Design of De Novo Mimetic Protein of Interleukin-21

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Abstract

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Interleukin-21 (IL-21) is crucial in coordinating immune cells and has pleiotropic effects in their immune responses. It has established clinical anti-tumor activity in many cancer models. Despite its efficacy, its therapeutic window has been limited due to its systemic toxicity and poor protein engineerability, resulting in the molecule with suboptimal biodistribution and pharmacokinetic properties. In addition, the limited cross-reactivity of human IL-21 in mice complicates the use of animal models to study the toxicity and activity of candidate IL-21 therapeutics. Here, we created de novo IL-21 mimetic proteins that can fully recapitulate its interaction with receptors and the biology of native IL-21 in both humans and mice.

Table of Contents

List of Figures

Agonistic mimic of interleukin-21

Affinity variants and antagonistic mimic of interleukin-21

1. Overall background, challenges, and motivation

IL-21, a pivotal member of the γ_c family of cytokines, occupies a central position in regulating a multitude of immune responses and maintaining immune homeostasis. Its mode of action involves the induction of heterodimerization between the IL-21 receptor (IL-21R) and the common γ_c receptor chain, leading to the activation of downstream signaling molecules, particularly phosphorylated STAT proteins. Significantly, IL-21 exhibits pleiotropic effects on various immune cell subsets, encompassing T cells, B cells, natural killer (NK) cells, macrophages, and dendritic cells. Within the context of CD8+ T cells, IL-21 plays a pivotal role in stimulating crucial processes such as cellular proliferation, survival, memory formation, and augmentation of effector functions[1]. In the context of B cells, IL-21 serves as a regulator of several fundamental processes, including cell proliferation, differentiation, and the production of immunoglobulins, which collectively contribute to the maintenance of the germinal center reaction[2].

Furthermore, IL-21 plays a pivotal role in the regulation of natural killer (NK) cells[3–5], influencing their activation, cytotoxicity, and apoptosis. These effects are closely associated with the production of essential cytokines, particularly interferon-γ, which plays a crucial role in mediating anti-tumor[6–8] and anti-viral[9–11] effector functions. IL-21's involvement extends to the orchestration of immune responses against infections, cancer, and autoimmune diseases. Consequently, it has garnered substantial attention in clinical research in recent years[12,13]. Clinical trials involving IL-21 have been conducted across a spectrum of cancer types, including melanoma[14–16], renal cell carcinoma[17], ovarian cancer, and non-Hodgkin's lymphoma. IL-21 has been explored as a standalone therapeutic agent and in combination with other immunotherapeutic modalities, such as immune checkpoint inhibitors and cancer vaccines. The development of IL-21-based cancer therapies represents an exciting frontier in research, holding significant potential for enhancing treatment outcomes for cancer patients. However, the mechanisms underlying IL-21's impact on anti-tumor responses remain incompletely elucidated. Additionally, realizing the full therapeutic potential of native IL-21 has posed challenges due to issues related to its stability and the limited cross-reactivity of human IL-21 in mouse models.

Computational protein design has emerged as a powerful tool for addressing longstanding challenges in protein-based therapeutics. One notable platform in this domain is Rosetta, a specialized computational molecular modeling suite tailored for protein design[18]. Rosetta has found widespread application in the creation of de novo proteins with diverse functionalities[18], including peptides[19], therapeutic proteins[20–23], biosensors[24–26], protein cages[27–29], and vaccines[30–32]. Previously, Rosetta demonstrated its capability in guiding the design of de novo mimetics of IL-2 for cancer therapeutics[20]. As evidenced by numerous recent instances, computational protein design empowers the development of entirely new proteins

customized to replicate known biological functions or even engender novel reactions previously unobserved in nature[20,28,33].

In this study, our objective was to engineer a de novo IL-21 mimetic protein. This protein was designed to recapitulate and enhance the structural features and intermolecular interactions of native IL-21, with an emphasis on enhancing stability while preserving the fundamental biology of native IL-21. Our research showed promising results, showcasing that this IL-21 mimetic protein possesses the potential to be successfully used for therapeutic agents.

2. Design, optimization, and characterization of de novo interleukin mimics

2.1. Agonistic mimic of interleukin-21

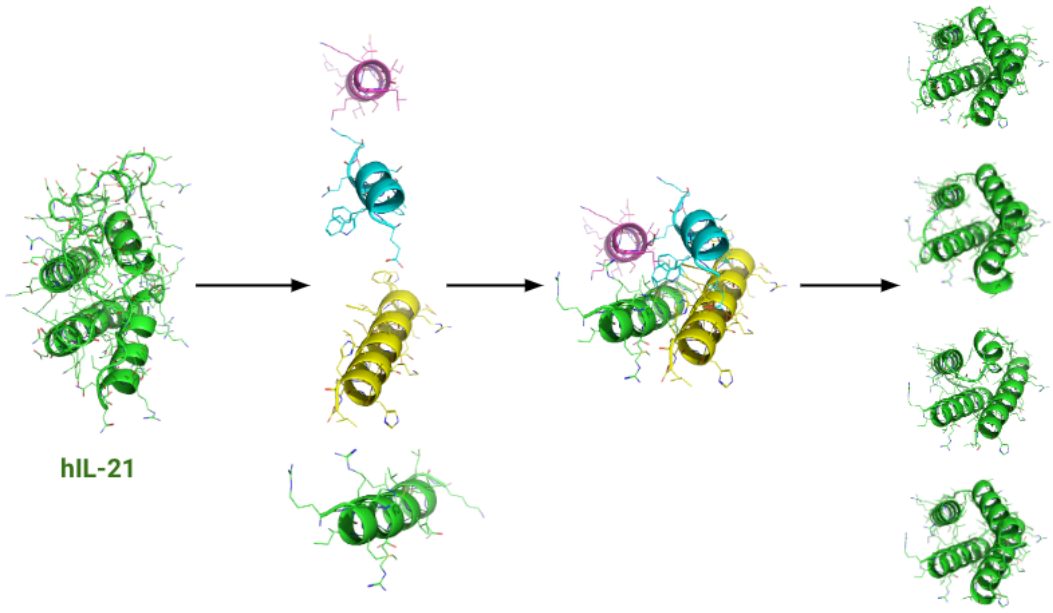

Fig. 2.1.A: The scaffold of the IL-21 mimic is optimized by removing its unstructured regions and extended on helices for ideal helical packing. The IL-21 mimics are designed by recapitulating the helical bundle structure of the native human IL-21. Unstructured regions in the hIL-21 are removed, and short helices are extended to accommodate improved intramolecular packing. Some of the native residues conserved from the native human IL-21. Some of its residues are further optimized, as shown.

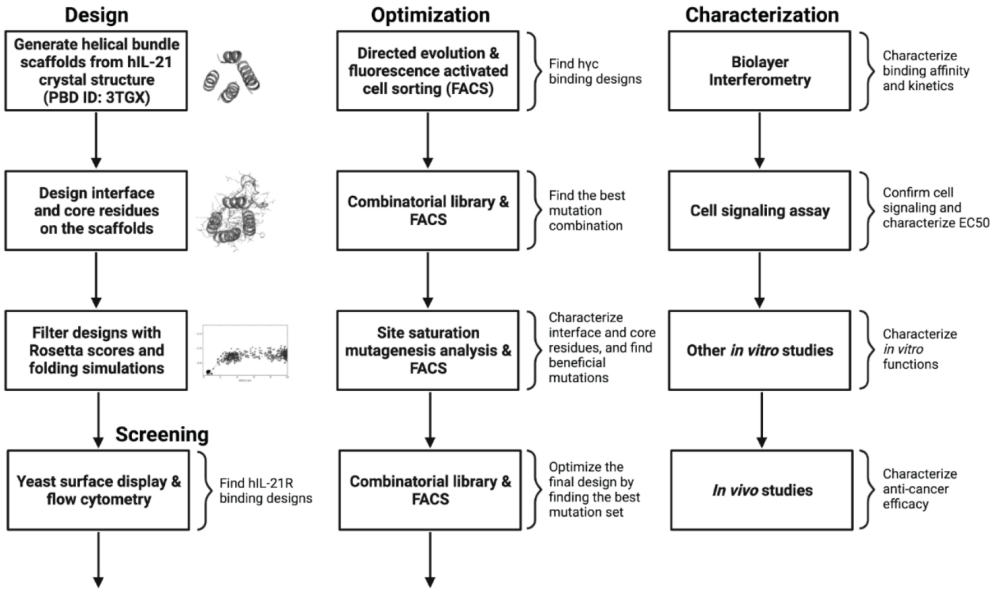

Fig. 2.1.B: Stepwise process for design, screening, optimization, and characterization of IL-21 mimics. The helical bundle scaffolds are generated from the hIL-21 structure, and their residues are designed in the context of hIL-21R (PDB ID: 3TGX). The designs are computationally screened using Rosetta scores and folding simulations. The designs are experimentally screened using yeast surface display with flow cytometry. The optimization processes incorporate directed evolution, site saturation mutagenesis analysis, and combinatorial library screening. Further characterizations use biolayer interferometry, cell signaling assay, other in vitro assays, and in vivo assays.

We employed a protein design approach to create IL-21 mimics. As the complete structure of the IL-21 receptor complex was unavailable at the time, we used computational modeling. To address the absence of the human γc ($h\gamma_c$) structure in the human IL-21/hIL-21R complex[34], we incorporated the $h\gamma_c$ chain from the human IL-2 complex[35] by using PyRosetta, a computational tool. We generated scaffolds that imitated the helical bundle structure of human IL-21 while enhancing structural stability[20]. Our optimization efforts included adjusting the lengths of helices, minimizing unstructured regions, and eliminating non-ideal long loops in native human IL-21. Native human IL-21 comprises four helices, with helices A (the first helix from the N-terminus) and C (the third helix) forming an interface to hIL-21R and helix D (the fourth helix) forming an interface to $h\gamma_c$[35,36]. The upper two helices, B and C, are too short to form ideal intramolecular and intermolecular interactions. To improve these helices, we extended them, substituting unstructured regions, including regions between helix pairs A/B and C/D (Fig. 2.1.A). Additionally, we adjusted helices A and D to enhance packing (Fig. 2.1.A). For interface design, we grafted interface residues from hIL-21 onto the scaffolds, designing the remaining residues within the context of hIL-21R using Rosetta. The designed proteins were evaluated and filtered based on Rosetta score metrics and folding trajectories using Fast Forward Folding (Fig. 2.1.B).

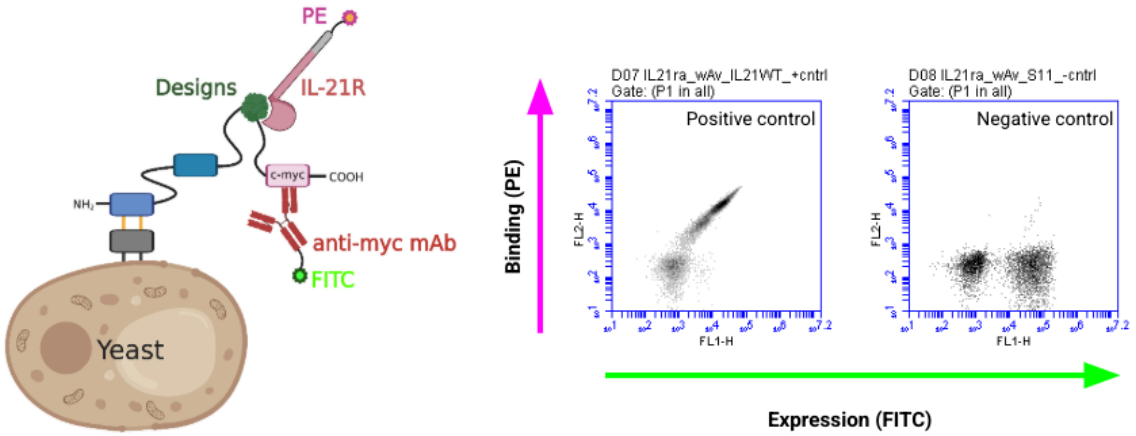

Fig. 2.1.C: Yeast surface display for binder screening. The x-axis represents expression of the protein, labeled as FITC, whereas y-axis represents binding signal, labeled as PE. The cells populate into two groups; the double negative ones are the ones that do not express the design on their cell surface and neither bind to the target receptor. The FITC-positive population is the population that expresses the designed protein on their surface and is able to bind their target receptor, showing diagonal distribution of the population.

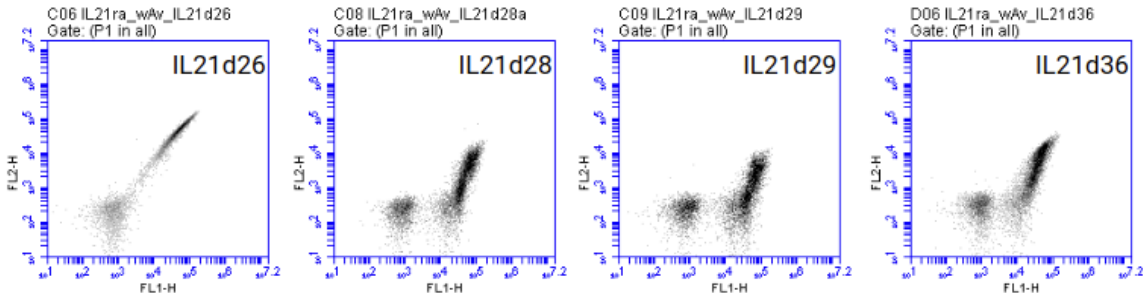

Fig. 2.1.D: Hits from initial designs that bind to human IL-21R. The designs are screened using yeast surface display and tested for receptor binding at 1 uM.

Four hits were identified out of 36 designs constructed with hIL-21R using yeast surface display as a screening platform (Fig. 2.1.C). These hits bound to hIL-21R but not to hγ_c (Fig. 2.1.D). To improve the binding capabilities of our best hit, 21d26, to hγ_c, we employed directed evolution. We isolated mutants of 21d26 that exhibited hγ_c binding in an hIL-21R-dependent manner. One variant, 21JC15, with four mutations (W15C, W49R, Q73H, H99G), displayed the strongest binding affinity. H99G was identified as the critical mutation for acquiring hγ_c binding, while the other three mutations enhanced the interface affinity. Incorporating all four mutations into 21d26, we created 21JC15, which exhibited significantly higher affinity than the parent and cross-reactivity between human and murine receptors.

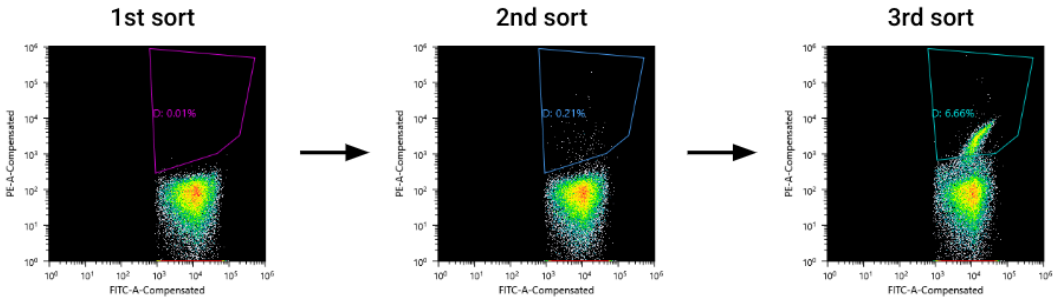

Fig. 2.1.E: Fluorescence-activated cell sorting of random-mutagenesis library of 21d26. The designs are screened using yeast surface display and tested for receptor binding at 1 uM.

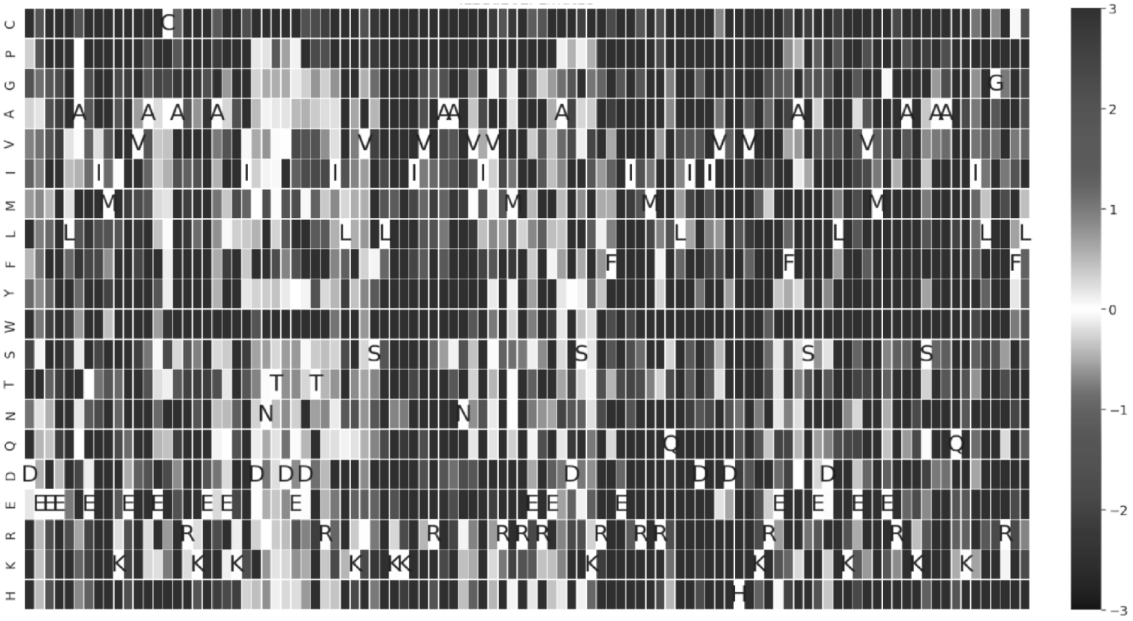

Fig. 2.1.F: Site-saturation mutagenesis of 21JC15.

We used site-saturation mutagenesis to analyze interface residues of 21JC15 for their individual impact on receptor binding affinity (Fig. 2.1.F). Given the combined mutations, we isolated 21h10, a mutant optimized from 21JC15, to exhibit significantly higher affinity than the parent while retaining cross-reactivity between human and murine receptors (Fig. 2.1.G).

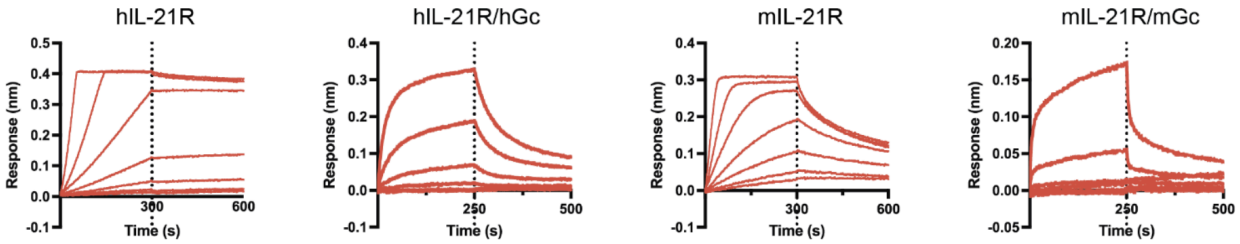

Fig. 2.1.G: Biolayer interferometry for 21h10's binding against human and murine IL-21 receptors. Association and dissociation of 21h10 to human and murine IL-21R and γ_c show concentration-dependent binding curves of 21h10.

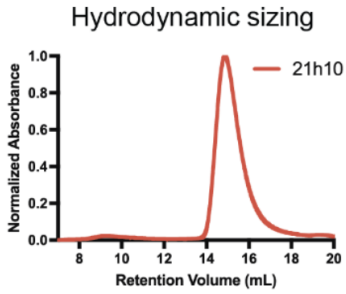

Fig. 2.1.H: Size exclusion chromatography for hydrodynamic sizing. 21h10 exhibits monodispersed distribution when sized through size-exclusion chromatography using Superdex 75 10/300 GL column.

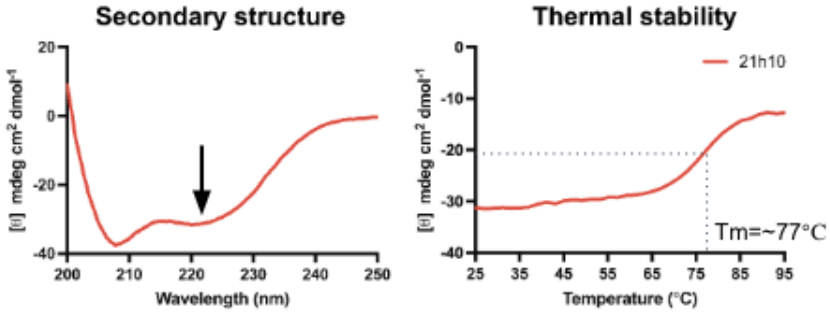

Fig. 2.1.I: Circular dichroism for secondary structure and thermal stability. Wavelength scan from 200 nm to 250 nm confirmed α-helical secondary structure present in 21h10. Thermal melt from 25°C to 95°C with 222 nm scan revealed superior thermal stability of 21h10.

Despite only 44.9% sequence identity to hIL-21 and 23.5% to mIL-21[37], 21h10 showed cross-reactivity and was tested in various in vitro and in vivo studies. Furthermore, the protein was highly stable and efficiently expressed in *Escherichia coli*. Size-exclusion chromatography confirmed monodispersity in size (Fig. 2.1.H), and circular dichroism analysis confirmed the presence of helical secondary structures and superior thermal stability, holding its helical structure up to 65°C (Fig. 2.1.I).

2.2. Affinity variants and antagonistic mimic of interleukin-21

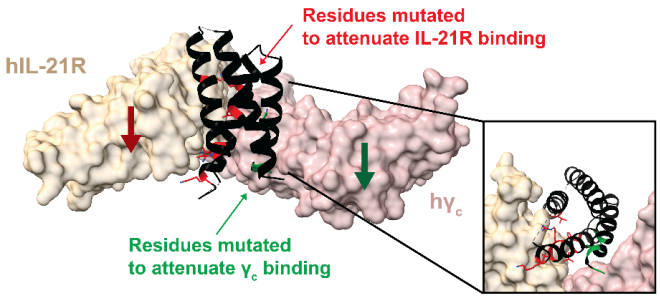

Fig. 2.2.A: Design of affinity variants of IL-21 mimic. Interface residues are mutated (either red or green) to modulate receptor binding affinity to design affinity variants that can bind to the two receptors (IL-21R and γ_c) in a range of affinities.

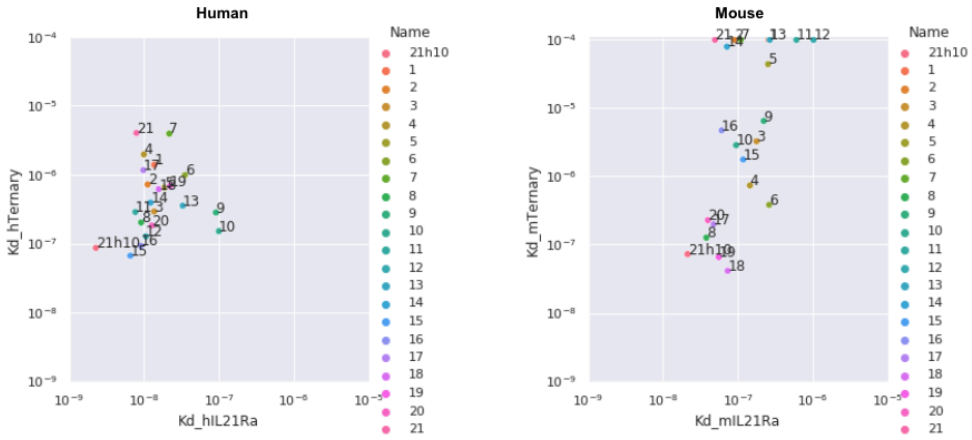

Fig. 2.2.B: Characterization of the affinity variants. Left, human receptor binding affinities of the affinity variants. Right, murine receptor binding affinities of the affinity variants.

Utilizing ProteinMPNN[38], we also utilized site-saturation mutagenesis to design affinity-attenuated mutants by weakening affinity from 21h10 (Fig. 2.2.A). A series of variants are acquired by implementing several mutations in the interfaces towards IL-21R and γ_c. The affinity variants with mutations on their receptor binding interfaces show a range of binding affinities to both human and mouse receptors, either IL-21R or IL-21R/γ_c complex (Fig. 2.2.B). This tuning of the interface affinity is feasible due to robustness of the designed *de novo* proteins with high stability which can withstand mutations. Their binding kinetics to each receptor with association/dissociation rates were measured using biolayer interferometry.

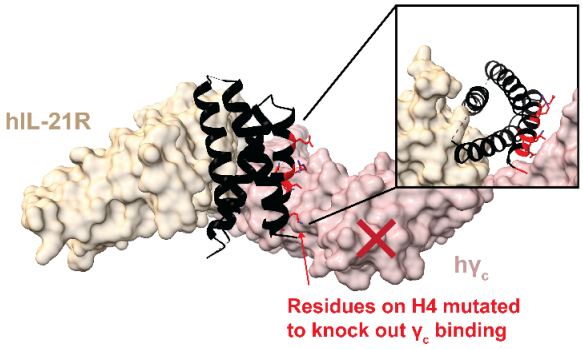

Fig. 2.2.C: Design of 21AT36, an antagonist of IL-21 mimic. Interface residues towards γ_c are mutated (red) to knock out the γ_c binding.

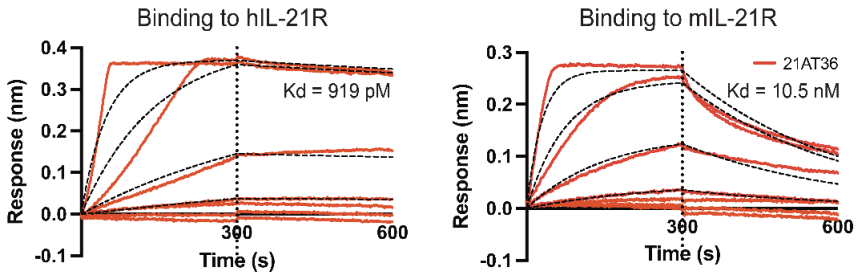

Fig. 2.2.D: Characterization of the antagonist, 21AT36. Association and dissociation of 21AT36 to human and murine IL-21R show concentration-dependent binding curves of 21AT36.

With extensive mutations on the γ_c-interface using ProteinMPNN[38], we strategically redesigned the γ_c interface of the most optimized and validated IL-21 mimic, 21h10, to abolish its receptor affinity to achieve antagonistic activity (Fig. 2.2.C). The antagonist hits were screened using yeast surface display, biolayer interferometry, and cell assay to confirm their ability to bind to IL-21R but not γ_c, and antagonism in cells. Their receptor binding affinities to IL-21R are characterized in Fig. 2.2.D.

2.3. Takeaways

Cytokine-based drug development has been a subject of exploration for various clinical applications. However, the engineering of these cytokines for enhanced druggability has been hindered by issues such as poor stability, limited cross-reactivity, and unpredictable behavior of native cytokines. Computational design of de novo proteins has emerged as a promising approach to overcome these limitations by

improving the stability and biochemical properties of native proteins while retaining their binding mechanisms.

Native IL-21 has attracted significant interest as a therapeutic target for conditions like cancer. IL-21 mimics offer a potential avenue to leverage the therapeutic properties of IL-21 while enhancing its molecular stability for optimized engineering. Our computationally designed de novo IL-21 mimic, 21h10, presents several structural and biochemical advantages. First, this mimic demonstrates significantly improved stability, making it highly suitable for further development as a therapeutic agent. Unlike native IL-21, which often faces challenges in expression, 21h10 can be readily produced using *E. coli* and mammalian expression systems. Furthermore, such enhanced stability allows more reliable and consistent data *in vivo* where native IL-21 is impacted by their molecular instability. Third, 21h10 exhibits full cross-reactivity, a feature lacking in native IL-21, and displays comparable receptor binding profiles to its natural counterpart. 21h10 underwent rigorous biochemical and structural validation, confirming its cross-reactivity with human and murine receptors. It recapitulated critical molecular interactions essential for receptor assembly, underscoring the precision of computational protein design.

In summary, the development of 21h10 as a versatile IL-21 mimic with broad immunological applications represents a significant advancement in immunotherapy. The mimic's full cross-reactivity with human and murine systems enables more effective use of animal models for predicting efficacy and toxicity, with full translational possibility. With its numerous advantages, the designed mimics stand as a valuable tool for exploring and harnessing the potential of IL-21-based immunotherapies across diverse disease contexts. Its engineering flexibility opens the door to immunocytokine approaches employing this novel IL-21 mimic and its variants.

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- Marion Pepper (Reading Committee)
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4. Competing interests

J.C. is one of the co-inventors on a patent application (49605.01US1; 49605.02WO2), which incorporates discoveries described in this thesis. UW FIDS Disclosure #D250035 CoMotion #54400A.

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