



Research Article

Simulation-Based Engineering of Humanized Scfv Antibody against hTNF- α

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Abstract

Background: Uncontrolled activity of tumor necrosis factor alpha (TNF- α) as pro-inflammatory cytokine has been linked with pathogenesis of autoimmune/inflammatory diseases. Therefore, modulating of TNF- α associated biological pathways is a promising strategy for alleviating of such diseases. In view of this, the use of antibody fragments such as single-chain variable fragments (scFv) in therapeutic applications has been gained much attention in terms of pharmacokinetic as well as production and therapeutic costs.

Methods: In the current investigation, the previously designed and humanized hD2 antibody was modeled and docked onto the TNF- α structure. The binding free energy was predicted for the complex of hD2-TNF- α using molecular dynamics calculation followed by per-residue energy decomposition for residues of hD2. In addition *in silico* mutations of important amino acids at the binding site of enzyme were performed and the binding free energy was calculated for mutant forms of scFv in complex with TNF- α .

Results: The analyses of the results proposed Y27F mutation in heavy chain CDR1 of hD2 scFv antibody may be considered as a promising substitution.

Conclusion: The results may be used for designing new anti-TNF- α antibody with improved activity.

Introduction

Tumor necrosis factor alpha (TNF- α) is a pleiotropic cytokine mainly secreted by stimulated macrophages to activate controlling systems involved in cell proliferation, differentiation, inflammation, death and immune regulation.^{1,2} The 17 kDa mature form of TNF- α is produced by proteolytic activity of TNF α -converting enzyme (TACE) on a 27kDa precursor protein. The biological functions of TNF- α are mediated via two distinct cell membrane receptors namely TNFR1 and TNFR2.³ Although normal serum level of TNF- α is very important to modulate physiological functions, its elevated level has been implicated in the pathogenesis of chronic inflammatory, autoimmune, infectious, and Alzheimer's diseases.^{1,2,4-7} Therefore, targeting TNF- α is an effective therapeutic strategy in control and treatment of such diseases. One of the effective strategies for targeting TNF- α is the use of monoclonal antibodies such as infliximab (Remicade)⁸, adalimumab (Humira)⁹, and golimumab (Simponi).⁸ In spite of high selectivity of full antibodies towards targets of interest, the associated obstacles with these therapeutics are their undesired pharmacokinetics, insufficient tissue penetration, immunogenicity, low stability, and high production cost.^{8,9} Moreover, large proportion of

individuals demonstrates insufficient clinical responses to these therapeutic agents during the pharmacotherapy period.^{9,10} Antibody fragments have attracted much attention in drug design and discovery projects over the past decades. Single-chain variable fragments (scFvs) are engineered antibody fragments in which heavy (V_H) and light chains (V_L) of immunoglobulins are connected via a short peptide linker. The advantages associated with these types of antibody fragments are improved pharmacokinetic, low immunogenicity, and high affinity making them as interesting agents for the therapeutic and diagnostic purposes.¹¹⁻¹³ Since the biological function of molecules such as proteins originate from their molecular structure, atomic-scale structural studies are of great importance for better understanding of their mechanism of action. Experimental methods such as X-ray crystallography and NMR spectroscopy are used to solve three dimensional structure of macromolecules, however, these techniques are time consuming, laborious, and costly, preventing them to be used for simple and fast structural studies. In the absence of experimentally solved structure, molecular modeling approaches can be utilized as an alternative method for predicting the 3D structural models of

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biomacromolecules. The aim of this study was to model previously designed anti-TNF- α hD2-scFv antibody¹⁴ and investigate its possible interactions with TNF- α . Moreover, for the identification of important key residues of scFv involved in antigen-antibody interaction, *in silico* alanine screening was conducted. The result of current study may provide preliminary information required for rationally designing experimental procedures, such as site directed mutagenesis, for developing TNF- α antagonizing agents where inhibition of TNF- α is needed.

Materials and Methods

Molecular modeling studies

Sequence of murine single chain antibody (D2)¹⁵ with highest binding affinity toward TNF- α was humanized virtually and named hD2 scFv.¹⁴ BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) search engine was used to search protein databank (PDB)¹⁶ for finding suitable template for homology modeling of hD2 protein. The sequences of hD2 and the selected templates were aligned using Clustal-Omega web server accepting default parameters (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The obtained alignment was used to built initial three-dimensional model of hD2 using Swiss PDB viewer software (SPDBV, version 4.1, Swiss Institute of Bioinformatics, Lausanne, Switzerland). The initial model was submitted to Swiss-Model server to generate energy minimized model structure (<https://swissmodel.expasy.org/>).¹⁷ The quality of the obtained model was evaluated using PROCHECK¹⁸ and Molprobit¹⁹ from their web sites.

Molecular dynamics (MD) simulation and docking studies

Energy minimization (AMBER-ff99 force field) and MD simulation calculations on the structural models were carried out using the Assisted Model Building with Energy Refinement (AMBER) suite of programs (version 11) operating on a Linux-based cluster (32 processors).^{20,21} After generating coordinates files (i.e. *.prmtop and *.inpcrd) using *leap* module of AMBER, the total charge of the system was neutralized by adding appropriate number of counter ions. Then, using TIP3P water molecules, the system was solvated in a rectangular box with the buffering distances set to 12 Å in all directions. In the next step, the solvated system was energy minimized using *Sander* module of the AMBER (500 steps of steepest descent followed by 500 steps of conjugate gradient) with subsequent 50 ps heating step in which the temperature continuously increased from 0 to 300 °K. After heating step, a 50 ps density equilibration was performed followed by 500 ps of constant pressure equilibration at 300 °K with a time step of 2 fs. SHAKE algorithm was used for constraining all hydrogen-containing bonds. Final MD simulation was performed for 5 ns by setting the non-bonded interaction cutoff to 8.0 Å, and applying the Particle Mesh Ewald (PME) method for calculation of long-range electrostatic interactions. All calculations were done using periodic

boundary conditions where no constraint was applied to the molecules. The trajectory of the MD simulation was obtained by writing out the coordinates every 10 ps. The model with the lowest energy during simulation period was extracted for molecular docking calculations. ZDOCK program (version 3.0)²² installed on Linux based workstation was used for the rigid-body docking of the selected model structure for hD2 scFv antibody with the crystal structure of TNF- α (PDB ID: 1TNF). The best top ranked solution was selected for investigating the protein-protein interactions and calculating binding free energy.

Binding free energy calculations for hD2-TNF- α complex

Following the molecular docking, the complex of hD2-TNF- α was submitted for MD simulation for 1 ns according to the procedure described above. Then, snapshots were extracted in a time interval of 10 ps for entire simulation. The dielectric constant values for the interior of solute and the surrounding solvent were set to 1.0 and 80, respectively. The interaction energies were calculated and averaged over a series of the extracted equilibrated snapshots by excluding counter ions and water molecules using Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method^{21,23} implemented in AMBER package. Moreover, the energy contribution of the individual binding site residues to the overall binding free energy was estimated using per-residue energy decomposition calculations embedded in MM-GBSA approach.

Binding free energy (ΔG_{bind}) is calculated as follows:

$$\Delta G_{\text{bind}} = G_{\text{water}(\text{complex})} - G_{\text{water}(\text{protein})} - G_{\text{water}(\text{ligand})}$$

In this equation, $G_{\text{water}(\text{complex})}$, $G_{\text{water}(\text{protein})}$, and $G_{\text{water}(\text{ligand})}$ refer to the free energies of the complex, protein, and ligand, respectively. Free energy, ΔG , for each species is calculated using the following equation:

$$G = E_{\text{gas}} + \Delta G_{\text{solvation}} - TS$$

$$\Delta G_{\text{solvation}} = \Delta G_{\text{GB}} + \Delta G_{\text{non-polar}}$$

$$E_{\text{gas}} = E_{\text{int}} + E_{\text{vdw}} + E_{\text{elec}}$$

$$E_{\text{int}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{tors}}$$

where G demonstrates the calculated average free energy, and E_{gas} , the standard force-field energy, consists of internal energy (E_{int}) in the gas phase, non-covalent van der Waals (E_{vdw}) and electrostatic (E_{elec}) energies. Internal energy (E_{int}) is composed of E_{bond} , E_{angle} , and E_{tors} which are the potential energies due to deviation of the bonds, angle, and torsion angle from their equilibrium values, respectively. $\Delta G_{\text{solvation}}$ denotes the solvation-free energy calculated with a Generalized Born equation and non-polar free energy estimation with a surface area using the LCPO method.²⁴ TS shows the vibrational entropy term which is ignored in this study assuming similar contribution of the entropy for all complexes to the binding free energy.

In silico mutagenesis studies

In this investigation, for the identification of key amino acids involved in the interaction of hD2 with TNF- α , *in silico* alanine scanning mutagenesis was used. To this end, the important residues of hD2 scFv at the interface

EVQLLESGGGLVQPGGSLRLSCAASGYNFTSYWMHWVRQAPGKGLEWVSALFPGNSDITTYKEMLKGRF
 CDR1 CDR2
 TISRDNKNTLYLQMNLSRAEDTAVYYCRGDFGAMDYWGQGTLLVTVVSSGGGSGGGSGGGGSTDI
 CDR3 Linker peptide
 QMTQSPSSLSASVGRVTITCRASQSIGTSIHWYQQKPGKAPKLLIYYTSESISGGVPSRFSGSGSGT
 CDR1 CDR2
 DFTLTISSQPEDFATYYISQSYNWPTFTFGQGTKVEIKRAAA
 CDR3

Figure 1. Sequence of humanized anti-human TNF-α D2 scFv antibody. Complementarity determining regions (CDRs) are underlined and located in the heavy and light chain variable regions, respectively.

of hD2-TNF-α complex were mutated to alanine using Swiss PDB viewer (SPDBV, version 4.1, Swiss Institute of Bioinformatics, Lausanne, Switzerland). Consequently, MD simulation and binding free energy calculation was also conducted for mutant forms of hD2 in order to estimate the contribution of the mutated residues to the binding energy.

According to the substitution frequencies in CDR rejoins observed in multiple sequence alignment of hD2 with other human anti-TNF-α scFv antibodies¹⁴ few substitutions were considered for further *in silico* mutation and binding free energy evaluation.

Results and Discussion

Although full length antibodies are important class of biopharmaceutical agents for therapeutic applications, antibody fragments such as scFv antibodies have gained considerable attention not only in pharmacotherapy of several diseases but also in preparation of immunotoxins and therapeutic gene delivery owing to their desirable features.²⁵ In the current study, comparative molecular modeling was utilized to build structural model for hD2 scFv fragment antibody. The sequence of humanized version of anti-TNF-α scFv hD2 shown in Figure 1 was used in BLAST search against PDB database to identify 2GHW, 2UZI, and 2YBR as suitable templates for the modeling purpose. The initial models were built guided by the alignments obtained using Clustal-Omega web server (Figure 2). Then, the models were submitted to Swiss-Model server for further refinement. The models were compared with each other in terms of RMSD (Table 1), and the results revealed that the models based on 2GHW and 2UZI templates are structurally very similar judged based on low RMSD value of 1.42 Å. Moreover, the quality and geometrical evaluation of the generated models using PROCHECK and MolProbity showed that more than 96% of the backbone PHI and PSI angles in all models are in the allowed region., The model with the highest percentage of dihedral angles in the allowed region (model I) was selected for further investigation (Figure 3). The selected model for hD2 scFv antibody (model I) was subjected to molecular dynamics simulation for 5 ns. Analysis of the MD trajectory demonstrated that the model was stable throughout 5 ns simulation deduced from the stable pattern of potential energy plot shown in

Table 1. The RMSD between models of hD2-scFv based on different templates.

Model (Template structure)	Model I (2GHW)	Model II (2UZI)	Model III (2YBR)
Model I (2GHW)	-	1.42 Å	6.00 Å
Model II (2UZI)	1.42 Å	-	3.82 Å
Model III (2YBR)	6.00 Å	3.82 Å	-

Table 2. List of interactions between TNF-α and hD2 scFv proposed by docking study.

Type of interaction	hD2 scFv		
	Interacting residue in TNF-α	Interacting residue	Located in CDR loop
Hydrophobic interactions	Leu ²⁹	Trp ²²⁷	L3
	Arg ³²	Phe ¹⁰¹	H3
	Arg ³²	Trp ²²⁷	L3
	Ala ³³	Phe ¹⁰¹	H3
	Leu ³⁷	Thr ⁵⁹	H2
	Ala ³⁸	Ser ⁵⁶	H2
	Ala ⁸⁴	Phe ¹⁰¹	H3
	Val ⁹¹	Phe ¹⁰¹	H3
Hydrogen bonds	Gln ²¹	Trp ²²⁷	L3
	Asn ³⁰	Tyr ²⁷	H1
	Tyr ⁸⁷	Ser ²²⁴	L3
	Glu ¹⁴⁶	Asn ²²⁶	L3

Figure 4. In the next step, the structure with the lowest potential energy was extracted from the trajectory and used in molecular docking study. ZDOCK program was utilized for the docking of hD2 scFv onto TNF-α trimer in a rigid-body manner. For this purpose, only residues in the CDR regions of hD2 model structure were included in the docking calculation. Ultimately, 2000 rigid body docking orientations were generated and ranked based on the scoring function implemented in ZDOCK program. Then, the solution with the highest score was selected for further analysis.

The result of docking study was analyzed and the important interactions were identified as seen in Table 2 and Figure 5. It seems that hydrophobic contacts and hydrogen bonds play critical role in stabilization of scFv-TNF-α complex. Molecular dynamics simulation for the length of 1 ns was also conducted on scFv-TNF-α complex followed by calculation of binding free energy using MM-GBSA algorithm. Subsequently, the individual residue energy

a)

hD2	1	AMAEVQLLES	GGGLVQPGGS	LRLSCAASGY	NFTSYWMHWV	RQAPGKGLEW
2GHW	1	EVQLVQS	GGGVVQPGKS	LRLSCAASGF	AFSSYAMHWV	RQAPGKGLEW
		. * .	***. * .	***** .	*. ** ***	*****
hD2	51	VSALFPGNSD	TTYKEMLKGR	FTISRDN SKN	TLYLQMN SLR	AEDTAVYYCA
2GHW	48	VAVISYDGSN	KYYADSVKGR	FTISRDN SKN	TLYLQMN SLR	AEDTAVYYCA
		*. . . *	* . .***	*****	*****	*****
hD2	101	RGDFGAMDYW	GQGTLVTVSS	GGGSGGGGS	GGGGSTDIQM	TQSPSSLSAS
2GHW	98	RDRSYLDYW	GQGTLVTVSS	GG-----	-----SETTL	TQSPATLSLS
		* .***	*****	**	. . .	***.*** *
hD2	151	VGDRVITICR	ASQSIGTSIH	WYQQKPGKAP	KLLIYYTSES	ISGVPSRFSG
2GHW	147	PGERATLSCR	ASQSVRSNLA	WYQQKPGQAP	RPLIYDASTR	ATGIPDRFSG
		*. * *..**	****. . . .	***** **	. *** .*	.*. * ****
hD2	201	SGSGTDFTLT	ISSPQPEDFA	TYYSQQSYNW	PTFTFGQGTK	VEIKRAAA
2GHW	197	SGSGTDFTLT	ISRLEPEDFA	VYYCQQRSNW	PP-TFGQGTK	VEVKSGLVP
		*****	** .*****	** ** **	* *****	**.* .

b)

hD2	1	AMAEVQLLES	GGGLVQPGGS	LRLSCAASGY	NFTSYWMHWV	RQAPGKGLEW
2UZI	1	EVQLLES	GGGLVQPGGS	LRLSCAASGF	TFSTFSMNWV	RQAPGKGLEW
		*****	*****	***** .	*..* .**	*****
hD2	51	VSALFPGNSD	TTYKEMLKGR	FTISRDN SKN	TLYLQMN SLR	AEDTAVYYCA
2UZI	48	VSYISRTSKT	IYYADSVKGR	FTISRDN SKN	TLYLQMN SLR	AEDTAVYYCA
		** . . .	* . .***	*****	*****	*****
hD2	101	RGDFGAMDYW	GQGTLVTVSS	GGGSGGGGS	GGGGSTDIQM	TQSPSSLSAS
2UZI	98	RGRF--FDYW	GQGTLVTVS-	-----	-----IQM	TQSPSSLSAS
		** * .***	*****		***	*****
hD2	151	VGDRVITICR	ASQSIGTSIH	WYQQKPGKAP	KLLIYYTSES	ISGVPSRFSG
2UZI	17	VGDRVITICR	ASQSISSYLN	WYQQKPGEAP	KLLIYSASVL	QSGVPSRFSG
		*****	****. . . .	***** **	***** .*	*****
hD2	201	SGSGTDFTLT	ISSPQPEDFA	TYYSQQSYNW	PTFTFGQGTK	VEIKRAAA
2UZI	67	SGSGTDFTLT	ISSLQPEDFA	TYYCQQSVMI	P-MTFGQGTK	VE
		*****	** * *****	** ** *	* *****	**

c)

hD2	1	AMAEVQLLES	GGGLVQPGGS	LRLSCAASGY	NFTSYWMHWV	RQAPGKGLEW
2YBR	2	VQLVES	GGGLVQPGGS	LRLSCTGSGF	TFDNYAMHWL	RQVPGEGLW
		. **	**	*****.*** .	* . * *** .	** ** *
hD2	51	VSALFPGNSD	TTYKEMLKGR	FTISRDN SKN	TLYLQMN SLR	AEDTAVYYCA
2YBR	48	VSGISRSSGD	IDYADSVKGR	FTISRDDAKK	TLSLQMN SLR	AEDTAVYYCA
	 *	* . .*	*****.*** .	** *****	*****
hD2	101	RGDFGAMDYW	GQGTLVTVSS	GGGSGGGGS	GGGGSTDIQM	TQSPSSLSAS
2YBR	98	RGGVGSFDTW	GQGTMTVTVSS	-----GGGGS	-----EIVL	TQSPATLSVS
		** * . * *	***.*** **	*****	. * .	***.*** *
hD2	151	VGDRVITICR	ASQSIGTSIH	WYQQKPGKAP	KLLIYYTSES	ISGVPSRFSG
2YBR	147	PGERATLSCR	ASQSVRSYLA	WYQQKPGQAP	RLLFSDASNR	ATGIPARFTG
		*. * *..**	****. . . .	***** **	.** . * .	.*.***.*

Figure 2. Sequence alignment used for building homology model of hD2 scFv antibody based on (a) 2GHW, (b) 2UZI, and (c) 2YBR structures used as templates.

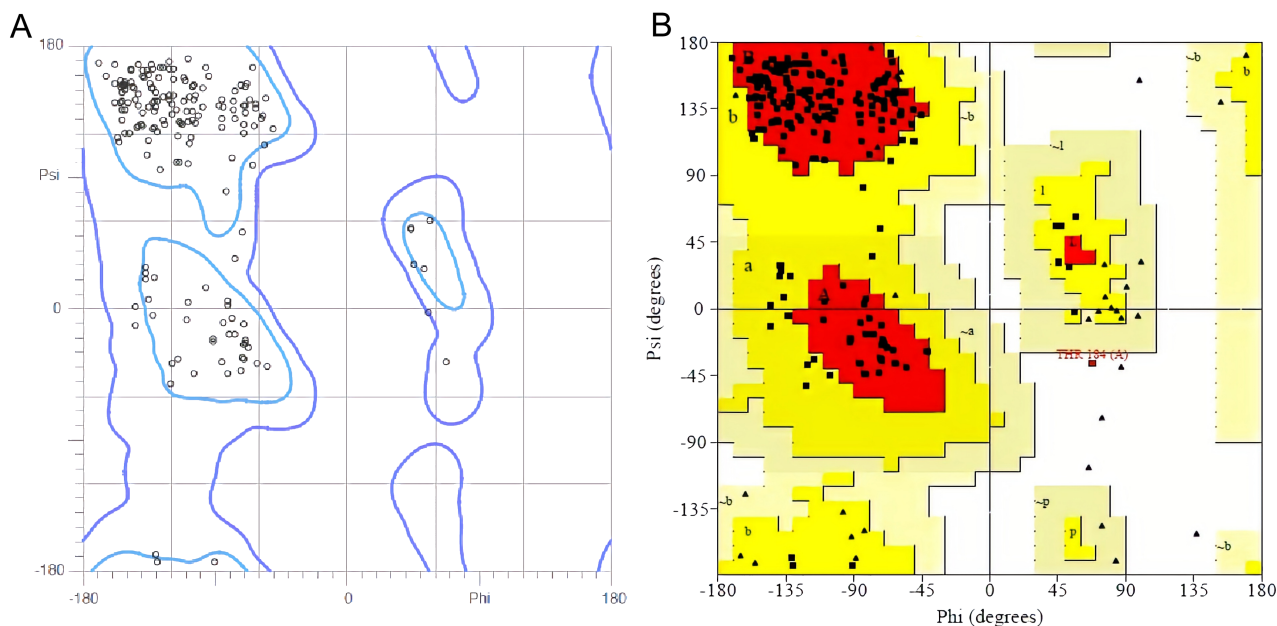


Figure 3. The Ramachandran plots for modeled hD2 scFv obtained from (A) Molprobdity and (B) PROCHECK model evaluation web servers. Both calculations showed that more than 96% of amino acids are in the allowed regions.

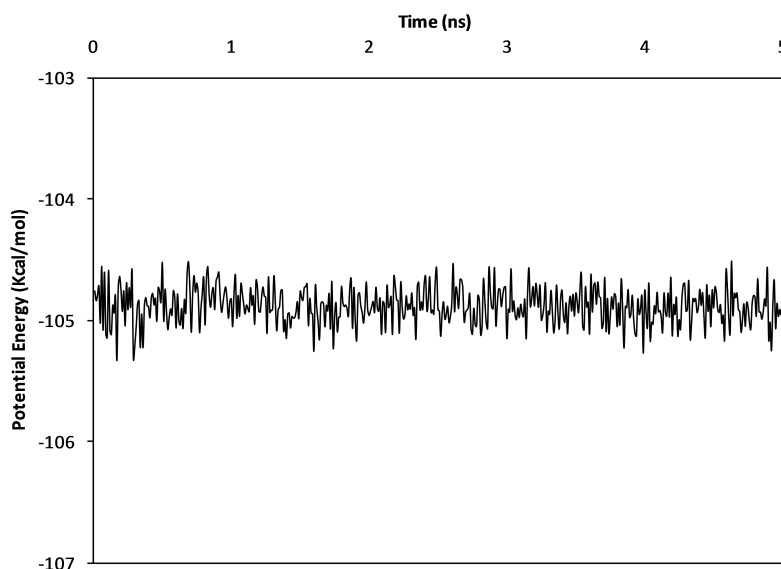


Figure 4. The result of molecular dynamics simulation analysis on the modeled hD2 scFv antibody. Plot shows potential energy for the modeled hD2 scFv antibody during 5 ns molecular dynamics simulation.

Table 3. Per-residue energy contribution for residues in CDR regions of hD2 scFv. The calculated binding free energy for hD2-TNF- α complex using MM-GBSA method is -28.04 kcal/mol.

Heavy Chain			Light Chain		
Amino acid (kcal/mol)	Contribution to BFE*		Amino acid (kcal/mol)	Contribution to BFE	
CDR1	Tyr ²⁷	-1.06	CDR1	-	-
	Asn ²⁸	-2.78			
	Tyr ³²	-3.36			
	Trp ³³	-5.12			
CDR2	Phe ⁵²	-7.18	CDR2	Tyr ¹⁸³	-3.91
	Phe ⁵³	-5.09		Thr ¹⁸⁴	-1.79
CDR3	Asp ¹⁰⁰	-1.11	CDR3	Tyr ²²⁵	-5.51
	Phe ¹⁰¹	-6.31		Trp ²²⁷	-4.73
	Ala ¹⁰³	-1.79		Phe ²³⁰	-1.24

*BFE: binding free energy

Table 4. Calculated binding free energies for the complex formed between hD2 (and its mutants) and TNF- α using Generalized-Born, $\Delta G_{(GB)}$, method based on 1 ns MD simulation.

Mutant	$\Delta G_{(GB)}$	$\Delta\Delta G_{bind(GB)}$
Y27A	-27.19	-12.72
N28A	-24.85	-3.19
Y32A	-21.61	-6.43
W33A	-20.35	-7.69
F52A	-21.37	-6.67
P53A	-26.45	-1.59
D100A	-17.28	-10.76
F101A	-19.43	-8.61
Y183A	-21.26	-6.78
T184A	-25.38	-2.66
T225A	-21.53	-6.51
W227A	-20.96	-7.08
F230A	-25.45	-2.59

$\Delta\Delta G$ values ($\Delta G_{wildtype} - \Delta G_{mutant}$) represent the relative binding energies for different mutants compared to wild type hD2.

contributions to the total binding free energy of the hD2 scFv in complex with TNF- α were calculated by decomposition module of MM-GBSA program. Table 3 provides the binding energy contribution for key residues in CDR regions. The identified amino acids with negative binding free energy values were subjected to *in silico* alanine scanning mutagenesis and the results shown in Table 4 indicated that all these residues play important role in stabilizing the formation of hD2-TNF- α complex.

To further narrow down the most promising residue substitutions in the process of rationally designing novel fragment anti-human TNF- α antibody, seven residues out of thirteen residues listed in Table 4 were investigated based on previously reported multiple sequence alignment of known anti-human TNF- α scFv antibodies.¹⁴ The mutations (Y27F, N28T, F52N, P53T, D100S, F101Y, W227Y) were suggested based on amino acid frequency of occurrences observed in the multiple sequence alignment.

The models built based on the mentioned mutations were underwent MD simulations to calculate binding free energy changes ($\Delta\Delta G_{bind(GB)}$) upon the performed *in silico* mutations. The analysis of the results (Table 5) showed that only Y27F mutation tend to improve the formation of complex between hD2 and TNF- α with a $\Delta\Delta G_{bind(GB)}$ of 12.72 Kcal/mol. The results obtained in this work can be useful for the design of novel humanized scFv antibody targeting TNF- α .

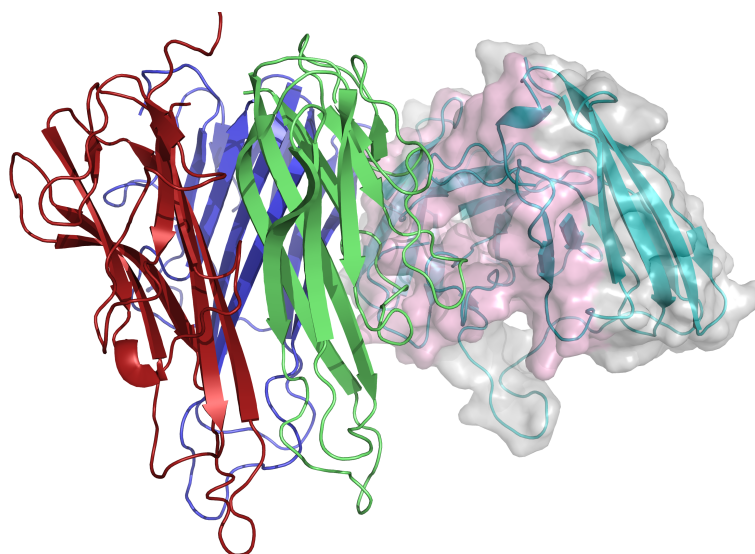
Table 5. Calculated binding free energies for the complex formed between hD2 (and its mutants) and TNF- α using Generalized-Born, $\Delta G_{(GB)}$, method for 200 ps MD simulation

Mutant	$\Delta G_{(GB)}$	$\Delta\Delta G_{bind(GB)}$
Y27F	-71.6	12.72
N28T	-51.36	-7.52
F52N	-51.02	-7.86
P53T	-48.37	-10.51
D100S	-55.92	-2.96
F101Y	-18.94	-39.94
W227Y	-59.52	0.64

$\Delta\Delta G$ values ($\Delta G_{wildtype} - \Delta G_{mutant}$) represent the relative binding energies for different mutants compared to wild type hD2.

Conclusion

In the current work, a model for anti-TNF- α scFv antibody (hD2-scFv) was generated and used for the docking with TNF- α structure. Then the complex was subjected to MD simulation with subsequent estimation of binding free energy. Moreover, per-residue energy decomposition calculations were conducted to estimate the individual binding site amino acids energy contributions to the total binding free energy. *In silico* mutation was also performed for determining the most promising substitution for the residues in the binding site of scFv. The analyses of the findings showed that Y27F mutation in CDR1 from heavy chain might be a suitable substitution for the designing

**Figure 5.** The Cartoon representation of docked hD2 onto the TNF- α structure generated in PyMol (version 1.7.5.0). The heavy and light chains of hD2 scFv have been shown in cyan while the corresponding CDRs are colored in pink. TNF- α chains have been illustrated in green, red, and blue.

of novel scFv antibody with enhanced binding affinity. However, experimental studies are necessary to verify the *in silico* results presented in the current work.

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Conflict of Interest

The authors declare no conflict of interest.

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