

Crystal structure of human interleukin-2 in complex with TCB2, a new antibody-drug candidate with antitumor activity

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ABSTRACT

Immunotherapy via interleukin-2 (IL-2) mediated activation of anti-tumor immune response is a promising approach for cancer treatment. The multi-potent cytokine, IL-2 has a central role in immune cell activation and homeostasis. Since IL-2 preferentially activates immunosuppressive T regulatory cells by IL-2R α dependent manner, blocking IL-2:IL-2R α interaction is a key to amplify the IL-2 activity in effector T cells toward anti-tumor response. Anti-IL-2 monoclonal antibodies are good candidates to control the IL-2:IL-2R α interaction. In a previous study, we developed a new IL-2R α mimetic antibody, TCB2, and showed that the human IL-2(hIL-2):TCB2 complex can stimulate T effector cells specifically and elicit potent anti-cancer immunotherapeutic effect, especially when administered in combination with immune checkpoint inhibitors. To understand the molecular mechanism, we determined the crystal structure of TCB2-Fab in a complex with hIL-2 at 2.5 Å resolution. Our structural analysis reveals that TCB2 binds to the central area of the hIL-2R α binding region on hIL-2, and binding angle and epitope are different from previously known hIL-2R α mimicking antibody NARA1 which recognizes the top part of hIL-2. TCB2 binding to hIL-2 also induces an allosteric effect that increases the affinity for the hetero-dimeric hIL-2 receptor, IL-2R($\beta + \gamma$), on effector T cells.

ARTICLE HISTORY

Received 19 January 2021
Revised 23 February 2021
Accepted 24 February 2021

KEYWORDS

Interleukin-2; TCB2; immunotherapy; crystal structure

Introduction

Interleukin-2 (IL-2) is a 15.5-kDa cytokine that has a central role in immune homeostasis by activating both immune-suppressing and activating responses.¹ IL-2 stimulates T lymphocyte by binding to IL-2 receptors (IL-2Rs) on the cell surface. IL-2Rs are composed of three different components; IL-2R α (CD25), IL-2R β (CD122), and IL-2R γ (γ_c , CD132).² As a functional unit, intermediate affinity heterodimeric IL-2R($\beta + \gamma$) ($K_D \approx 1$ nM) and high affinity heterotrimeric ($\alpha + \beta + \gamma_c$) receptors ($K_D \approx 10$ pM) are expressed in CD8⁺ effector T (T_{eff}) and CD4⁺ FoxP3⁺ regulatory (T_{reg}) cells, respectively, and both dimeric and trimeric receptors are capable of activating the cytoplasmic signaling cascade upon IL-2 binding.^{3,4}

Since IL-2 showed the clinical efficacy in metastatic cancer treatment such as immunotherapy in the early 1990s,⁵ various efforts are applied to improve IL-2's therapeutic function and reduce the undesired side effects. The main shortcomings of IL-2 as a therapeutic candidate are short half-life, cytotoxicity at high doses, and intrinsic ability to activate not only T_{eff} but also T_{reg} cells.^{6,7} To make IL-2 activate only T_{eff} or T_{reg} cells, many different IL-2 variants have been developed including introducing mutation, chemical modification, making IL-2 fusion protein, and so on.⁸⁻¹⁰ Recently, Garcia and colleagues developed an IL-2 mutant, "superkine" with increased binding affinity for IL-2R β ⁸ and also designed a *de novo* molecule with

desirable affinity against IL-2R($\beta + \gamma$)^{hi} T_{eff} cells.⁹ Although some approaches using IL-2-based modification showed impressive antitumor effects via selective expansion of T_{eff} cells with reduced cytotoxicity, unnatural amino acid sequence in modified IL-2 often recognized as a foreign molecule and elicit an immunogenic response after multi-dose injection. Besides, manufacturing chemically modified or multi-complexed protein complicates the regulatory approval as a therapeutic drug. Therefore, none of them has been approved for validated human therapeutics to date.

The alternative way is a complex form of IL-2 with a monoclonal antibody. Based on the structural information of hIL-2:IL-2R complex² and the different IL-2 receptor subunit expression profile between T_{reg} and T_{eff} cells,⁴ the strategy of using monoclonal antibody which blocks an IL-2R α binding site of IL-2 can specifically stimulate the T_{eff} cells proliferation by skewing IL-2 binding toward heterodimeric IL-2R($\beta + \gamma$) on T_{eff} cells. Furthermore, antibody binding also increased the half-life of IL-2 in serum dramatically.¹¹ Several IL-2 specific monoclonal antibodies are developed to modulate the IL-2 activity. Especially, the structures of NARA1:hIL-2 complex (PDB ID:5LQB) and S4B6: mouse IL-2 complex (PDB ID: 4YUE) explained the mechanistic role of monoclonal antibody. Since S4B6 recognizes mouse IL-2 which has only 56% sequence identity with hIL-2, its interaction with IL-2 cannot be directly compared with NARA1 but the structural analysis

Table 1. Data collection.

Cell Dimensions	Space group	P1 21 1
	a,b,c (Å)	79.33, 72.26, 210.93
	α,β,γ (°)	90.00, 92.71, 90.00
Resolution (Å)		50–2.5 (2.54–2.50)
Rmerge		0.158(1.146)
I/ σ		9.57(1.56)
Completeness(%)		96.3(92.4)
Redundancy		4.3(3.7)
Refinement	Resolution	29.79–2.49
	No. reflections	80389
	R _{work} /R _{free}	0.1809/0.2494
No. atoms	Protein	17348
	Ligand/ion	16925
	Water	-
β -factor	Protein	42.39
	Ligand/ion	-
	Water	33.89
R.m.s. deviations	Bond lengths(Å)	0.008
	Bond angles(°)	0.984
	Ramachandran favored(outliers) %	97.37(0)

clearly showed that both antibodies are effectively but partially covering the IL-2R α binding site on IL-2 and consequently blocks the IL-2:IL-2R α interaction. Furthermore, NARA1 or S4B6 binding to IL-2 increases the affinity to IL-2R β by inducing allosteric conformational change in IL-2.^{12,13}

Encouraged by a clear mechanistic basis of antibody-complexed IL-2, we also developed a new anti-human IL-2 antibody, TCB2.¹⁴ In a previous study, we showed that TCB2 has a strong binding affinity for hIL-2 ($K_D = 81.1$ pM) and has a half-life of about 48 hours in the serum much longer than hIL-2 alone. In mouse model experiments, the hIL-2:TCB2 complex induced an average eightfold increase in the ratio of memory phenotype CD8 to T_{reg} cells, and as a result, the hIL-2:TCB2 complex exhibits strong antitumor activity alone and is more potent when applied with immune checkpoint inhibitors such as anti-PD1 and anti-CTLA-4.¹⁴ Because of the amino acid sequence difference in complementary determining regions (CDRs) of TCB2 from NARA1, we expect that TCB2 may recognize a different epitope on hIL-2 surface, but still can block the IL-2R α interaction effectively. In this study, we determined the crystal structure of the hIL-2:TCB2-Fab complex at 2.5 Å. The hIL-2:TCB2 complex structure explains the detailed molecular interaction between TCB2 and hIL-2 and identifies a new epitope on hIL-2, which is overlapped with a central area of the IL-2R α binding region.

Results

Crystal structure of the hIL-2:TCB2-Fab complex

In a previous study, we developed a new anti-hIL-2 monoclonal antibody named TCB2, which can selectively stimulate CD8⁺ T or NK cells by blocking the interaction between hIL-2 and IL-2R α , and hIL-2:TCB2 complex showed a synergistic

effect on anti-tumor therapeutic response when administered in combination with the immune checkpoint inhibitors such as anti-CTLA-4 or anti-PD1 antibodies in mouse tumor model.¹⁴ To understand the structural and mechanistic properties of TCB2, we determined the crystal structure of hIL-2 in complex with the antigen-binding fragment (Fab) of TCB2. hIL-2 was expressed as inclusion bodies in *Escherichia coli* (*E.coli*) without signal sequence and purified using refolding procedure as previously described.¹⁵ The full-length TCB2 antibody was expressed from hybridoma cells and the Fab domain was prepared by treating papain protease followed by size exclusion purification (details in Method). Size exclusion analysis showed that hIL-2 and Fab of TCB2 formed stable 1:1 complex in solution (Supplementary Figure 1a), corroborating previously determined low dissociation constant ($K_D = 8.11 \times 10^{-11}$ M) measured by SPR experiment.¹⁴ After making complex, hIL-2:TCB2-Fab was stable for more than a week at room temperature (Supplementary Figure 1a) and complex crystals were showed up in 3 days and grown to full size in two weeks. For molecular replacement (MR), the crystal structures of hIL-2 (PDB ID: 3INK) and NARA1-Fab (PDB ID: 5LQB) without CDR loops were used as a search model. After finding MR solution, the electron density of CDR regions of TCB2 was readily traceable. The atomic model structure of hIL-2:TCB2-Fab was determined to a resolution of 2.5 Å and refined to a final R_{work}/R_{free} = 0.18/0.25 (Table 1). Total four hIL-2:TCB2-Fab complexes are found in the asymmetric unit of crystal. Superposition of four hIL-2s and variable regions of TCB2 in asymmetric unit results in C α root mean squared deviation (RMSD) of 0.44 Å and 0.38 Å respectively (Figure S2), indicating that four hIL-2 and TCB2-Fab structures are almost identical. Since there is no major difference between these four copies, the chain K of hIL-2 and the chain H and L of TCB2 Fab, which has the least non-traceable region, are used here as

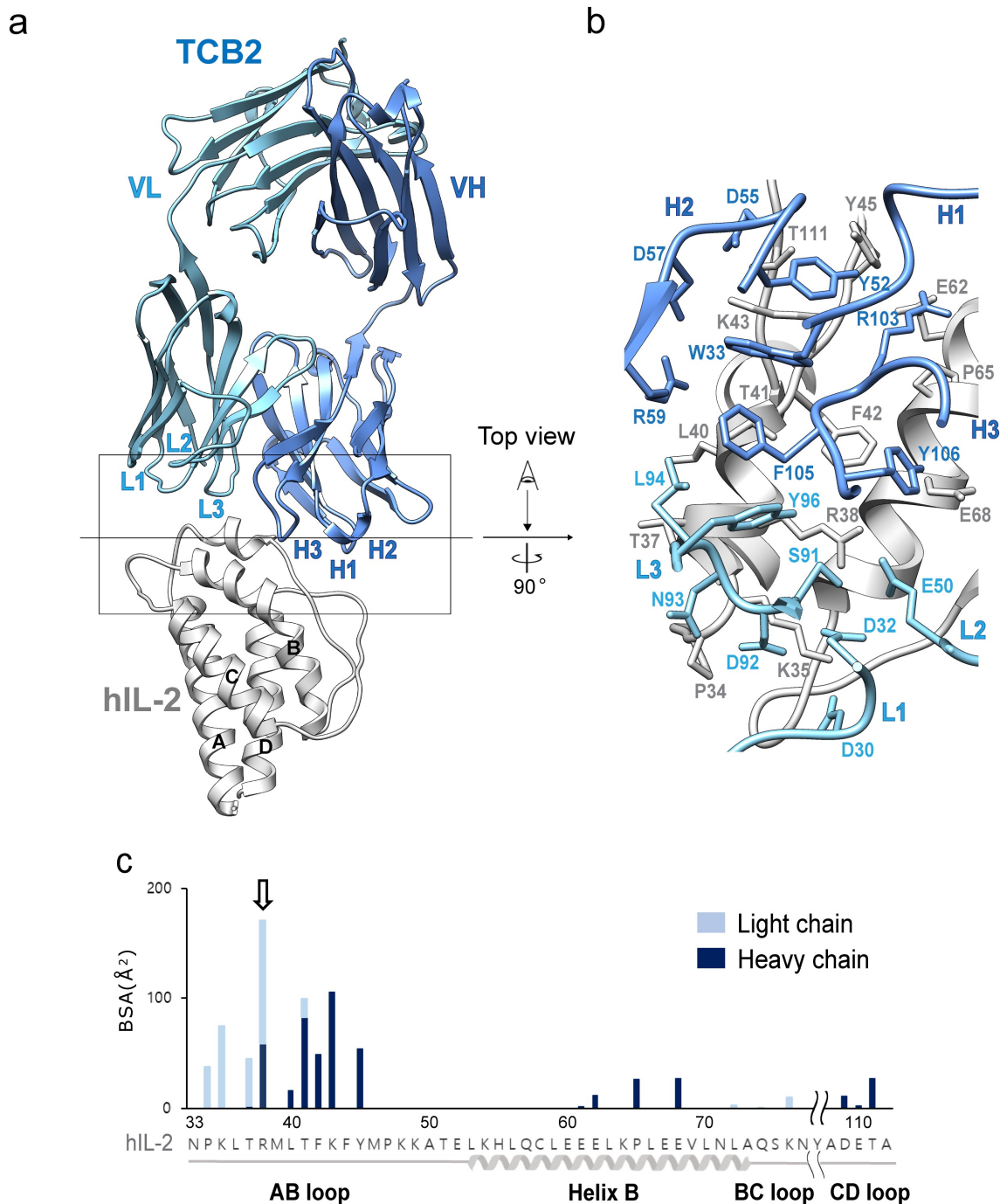


Figure 1. TCB2 interaction with human IL-2. **a.** Crystal structure of the human IL-2 and TCB2-Fab complex. The antigen-binding fragment (Fab) of the heavy chain and light chain are shown in blue and pale blue respectively. The human IL-2 is shown in white. The CDRs of TCB2 and α -helices of hIL-2 are labeled. **b.** Close-up view of the interface interaction between TCB2-Fab and human IL-2. Residues within 4 Å of each other are labeled and shown as ball-and-stick model. Secondary structure elements and CDRs from TCB2 are labeled. **c.** Epitope mapping of hIL-2. Residues of hIL-2 within 4 Å of TCB2 indicating direct inter-molecular contacts. The color-coded bar chart shows the BSA per human IL-2 residues contributed by heavy chain (blue) and light chain (pale blue) residues of TCB2 and is shown in a cumulative graph. R38 is labeled with an arrow.

the representative for illustration and discussion of structural features.

Molecular details of the interaction between hIL-2 and TCB2

The crystal structure of the hIL2:TCB2-Fab complex reveals that TCB2 binds on a slightly skewed top of IL-2 (Figure 1a), and

covered well the central part of IL-2R α binding sites (Figure 2b) as expected, explaining the steric blockade against IL-2R α binding without affecting IL-2R β or IL-2R γ -binding sites. The buried surface area (BSA) on hIL-2 by TCB2 spans about 800 Å², within the average value for antibody-antigen complexes.¹⁶ Close analysis of the binding interface between hIL-2 and TCB2 showed that the epitope of hIL-2 is mainly composed of residues from AB loop (P34, K35, T37, R38, L40, T41, F42, K43,

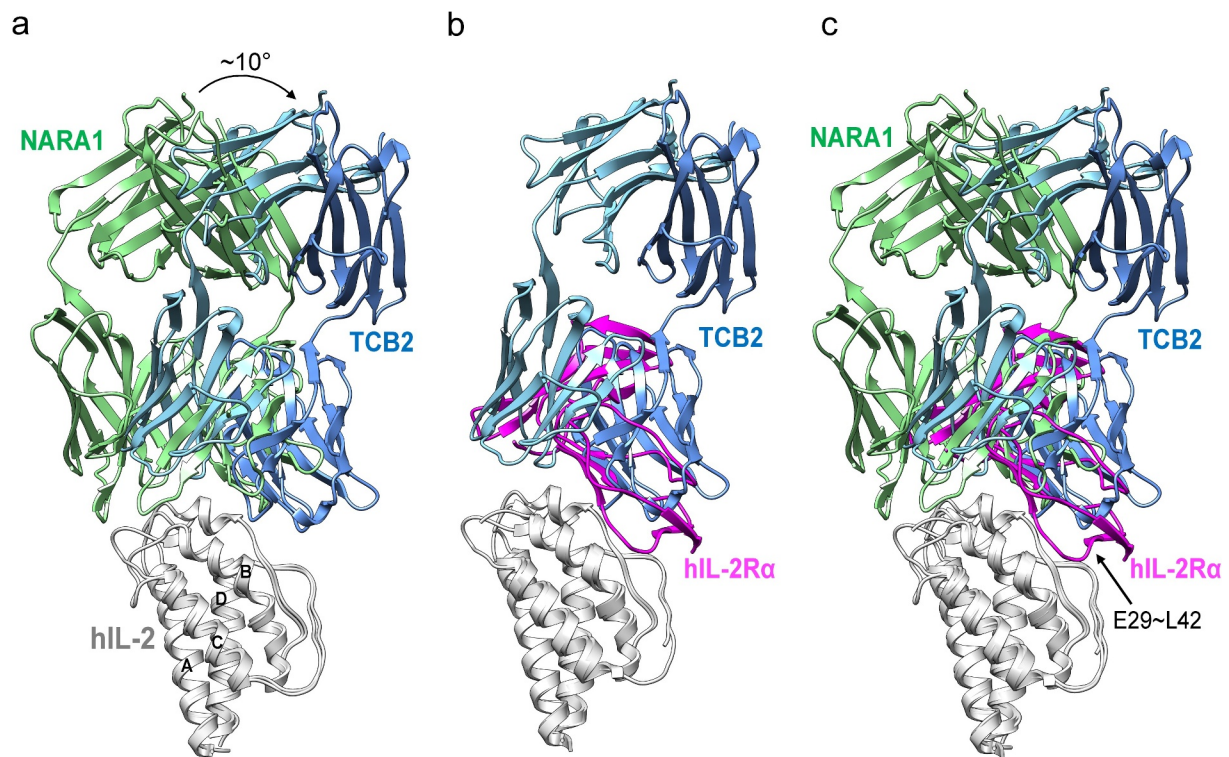


Figure 2. TCB2:hIL2 complex shows overlapped binding mode with NARA1 and IL-2Ra. a. Superposition of hIL-2:TCB2-Fab complex with hIL-2:NARA1 complex. For a clear comparison, all superpositions are done base on hIL-2. hIL-2s are shown in white color with an indication of α -helices a, b, c, and d. NARA1 is shown in green and TCB2 is colored as in Figure 1. b. Superposition of hIL-2:TCB2-Fab complex with hIL-2:hIL-2Ra complex. IL-2 Ra is shown in purple. c. Superposition of hIL-2:TCB2-Fab complex, hIL-2:hIL-2 Ra complex, and hIL-2:NARA1 complex. E29 ~ L42 loop of IL-2 Ra is indicated with arrow and this interaction is covered only by TCB2, not NARA1.

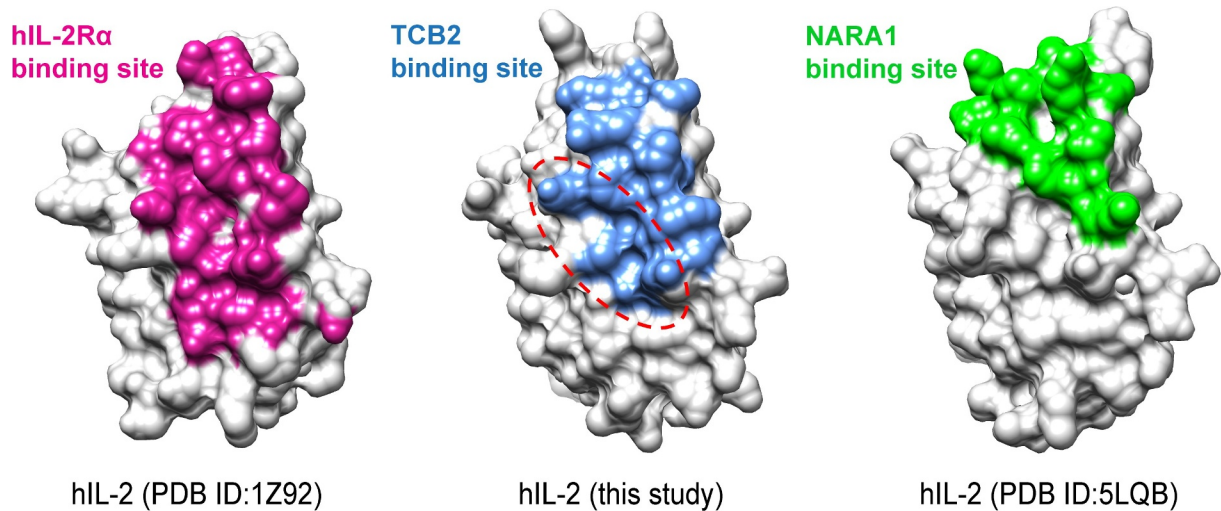


Figure 3. Analysis of binding epitopes on hIL-2. Surface representations of hIL-2 from hIL-2:hIL-2 Ra complex(left), hIL-2:TCB2-Fab (middle), and hIL-2:NARA1 complex(right). All hIL-2s are aligned in the same orientation for better comparison. hIL-2 Ra interacting area, TCB2 binding epitope, and NARA1 binding epitope are colored as violet, blue, and green respectively. TCB2-specific blockage for IL-2 Ra interaction is indicated by a red dotted circle.

Y45), helix B (E62, P65, E68, K76) and T111 from CD loop. These residues are located within 4 Å from the paratope of TCB2 (Figure 1b, 1c). Among the epitope on hIL-2, R38 has a central role in recognition by TCB2. About 144 Å² of accessible surface area from R38 buried after binding of TCB2. F105 from CDR3 of the heavy chain and D32, E50, Y96 from the light chain are involved in recognition of R38 of hIL-2 (Figure 1b). It is noteworthy that R38 and its neighboring residues of hIL-2 are also important for IL-2Ra interaction as well as NARA1

antibody recognition.^{2,12} Interestingly, even with differences in degree, all 6 CDR loops from the heavy and light chains of TCB2 participated in recognition of hIL-2 (Figure 1b), with heavy and light chains contributing 60.7% and 39.3% of total BSA respectively. From the viewpoint of TCB2's paratope, the most prominent interaction is provided by R103 from CDR3 of the heavy chain. The R103 is accommodated nicely into the cleft formed by Helix B (P65) and AB loop (F42, K43, and Y45) of hIL-2. Besides, R103 is further stabilized by the salt bridge

between the guanidine group of R103 and the γ -carboxyl group of E62 from the B helix of hIL-2 (Figure S3).

TCB2 blocks the IL-2Ra binding site of hIL2 in a different way from NARA1 or S4B6

To understand the distinct binding mode of TCB2, we compared the hIL-2:TCB2 complex structure with hIL-2:IL-2Ra and hIL-2:NARA1 complex. In the structure of the hIL-2:NARA1 complex, mainly CDRs from the heavy chain of NARA1 occupied the IL-2Ra binding site of hIL-2.¹² As expected from the difference in CDR sequences,¹⁴ TCB2 recognizes different but overlapped epitopes with NARA1, on the surface of hIL-2, about 55% of amino acids recognized by TCB2 are also recognized by NARA1. Superposition of complex structures showed that NARA1 binds hIL-2 “on top” position, while TCB2 recognizes slightly downshifted area of hIL-2, as a result, the binding angle is about 10 degrees different (Figure 2a). Because of this shift, TCB2 can cover the central part of IL-2Ra interaction area on hIL-2 more than NARA1. Especially, the extended loop composed of Glu29~Leu42 from N-terminal D1 domain of IL-2Ra interacts with AB loop of hIL-2¹⁷ and TCB2 can partially cover this area while NARA1 leaves this area of IL-2Ra accessible (Figures 2c and 3). Next, we also compared the hIL-2:TCB2 structure with mouse IL-2:S4B6 complex (Figure S2). Given the difference in amino acid sequence between human and mouse IL-2, each structure cannot be compared exactly. However, superposition based on sequence homology showed that S4B6 mainly recognized the “low-side” part of mouse IL-2, while TCB2 binding

epitope is partially overlapped with the upper part of the S4B6 binding epitope. As a result, TCB2 binding epitope on hIL-2 is located in between that of NARA1 and S4B6, therefore the three antibodies recognized the somewhat overlapped but distinctively different epitopes on IL-2, and epitopes of all three antibodies are overlapped partially with IL-2Ra binding site, which leads to structural blockage of IL2: IL-2Ra interaction.

TCB2 induces allosteric effects on IL-2 to increase IL-2R β affinity

The structure of the IL-2:IL-2Ra complex illuminates the subtle but obvious allosteric effects on IL-2 structure, which is induced by IL-2Ra binding, has an important role in IL-2R β interaction.^{2,17} One of the main structural changes in IL-2 after IL-2Ra binding is a small shift of the C-helix to the helical core, especially around Asp84 residue (Figure 4a). This allosteric conformational change makes IL-2 a more favorable form for IL-2R β . Interestingly, this allosteric effect was also observed in NARA1 complexed hIL-2,¹² S4B6 complexed mouse IL-2,¹³ as well as an activated variant of IL-2, “Superkine”.⁸ The close examination after superimposing TCB2-bound hIL-2 structure with hIL-2 alone or IL-2Ra-bound hIL-2 clearly showed that TCB2 binding also induced the allosteric effect on the C-helix of hIL-2 (Figure 4a) and this subtle movement was detected from all four TCB2-bound hIL-2 protomers in the crystallographic asymmetric unit. Therefore, TCB2 not only engages the steric blockade for IL-2Ra binding but also imposes an allosteric effect on hIL-2, which prompts IL-2R β preference of TCB2-bound hIL-2 and ultimately results in a ternary complex

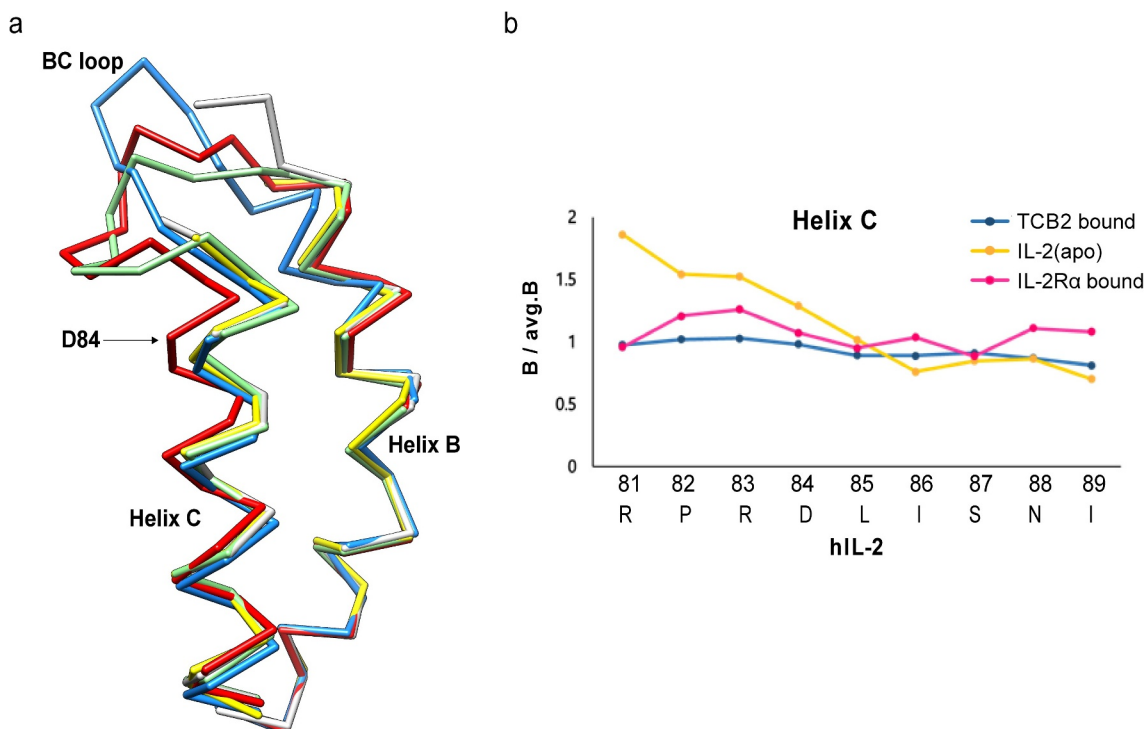


Figure 4. TCB2 induced allosteric effect on Helix C of hIL-2. a. Superposition of Helix B and Helix A from unliganded hIL-2 (Red, PDB ID:1M47), TCB2-bound hIL-2 (Blue, This study), IL-2 Ra-bound hIL-2 (Yellow, PDB ID:1Z92), and IL-2 R(α,β,γ)-bound hIL-2 (Gray, PDB ID:2B5I). Ca position of D84 was labeled. b. Crystallographic B-factor analysis in B and C helix of hIL-2. Average B factors of the main chain atom of helix C relative to the other part of the molecule are calculated. Flexible BC loop and elevated B-factors in Helix C observed in hIL-2 alone crystal structure (Yellow, PDB ID:1M47). After IL-2 Ra binding (Pink, PDB ID: 1Z92) or TCB2 binding (Blue).

of hIL-2:TCB2 and IL-2R($\beta + \gamma$). The conformational flexibility in the C-helix of hIL-2 comes along with higher crystallographic B-factors than the average value, and becomes much lower after IL-2Ra binding.¹⁷ TCB2-complexed IL-2 showed a lower B factor of C-helix than hIL-2 alone and the degree of B-factor stabilization is comparable to IL-2Ra binding, corroborating allosteric stabilization of C-helix upon TCB2 binding (Figure 4b). We also detected that the BC loop of IL2 in the IL-2:TCB2 complex is well defined and has more extended conformation even without the direct interaction with TCB2 or neighboring molecules in crystal packing, while the BC loop (of IL-2) is untraceable in the IL-2:IL-2R($\alpha + \beta + \gamma$) complex or IL-2:IL-2Ra complex structure (Figure 4a).

Discussion

A recombinant hIL-2 (aldesleukin) was approved by FDA in 1992 as the first immunocytokine drug to treat metastatic renal carcinoma and malignant melanoma.⁵ But hIL-2 has an extremely short half-life in serum and its pleiotropic function leads to cytotoxicity and also activates the unwanted immunosuppressive T_{reg} cells. To overcome the limitations of hIL-2 as therapeutics, using antibody-complexed hIL-2 showed quite promising results to improve pharmacokinetic values as well as to modulate hIL-2 specificity. Anti-IL-2 therapeutic antibodies can be divided into two groups base on purpose.¹¹ The first group of antibodies is used to reinforce the efficiency of IL-2 in cancer immunotherapy by specific activation of T_{eff} cells. NARA1, S4B6, and MAB602 belong to this category. The structural analysis explained that NARA1 and S4B6 block the IL-2Ra binding epitope on human IL-2 and mouse IL-2 respectively. The second group of antibodies is used to amplify the immune suppressive function of IL-2 by activating T_{reg} cells.¹⁸ JES6-1,¹³ UFKA-20,¹⁹ and 5344¹⁸ (anti-hIL-2 antibody, BD biosciences) are good examples. JES6-1 antibody blocks IL-2R β binding site and left IL-2Ra binding site as accessible, therefore JES6-1 antibody complexed mouse IL-2 can bind to IL-2Ra on immunosuppressive T cells, and then JES6-1 antibody is released from IL-2 to allow IL-2 interaction with IL-2R β and IL-2R γ for activation of IL-2 signaling. UFKA-20 binds hIL-2 and interferes with IL-2R β binding primarily as well as with IL-2Ra binding to a lesser extent. Both groups of IL-2 specific monoclonal antibodies can increase the serum half-life of IL-2 up to 2 weeks by reducing the size-dependent protein filtration in the kidney and by neonatal receptor (FcRn)-dependent antibody recycling mechanism.¹⁸

To utilize and maximize the benefits of antibody complexed IL-2 therapy, we developed a new anti-hIL-2 monoclonal antibody, TCB2 in a previous study¹⁴ and confirmed that hIL-2:TCB2 complex administration in mouse tumor model can successfully stimulate CD8⁺ T and NK cells rather than immunosuppressive T_{reg} by virtue of characteristic properties of preferential binding to dimeric IL-2R($\beta + \gamma$) receptor. Especially, when combined with a checkpoint inhibitor, the hIL-2:TCB2 complex showed a promising effect on tumor removal in a mouse model. In this study, we determined the crystal structure of the hIL-2:TCB2-Fab complex and explained that TCB2 binds to a new epitope, which is located between NARA1 and S4B6's epitope on IL-2 and covered the central

part of IL-2Ra binding site relatively more than other antibodies without hindering any of IL-2R β or IL-2R γ binding. And we also identified that TCB2 binding maintained the allosteric modulation of hIL-2 to increase the affinity for IL-2R β .

When the complex of IL-2 and IL-2Ra-mimicking antibody is used for cancer immunotherapy, the main shortcoming is unexpected activation of immunosuppressive T_{reg} cell proliferation even with a relatively low efficiency as compared to its effect on T_{eff} cells. There are several possible explanations for this phenomenon. First, IL-2 could be released from antibody complex formed in the serum. Second, partially exposed areas on the IL-2:antibody complex can have weak or transient interaction with IL-2Ra and results in antibody substitution with IL-2Ra on T_{reg} cells. The IL-2Ra binding area on IL-2 is about 893 Å,¹⁷ which is similar to the typical antibody-binding area. But there are still differences between the IL-2Ra binding region on hIL-2 and epitopes of IL-2Ra mimicking antibodies. In this regard, the extracellular domain (ECD) of IL-2Ra itself will be the best antagonistic candidate to block the endogenous IL-2Ra binding. Natacha *et al.* tested this hypothesis by making IL-2:ECD of IL-2Ra fusion protein.²⁰ However, this fusion protein preferentially activates the proliferation of T_{reg} cells rather than T_{eff}. The author explained that IL-2:IL-2Ra-ECD fusion protein formed face to tail dimerization and slow dissociation of the dimeric complex acts like low-dose IL-2 injection, which is often used for T_{reg} stimulation. This result implies that when making fusion protein of IL-2 and IL-2Ra blocker, an appropriate three-dimensional configuration should also be considered to maintain IL-2 specificity. Another group produced NARA1leukin, which is the fusion protein between hIL-2 and NARA1. In this case, they inserted hIL-2 in the frame of light chain CDR1 of NARA1 instead of making N – to C – terminus fusion between two proteins. NARA1leukin conserved interaction between hIL-2 and NARA1 and showed successful activation of CD8 T cell and NK cell over Treg cells.²¹

To cover the larger area of IL-2Ra binding region on IL-2 by a monoclonal antibody, CDR improvement of NARA1, S4B6 or TCB2 can be tried. The binding affinity of these antibodies for IL-2 is already high enough, therefore improvement of antibody could be focused on increasing the area of epitope and matching the IL-2Ra binding region even with a small compensation of affinity. Since all six CDRs of TCB2 are involved in interaction and interacting epitope is close to the center of IL-2Ra binding region rather than NARA1 and S4B6, TCB2 is a good candidate for starting model for improvement of antibody. For this purpose, a structure-based CDR design could be employed. Otherwise, using two different antibodies which recognize a non-overlapped region of IL-2Ra binding site could be next choice.

Our study provides detailed molecular information on a new way to harness pleiotropic hIL-2 activity by TCB2 and this information could be used for better IL-2Ra blocker design in the forms of antibody or a with new platform. Given the clear mechanistic role of TCB2 described by our structural studies and the fact that humanized TCB2 maintains an adequate binding affinity for hIL-2 in a previous study, it is worth

testing the hIL2:TCB2 complex for further clinical trials in patients with various types of cancer.

Methods

Protein expression and purification (hIL-2 purification)

Full-length TCB2 was purified as previously¹⁴ with modification. Hybridoma cells are adopted in suspension culture with Hybridoma medium (WELGENE) supplemented with 1% fetal bovine serum (FBS) and 1 mM Glutamine. TCB2 antibody from expression medium purified using Hitrap protein G column (GE Healthcare). The Fab fragments of TCB2 are generated by 4 hours incubation with papain and further purified with Hitrap protein G column followed by size exclusion chromatography (HiLoad S75, GE Healthcare). All purification steps were performed in 4°C unless specified. hIL-2 was purified by the refolding procedure as described previously.¹⁵ Briefly, hIL-2 (amino acid 1 ~ 133 without signal sequence) was overexpressed in *E. coli* as inclusion bodies and solubilized using 8 M Guanidine Hydrochloride (GuHCl) as a denaturing reagent. Denatured IL-2 was mixed with refolding buffer containing 1.1 M GuHCl, 110 mM Tris-HCl (pH 8.8), 6.5 mM Cysteamine, and 0.65 mM Cystamine and then stirred overnight at room temperature. After removing aggregation by spin down, the supernatant was dialyzed against buffer containing 10 mM Ammonium acetate (pH 6.0) and 25 mM NaCl for overnight. After refolding process, hIL-2 was further purified with Hitrap Q column (GE Healthcare) followed by size exclusion chromatography (HiLoad S75, GE Healthcare).

Crystallization and data collection

Purified TCB2-Fab fragments and hIL-2 were mixed at a ratio of 1:1.2 and incubated for 10 min at room temperature, and TCB2-Fab/hIL-2 complex was purified by size exclusion chromatography (HiLoad 75, GE Healthcare). For crystallization, purified TCB2-Fab:hIL-2 complex was concentrated to 12 mg mL⁻¹ in a buffer containing 20 mM HEPES (pH 7.5), and 300 mM KCl and mixed with equal volumes of reservoir solution containing 0.1 M MES (pH 4.8), 0.2 M Ammonium nitrate, and 25% PEG 3,350. Crystals were grown by the hanging drop vapor diffusion method at 20°C for two weeks. Before data collection, crystals were briefly cryoprotected in reservoir solution supplemented with 15% Ethylene glycol and rapidly frozen in liquid nitrogen. The diffraction data were collected at the PLS-II (Pohang Accelerator Laboratory, Pohang, Korea), beamline 11 C.

Structure determination and refinement

The diffraction data were processed and scaled using HKL2000 software package.²² The structure of the TCB2-Fab:hIL-2 complex was solved by molecular replacement with program PhaserMR²³ using a structure of hIL-2 (PDB ID: 3INK) and anti-hIL-2 antibody NARA1 (PDB ID: 5LQB) as a search model. The initial electron density map corresponding to four copies of the TCB2-Fab:hIL-2 was traceable

for CDR regions. Model building and refinement were carried out using Coot and PHENIX.^{24,25} Statistics for data collection and refinement are described in Table 1. Ramachandran plot analysis was performed with PROCHECK and the buried solvent accessible area was analyzed by ePISA.²⁶ All structural figures were generated using Chimera.²⁷ The coordinates and structure factors of TCB2-Fab:hIL-2 complex structure have been deposited in Protein Data Bank (PDB, <http://www.rcsb.org>) (PDB ID: 7DR4).

Data availability

The crystal structure of the hIL-2:TCB2-Fab complex from this study is available in the PDB with the accession code: 7DR4.

Acknowledgments

We thank Sin-Hyeog Im, and Yun-Sil Lee for helpful discussions. This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation (NRF) & funded by the Korean government (MSIT) (Grant NRF-2019M3E5D6066058), and 4th BK21 program funded by the Ministry of Education, Korean government.

Author contributions

J.K. performed the overall experiments. J.K., J.-Y.L, Y.J.L, and M.-S.K. conceived ideas and experimental design. S.-Y.P helped x-ray diffraction data analysis. All authors analyzed data. J.K. and M.-S.K. wrote the manuscript.

Disclosure of potential conflict of interest

J.Y.L works for Selexcine. Other authors declare no competing interests.

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