Q, T, and Y are denoted with ".", while "|" indicates no change in residues between the 2 sequences. The Q, T, and Y amino acid substitutions are colored in red. The N-terminus, extracellular loops are block, transmembrane is blue and intracellular components of the receptors are yellow. Characteristics of native, QTY variant-Fc fusion receptor proteins' pI, molecular weight, and overall variation rate and that % changes only transmembrane segments are presented.

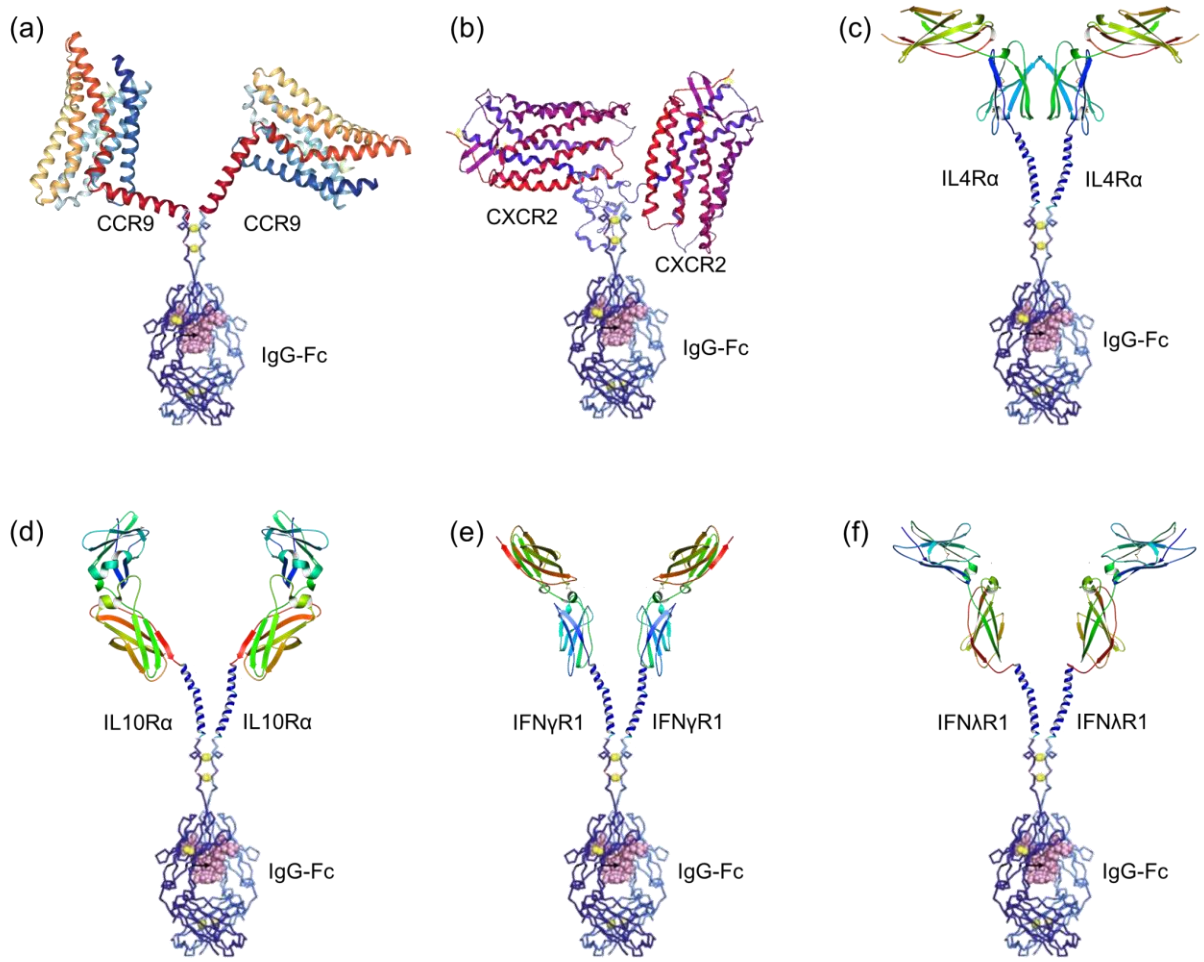

Figure 2. Schematic illustration for Fc fused QTY variant cytokine receptors with antibody-like structure. (a) CCR9^{QTY}-Fc; (b) CXCR2^{QTY}-Fc; (c) IL4Rα^{QTY}-Fc; (d) IL10Rα^{QTY}-Fc; (e) IFNγR1^{QTY}-Fc; (f) IFNλR1^{QTY}-Fc. These illustrations are not to scale and the receptors parts are significantly emphasized for clarity.

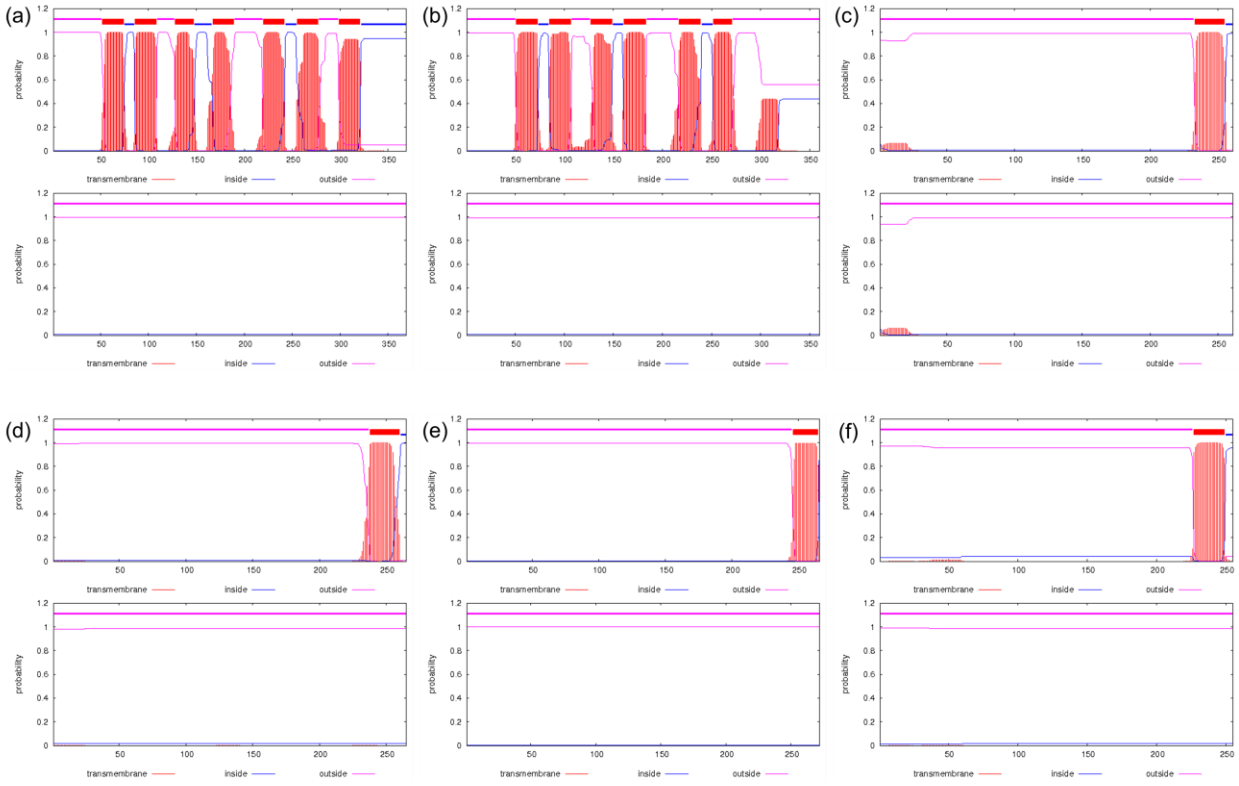

Figure 3. Bioinformatic predictions of cytokine receptors with hydrophobic segment of native (top) and QTY variant (bottom). The hydrophobicity probability of a protein is plotted *vs* the sequence. (a) CCR9^{QTY}-Fc; (b) CXCR2^{QTY}-Fc; (c) IL4Rα^{QTY}-Fc; (d) IL10Rα^{QTY}-Fc; (e) IFNγR1^{QTY}-Fc; (f) IFNλR1^{QTY}-Fc. Color code: Pink line= extracellular regions, red line = transmembrane regions, and blue line = intracellular region.

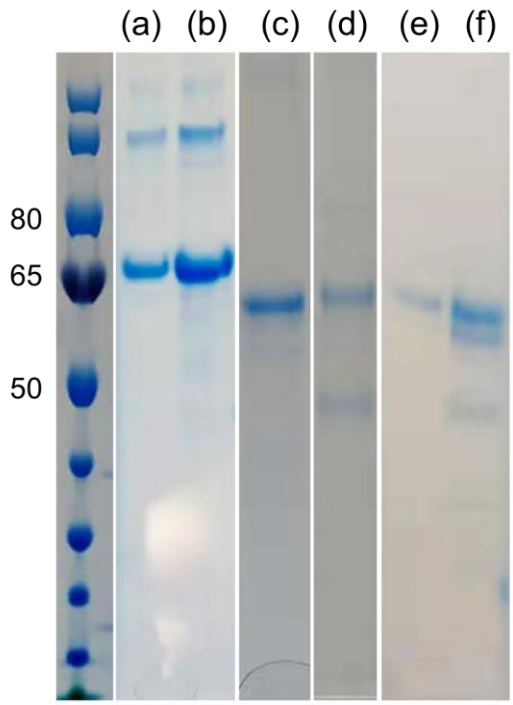

Figure 4. Gel-electrophoresis of purified QTY code designed Fc-fusion receptors. (a) CXCR2^{QTY}-Fc; (b) CCR9^{QTY}-Fc; (c) IL4Rα^{QTY}-Fc; (d) IL10Rα^{QTY}-Fc; (e) IFNγR1^{QTY}-Fc; (f) IFNλR1^{QTY}-Fc. The molecular weight of the ladder is labelled on the left in KDa. It is plausible that these bands can be attributed to dimeric or higher order of multimeric receptors. For panels D and F, the bands are likely to be impurities that are too close to the target band which we were not able to separate with either His-tag or gel-filtration purification. These bands might be eliminated with further Protein A/G purification in future experiments

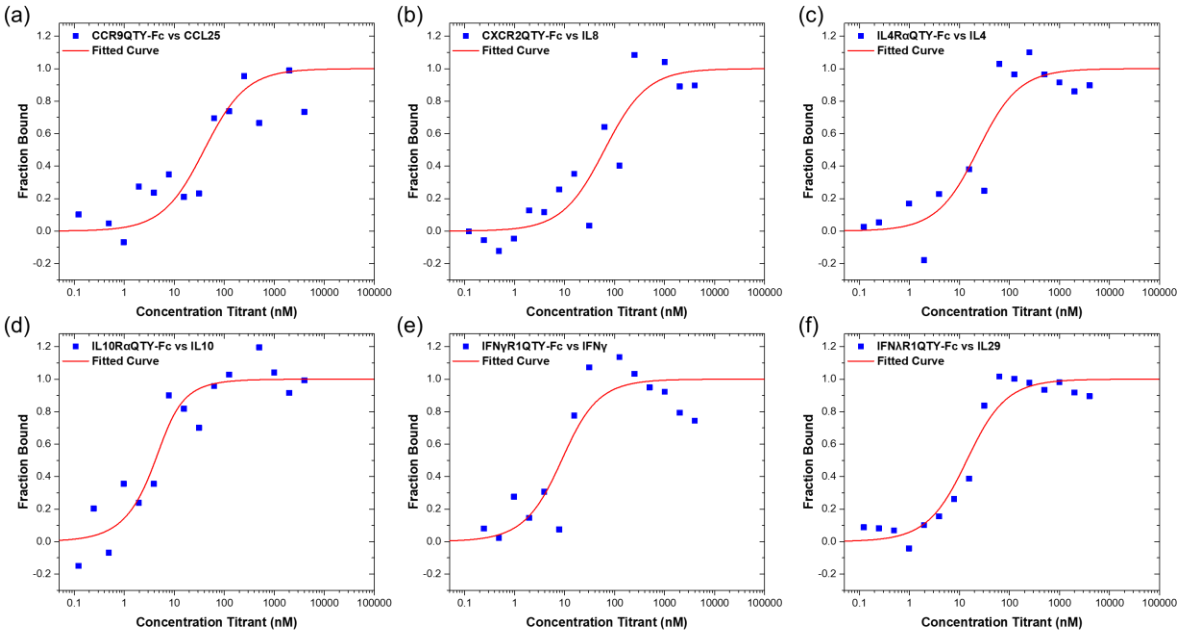

Figure 5. MST ligand binding measurements. The receptors were labeled with fluorescent dye. The ligands were purchased commercially from and dissolved in di water. (a) $CCR9^{QTY}$-Fc with CCL25; (b) $CXCR2^{QTY}$-Fc with IL8; (c) $IL4R\alpha^{QTY}$-Fc with IL4; (d) $IL10R\alpha^{QTY}$-Fc with IL10; (e) $IFN\gamma R1^{QTY}$-Fc with IFNγ; (f) $IFN\lambda R1^{QTY}$-Fc with IL29. The K_d values calculated from the graphs are listed in Table 1.

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