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Antibody Engineering Toward Enhancement of Teplizumab Anti-CD3 Binding Affinity in Type 1 Diabetes Prevention and Treatment

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Abstract

Objective: The monoclonal antibodies with CD3 target have the potential to change the progression of type 1 diabetes (T1D) and enhance the longevity of beta-cell function. The main objective of the study is antibody engineering toward Enhancement of Teplizumab Anti-CD3 Binding Affinity in T1D prevention and treatment.

Materials and Methods: We aimed at finding the important amino acids of this antibody, and then replaced these amino acids with others to improve their binding affinity, and examine the binding affinity of antibody variants to antigens. In the end, we selected high-affinity variants of the antibody according to results of High Ambiguity Driven biomolecular DOCKing (HADDOCK).

Results: Our research indicated that 14 mutated variants were able to enhance the binding characteristics of Ab in comparison to standard antibodies.

Conclusion: The altered antibodies could serve as promising options for enhanced affinity binding to antigens, which could affect the specificity and sensitivity of antibodies.

Keywords: Teplizumab, Antibody Engineering, Type 1 diabetes

QR Code:

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Introduction

Type 1 diabetes (T1D) is a long-term metabolic condition marked by the autoimmune destruction of insulin-secreting beta cells in the pancreas by T-cells (1-3). The occurrence of T1D has risen globally, experiencing an average annual growth of 3-4% over the past thirty years (4). CD4+ T cells assist CD8+ cytotoxic T cells, which are generally recognized as the primary effectors responsible for the destruction of human islet beta cells in T1D autoimmunity (5,6). In the initial phase (stage 1), T1D typically shows no symptoms, yet autoimmune activity is often identified early in life through the presence of autoantibodies that attack insulin or other related proteins. Once a significant portion of the beta cell mass has become impaired or lost, asymptomatic dysglycemia occurs (Stage 2), followed by noticeable symptoms of hyperglycemia (Stage 3) due to a lack of adequate insulin secretion. (7). Clinical symptoms typically arise after a significant portion of functional beta cell mass has been depleted (8), Individuals with T1D rely on external insulin and lifestyle modifications as their main form of treatment (9). Despite receiving intensive insulin treatment, people with T1Dface hazards of immediate complications like hypoglycemia and ketoacidosis, as well as long-term microvascular and macrovascular issues (10,11).

Therapies aimed at T cells are a significant focus in the early stages of T1D. Specifically, monoclonal antibodies that target CD3 have the potential to change the typical progression of T1D and promote a longer retention of beta-cell function (12-14).The discovery of hybridoma technology for production of murine monoclonal antibodies (mAbs) caused the success story of mAbs (15). Researchers illustrated that long-lasting remission from disease induced by that administration of anti-CD3 mAb to overt diabetic NOD (developing spontaneous autoimmune diabetes, non-obese diabetic) (16). Investigations into NOD mice, which naturally progress to autoimmune diabetes, provided us with insights regarding the therapeutic possibilities of anti-CD3 monoclonal antibodies (17,18).

One of the most important objects of antibody engineering method is to optimize the characteristics of the antigen-binding domains of antibody including increasing the affinity of the antibody to the targets (19,20). Antibody Engineering to increase the binding affinity of antibodies to antigens is important, because regardless of whether antibodies is developed by hybridoma, phage libraries or other technologies, there is a need to improve antibody affinity to its target (21).

Improved affinity enhances the biological activity of the antibody and thus improve treatment efficacy (22). Furthermore, the increased affinity of antibodies to its target reduces the amount of consumed antibody, resulting in lower toxicity and cost to consumers (23). In addition, the strength of an antibody depends on the antigen-antibody binding kinetics. Antibody affinity is the binding strength of a molecule to another in a certain position. A high-affinity antibody binds to its ligand more quickly or remains attached to its ligands for a longer time or show these both features simultaneously (24). These characteristics are determined by binding constant (K_a or K_{on}) and dissociation constant (K_{off} or K_d). Better binding is associated with improved clinical effects. Research has shown that increasing the antibody affinity is associated with their selective delivery to the tumor which increases the likelihood of their use in the treatment of tumors. Regarding diagnosis, the increased affinity reduces the amount of antigen needed to cause a response in immunoassay and increases test sensitivity. The use of antibodies and their fragments in research, diagnosis and treatment, has caused the increase in affinity improving methods, specificity of antibodies and a growing demand for efficient and effective technics to engineer protein variants in order to study biological and

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structural function as well as drugs manufacturing (25).

In the present study, we aimed at finding the important amino acids of Anti-cd3 antibody, then replaced these amino acids with others to improve their binding affinity, and examine the binding affinity of antibody variants to antigens. In the end, we selected high-affinity variants of the antibody according to results of High Ambiguity Driven biomolecular DOCKing (HADDOCK).

As a result considering the appropriate features of Anti-cd3 antibody in the treatment and prevention of diabetes (26-30), the aim of this research is to improve the diagnosis and treatment of diabetes using Anti-CD3 monoclonal antibody engineering.

Material and Methods

Prediction of complementarity-determining regions in Teplizumab

The primary function of the antibody's binding is facilitated by the complementarity-determining region (CDR). Paratome, available at http://ofranservices.biu.ac.il/site/services/paratome, forecasts the antigen-binding regions (ABRs) of an antibody using its amino acid sequence or three-dimensional structure. Paratome was created by systematically aligning a comprehensive, non-redundant collection of all known antibody-antigen complexes in the PDB, which led to the identification of structural consensus elements frequently engaged in antigen binding across various antibodies.

Teplizumab conservation of amino acid positions evolution

ConSurf available at https://consurf.tau.ac.il/ was employed to assess the evolutionary conservation of amino acid locations within a protein based on the phylogenetic relationships among homologous sequences. The extent to which an amino acid location is conserved over evolutionary time (i.e., its evolutionary rate) heavily depends on its structural and functional significance. Therefore, analyzing the conservation of positions within members of the same family can often highlight the importance of each position in relation to the protein's structure or function. In ConSurf, the evolutionary rate is calculated by examining the evolutionary connections between the protein and its homologues, while also taking into account the similarities between amino acids as detailed in the substitutions matrix.

Teplizumab Interfaces prediction

XGBoost-based Interface Prediction of Specific Partner Interactions (BIPSPI) at http://bipspi.cnb.csic.es/xgbPredApp/ was used for predicting protein interfaces that are specific to partners, utilizing PDB files or input sequences. BIPSPI utilizes Extreme Gradient Boosting (XGBoost) models, which have been trained on the residue pairs of protein complexes gathered in the Protein-Protein Docking Benchmark version 5, along with a scoring function that transforms pair predictions into interface residue predictions.

Teplizumab binding sites prediction

InterProSurf at http://strcomp.protein.osaka-u.ac.jp/ghecom/ was used to identify multi-scale cavities on protein surfaces through the use of mathematical morphology. InterProSurf was utilized to predict interaction sites on protein surfaces, while statistical analysis of physicochemical properties helped in anticipating protein-protein interfaces and identifying functionally critical amino acid residues.

Significant residues selection

Interfaces amino acids were selected as significant residues in the Teplizumab structure by employing the results of different software. These residues are located in one of three CDR regions predicted by the Paratome server.

SIFT analyses

The SIFT server, which is accessed at http://sift.jcvi.org/, predicts if an amino acid substitution would influence protein function. The conservation degree of residues in sequence alignments obtained from closely

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similar sequences acquired using PSI-BLAST is used to predict SIFT.

Teplizumab variants sketching

Twenty variants were created, including mutations in at least one of the three ABRs. The 3D structure of all offered variants is determined by SAbPred at http://opig.stats.ox.ac.uk/webapps/sabdab-sabpred/ Welcome SAbPred.php (The Oxford Protein Informatics Group (OPIG) created an antibody modeling and prediction software tool).

Antibody-Antigen docking

HADDOCK at http://haddock.science.uu.nl/services/HADDOCK/haddock.php uses the 3D structures of each variation and CD3 subunit as input. HADDOCK is a docking approach that is informed by data and allows flexibility in the modeling of biomolecular complexes.

Ethical considerations

As the present study involved no experimental with animals or human, hence there was no need for approval by the Ethics Committee.

Results

Teplizumab CDR prediction

Paratome is a browser server for identifying Antigen Binding Regions (ABRs) in antibodies. This server predicted three ABRs in the Teplizumab heavy chain and three ABRs in the Teplizumab light chain. These regions are YTFTRYTMH (27-35) as ABR1, WIGYINPSRGYTNY (47-60) and RYYDDHYCLDY (98-108) as ABR2 and ABR3 in Teplizumab heavy chain, and SSVSYMN (27-33), RWIYDTSKLAS (45-55), and QQWSSNPF (88-95) as ABR1, ABR2, and ABR3 in Teplizumab light chain. Paratome results are shown in Figure 1.

Paratome identifies three areas as ABRs within the heavy chain of Teplizumab and three areas as ABRs in the light chain of Teplizumab.

Teplizumab conservation of amino acid positions evolution

The ConSurf server was used to identify functional regions within proteins. The conservation scores, presented in nine colors, are mapped onto the sequences of both antibodies and antigens, with the colored protein structure visualized using FirstGlance in Jmol, as displayed in Figure 2

The conservation scores, represented in nine colors, are mapped onto the antibody's 3D structure, which is displayed through FirstGlance in Jmol.

Teplizumab Interfaces prediction

BIPSPI is a tool for predicting partner-specific protein-protein interfaces. Residues whose score has an expected precision greater than or equal to the precision threshold (0.500) are **:**H104, H102, H103, H105, H56, H101, H54, L47, L89, L30, L48, L212, L91, L90, L211, L92, L46, L29, L88, and L94.

These thresholds are useful to explore different expected precision/recall values.

Figure 1. Teplizumab CDR Prediction

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Teplizumab binding sites prediction

InterProSurf is a server for protein-protein interaction site prediction. The First Five cluster residues are 4, 107, 108, 109, 164, 99, 100, 101, 102, 103, 104, 150, 209, 130, 131, 132, 201, 223, 224, 52, 53, 54, 55, 56, 57, 93, 114. And the Next Five cluster residues are 10, 11, 12, 116, 5, 21, 23, 80, 17, 18, 19, 1, 2, 3, 25, 26, 27, 33, 50, 51, and 58 (Figure 3).

The first five clustered residues are illustrated in red (stick representation), while the next five clustered residues are depicted in green (stick representation)

Significant residues selection

We select L:30S, L:31Y, L:47I, L:48Y, L:49D, L:52K, L:88Q, L:89Q, L:90W, L:91S, L:92S, L:93N, and L:95F residues by employing the results of different software. These residues are located in one of three CDR regions predicted by the Paratome server. The specially selected residues were confirmed by at least four softwares. In this regard, BIPSPI, InterProSurf and ConSurf predicted residues for research to select the significant amino acids.

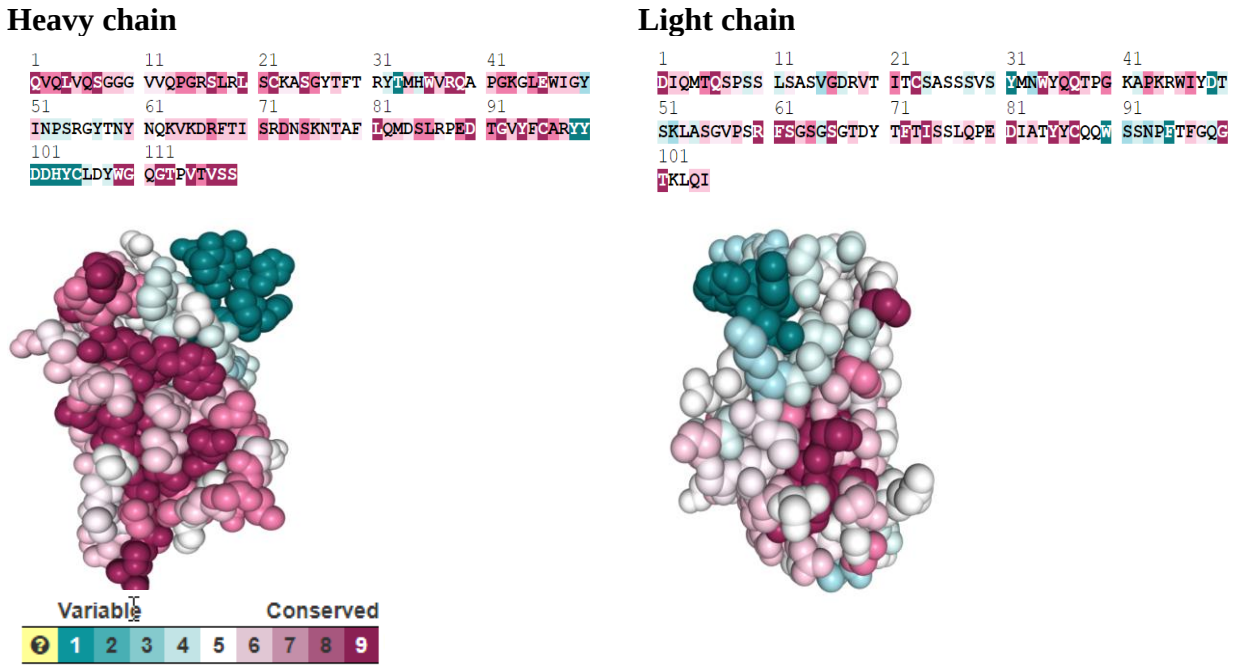

Figure 2. The conservation of amino acid positions in Teplizumab was analyzed using the ConSurf server

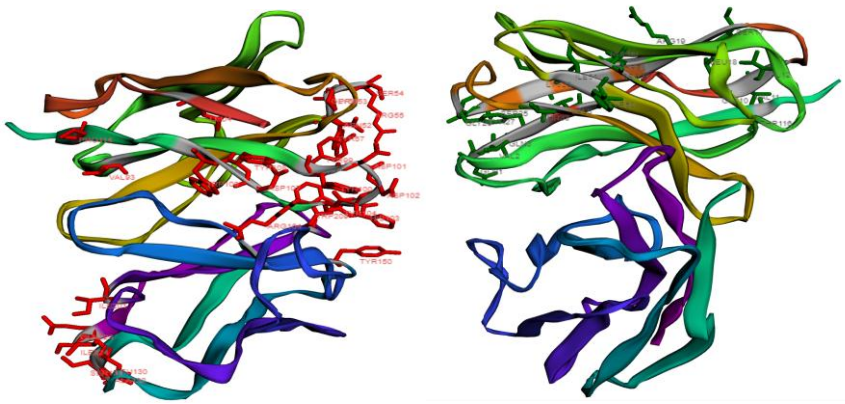

Figure 3. The results from InterProSurf display a Jmol representation of the pocket structure

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SIFT analyses

SIFT is a tool that analyzes sequence homology to differentiate between amino acid substitutions that are tolerant and those that are intolerant, predicting the potential phenotypic effects of these substitutions in a protein. The SIFT results for chosen residues are presented in Table 1.

Teplizumab variants sketching

20 variants including mutations in at least one of 3 ABRs offered. L:30S, L: 31Y, L: 47I, L: 48Y, L: 49D, L: 52K, L: 88Q, L: 89Q, L: 90W, L: 91S, L: 92S, L: 93N, L: 95F residues which were confirmed by different software mutated in suggested variants randomly. Mutated sequences are aligned and illustrated in Figure 4.

Table 1. SIFT results for selected residues

Predict not tolerated	Position	Seq rep	Predict tolerated
w c	30S	0.98	m f H i Y p V G R Q D T A N L K E S
c p m	31Y	0.99	W D k q E G R T I v A H L N F S Y
d h g n e c w s r k y q t a P F V L M	47I	1.00	I
c w m p d i n t v a l R E Q H K S G F	48Y	1.00	Y
c m W	49D	1.00	i p H V F Y T Q L R K A S G E N D
w f c m h p v g L I	52K	0.99	a Y D R Q S T N E K
w p i d n v f t H k y R C E	88Q	0.96	M G S L A Q
w c m f i y p r V N k E D H G	89Q	0.92	L T A S Q
p d m e k i v Q R	90W	0.94	N T C G A L H F S Y W
c p m W	91S	0.94	e Q I K R H G T V F L D N Y A S
w c p M I v F H K Q L D A	92S	0.94	Y G R T N E S
	93N	0.94	c M W Q E P K G D R I h A V T F L Y S N
c d m e n K Q	95F	0.89	G P T A H S I R W V Y L F

Amino acid color code: nonpolar, uncharged polar, basic, acidic. Uppercase letters signify residues present in the alignment, lowercase letters are the result of predictions.

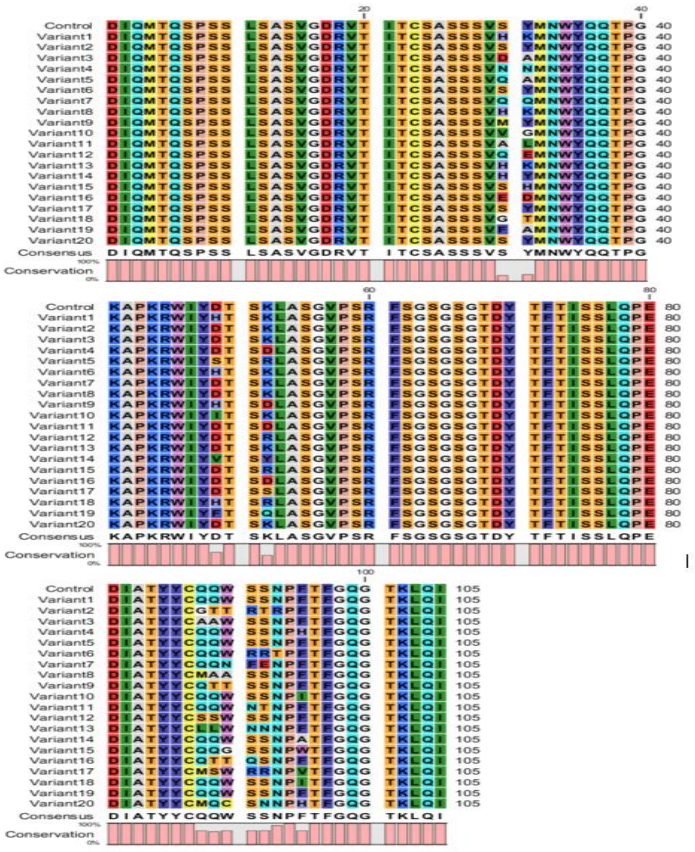

Figure 4. Illustration of mutated sequences alignment

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Antibody-Antigen docking

The HADDOCK (High Ambiguity Driven Biomolecular Docking) server evaluates the integration of ligands and receptors based on biochemical and/or biophysical data. Table 2 presents details on variants where the HADDOCK score exceeds that of the control. The arrangement of complex structures is determined by their HADDOCK scores. HADDOCK provides a completely flexible scoring system, allowing for the individual adjustment of the weight assigned to different energy terms for each stage of the docking process. The scoring is conducted using the weighted sum (HADDOCK score) of the specified terms:

Evdw, Eelec, Eair, Erg, Esani, Evean, Epcs, Edani, Ecdih, Esym, BSA, dEint and Edesol which stands for the following, respectively:

Van der Waals energy, electrostatic energy, distance restraints energy, radius of gyration restraint energy, radius of gyration restraint energy, direct RDC restraint energy, intervector projection angle restraints energy, pseudo contact shift restraint energy, diffusion anisotropy energy, dihedral angle restraints energy, symmetry restraints energy, buried surface area , binding energy and desolvation energy.

The HADDOCK scoring function consists of a linear combination of various energies and buried surface area.

The best-ranking structure will have the lowest weighted total. In this table, the Van der Waals and electrostatic energy values as well as the buried surface between two complexes are shown. The HADDOCK Score for all versions is shown in Figure 5.

Antibodies are significant tools for use in the research laboratories and clinics. The base notion of antibody's utility is its unique molecular structure by which bivalent binds to a broad range of antigenic epitopes (on,e.g, proteins, carbohydrates, and nucleic acids). The concept of a "magic bullet" was introduced based on antibodies use as research, diagnostic, and therapeutic reagents (23).

Monoclonal antibodies are used in various fields such as biotechnology, industry, treatment, biosensor design and straightforward research. In the last two decades, monoclonal antibodies have been recognized as one of the utmost important biological drugs (31). Teplizumab is an anti-CD3 monoclonal antibody (3,32-37), humanized which has been mutated to greatly decrease FC receptor and complement binding (38).

Table 2. Docking analysis of the standard Teplizumab human antibody variants and the five most effective mutated variants with the CD3 antigen

Variable	control	variant 1	variant 2	variant 4	variant 5	variant 6	variant 8	variant 9	variant 12	variant 13	variant 14	variant 15	variant 16	variant 17	variant 19
HADDOCK score	-113.0 (±9.2)	-125.4 (±12.5)	-122.2 (±10.8)	-126.2 (±8.0)	-141.9 (±3.0)	-122.5 (±4.2)	-131.6 (±7.4)	-143.2 (±13.5)	-122.6 (±8.9)	-136.5 (±7.6)	-125.6 (±24.2)	-127.8 (±2.1)	-141.8 (±9.7)	-117.2 (±11.7)	-119.2 (±5.8)
Cluster size	17	8	13	104	39	31	28	44	9	13	12	96	38	30	11
RMSD	0.7 (±0.4)	1.1 (±0.8)	1.0 (±0.6)	0.4 (±0.3)	0.4 (±0.3)	3.0 (±0.3)	1.6 (±1.3)	1.0 (±0.8)	0.5 (±0.3)	0.6 (± 0.4)	0.7 (±0.5)	3.5 (± 0.4)	1.0 (±0.7)	1.0 (±0.6)	0.8 (±0.5)
Van der Waals energy	-53.9 (±10.2)	-62.0 (±4.4)	-59.6 (±4.3)	-35.2 (±2.4)	-82.7 (±2.6)	-48.4 (±2.7)	-59.3 (±4.2)	-69.6 (±10.5)	-66.7 (±4.2)	-53.5 (±7.6)	-68.7 (±10.8)	-55.9 (±4.1)	-60.5 (±6.4)	-51.7 (±6.5)	-67.7 (±4.7)
Electrostatic energy	-391.3 (±14.4)	-300.8 (±62.2)	-522.9 (±40.4)	-494.6 (±15.7)	-338.4 (±56.8)	-508.2 (±12.5)	-471.2 (± 89.5)	-490.4 (±76.1)	-438.6 (±42.6)	-501.5 (±32.7)	-205.0 (±43.3)	-362.7 (±31.7)	-490.9 (±41.9)	-472.1 (±29.4)	-164.8 (±45.0)
Desolvation energy	3.7 (±12.9)	-13.0 (±6.5)	22.0 (± 14.7)	-6.5 (±6.6)	-14.2 (±11.2)	18.4 (±5.7)	8.5 (±10.5)	5.5 (±7.9)	19.4 (±7.2)	7.3 (±6.4)	-35.8 (±9.3)	-14.4 (±5.7)	-1.1 (±4.3)	8.5 (±5.7)	-40.8 (±5.7)
Restraints violation energy	154.2 (±5.84)	97.0 (±44.16)	199.7 (±39.37)	144.0 (±32.30)	226.6 (±6.85)	91.2 (±37.24)	133.9 (±89.13)	190.3 (±17.11)	124.8 (±31.54)	99.6 (±22.59)	199.0 (±75.09)	151.3 (±35.38)	179.9 (±32.54)	205.2 (±75.72)	222.9 (±56.45)
Buried Surface Area	2053.8 (±144.6)	2379.2 (±159.3)	2529.2 (±153.2)	1654.3 (± 45.5)	2540.0 (±125.8)	2030.3 (±77.3)	2259.2 (±180.0)	2502.1 (±192.3)	2676.5 (±99.0)	2287.6 (±193.3)	2310.9 (±203.7)	1978.6 (±189.2)	2362.0 (±110.7)	1920.6 (±130.8)	2317.5 (±72.7)
Z-Score	-1.3	-2	-1.8	-1.5	-1.8	-2	-1.5	-1.4	-2.5	-2.4	-1.8	-1.3	-2.2	-1.9	-2.2

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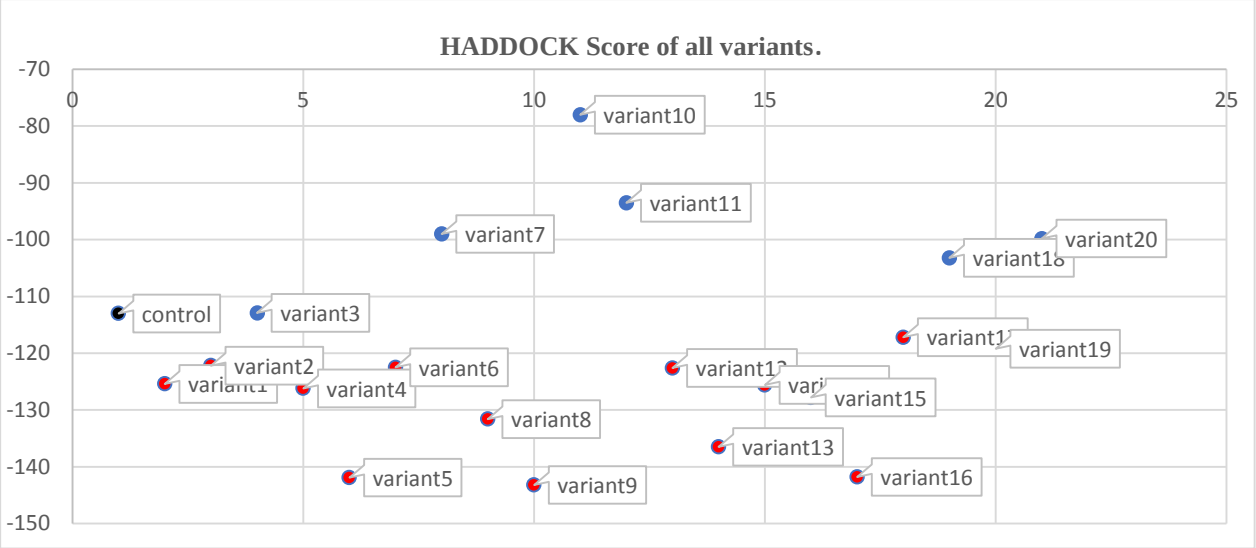

Figure 5. HADDOCK scores were evaluated for all variants. Variants with scores lower than the threshold (control score is -113) are anticipated to exhibit improved binding affinity for the Teplizumab human antibody.

Multiple studies in patients with T1D have shown that teplizumab treatment diminishes the loss of beta-cell function even up to 7 years after diagnosis (39-43).

Optimization the features of antigen-binding domains of antibody, including improving the affinity of the antibody to its target, is one of the most important purposes of antibody engineering methods. This is important, because, regardless the method used to antibodies development including hybridoma, phage libraries or other technologies, the need to improve antibody affinity to its target still remains important. The biological activity and treatment efficacy of the antibody increases by improving in antibody affinity. Furthermore, this improvement in antibody affinity reduces the needed amount of antibody, resulting in lower toxicity as well as the lower cost to consumers (44). CDRs largely mediate the binding activity of the antibody. There are several novel technics developed for designing CDRs extended from de novo design methods to methods based on the redesign of existing antibodies. One interesting study performed with the purpose of improvement the antibody affinity, showed the potential of optimizing electrostatic interactions in this regard (45). In order to expand the healing properties of monoclonal antibodies, numerous features such as binding affinity, geometry among heavy and light chains, and constancy in various pharmacokinetic properties should be considered for better efficacy of monoclonal antibodies (46). Optimizing these features with laboratory approaches is costly and slow (47). By the aid of in silico calculations, the expansion of antibody properties was done more accurately and faster (48).

Over the past two decades, numerous remedial mAbs have been accepted by the FDA for the cure of several tumors. Bevacizumab is one of the mAbs which have been effectively utilized in the cure of ovarian cancer and several other cancers. In 2018, Shirin Eyvazi and colleagues found the significant amino acids of the bevacizumab antibody and thereafter to better the affinity of the antibody, they replaced these amino acids with other amino acids. They investigated the binding affinity of different types of antibodies to antigens. The results of this study at that time could be considered as the beginning step towards the expansion of ameliorated anti-VEGF antibodies against ovarian cancer (49).

The mutagenesis methods have important roles in understanding the relationship between structure and function of proteins and were involved in modifying their physicochemical properties (44). Studies show that antibody

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affinity and specificity can be altered by replacing amino acid in the binding region. Since several central amino acids in the antigen-antibody binding region are responsible for binding affinity to antigen, replacing such key amino acid can significantly increase antibody affinity (45). Amino acids effective in binding affinity, can be identified using bioinformatics tools and replaced with more effective amino acids. Nowadays, bioinformatic tools are of interesting advantages for biologists (50-60).

Theory and computational methods accompanied by advanced laboratory technics will accelerate our understanding about interaction between molecules (23,24). Molecular docking strategy, which consider two or more structures as input, and predict their complex structure is used for this purpose. Docking is a computational method which puts a ligand molecule in target molecule location and estimates the binding affinity. In this method, the thousands of possible ways for protein-ligand interaction are checked, and the state which has the lowest score of binding energy is predicted as the best match and the molecular with high binding affinity (25).

A systematic in silico method of discovery of newer mAb variants with ameliorated binding attributes was suggested. Since the Adalimumab mAb is one of the greatest candidates' therapies for rheumatoid arthritis and other autoimmune illnesses, they have recognized the six utmost considerable residues on Adalimumab mAb, implicated in the antigen antibody contacts (61).

Antibody engineering projects have been carried out to increase antibody affinity (54-56,58,62). They identified important amino acids, then changed the amino acids by other amino acids to expand the binding affinity of different antibodies to antigens (56).

In this research, we sought to expand the binding affinity of the antibody with an in-silico approach. We used a Paratom web server to foresee the ABRs of the teplizumab antibody in this paper. This server's alignment algorithm relies on a non-redundant set of all identified antibody-antigen complexes in the PDB database. The Paratom web server foretold three areas as ABRs in each chain. For the teplizumab heavy chain, YTFTRYTMH (27-35) by means of ABR1, WIGYINPSRGYTNY (47-60) by means of ABR2, and RYYDDHYCLDY by means of ABR3. The Paratom also predicted SSVSYMN (27-33), RWIYDTSKLAS (45-55), and QQWSSNPF (88-95) as ABR1, ABR2, and ABR3 for the light chain of this antibody, respectively.

BIPSPI predicts partner-specific protein-protein interactions. H104, H102, H103, H105, H56, H101, H54, L47, L89, L30, L48, L212, L91, L90, L211, L92 and L46 were selected based on the considered threshold. We used ConsSurf, Paratom, and InterProsSurf to identify important structural and functional residues. We used ConSurf to find residues that were conserved during evolution. InterProSurf was used to predict protein-protein interaction sites. Paratom predicted the residues located in the CDR regions. In addition, we used BIPSI, ConSurf, and InterProSurf to confirm the residual selection. Finally, the selected residues were: L:30S, L:31 Y, L:47 I, L:48 Y, L:49 D, L:52 K, L:88 Q, L:90 W, L:91 S, L:92 S, L:93 N and L:95 F.

These residues were discovered using the mentioned software in antigen-antibody contacts. These amino acids can be substituted with other amino acids which increase the binding affinity to the mark antigen. SHIFT is used to predict an amino acid replacement will have a phenotypic influence.

After identifying the functional and conserved amino acids in the CDRs that potentially be substituted with another amino acid, such that this substitution with another amino acid boosts the antibody's affinity. Based on this, we designed several variants of the cited antibody. We analyzed the affinity of new variants that have mutations using the HADDOCK server. HADDOCK's input was the 3D structure of the variants. HADDOCK scoring was based on several different terms and the Z-score was the sum of these terms, the more negative it was, the higher and the

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affinity. The docking score of the natural antibody was considered as a control and the docking score of the new variants was compared with it. In this study 3 variants of teplizumab obtained a more negative score than the control as a result of docking with CD3 antigen, which indicates the higher affinity of these variants compared with the control sample. According to our results, variants 5 (Z-score= -2.4), 11 (Z-score=-1.9) and 16 (Z-score=-2.2) had more negative Z-scores than the control sample. The buried area among the two complexes in desired variants was further than the control, indicating that the mutations increased the binding ability of antibodies compared to the wild sample.

Our results, suggest that Teplizumab can be a therapeutic candidate for the inhibition or cure of type 1 diabetes. Optimizing binding properties and affinity maturation of Teplizumab to reduce cross-reactions and select suitable antibodies via using new mutations through addition of new mutations to produce the wanted antibody will be beneficial.

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Conflict of Interest

The authors have received no financial support for the elaboration of this manuscript. Shahid Sadughi University of medical science did not play any decision-making role in the study analysis or writing of the manuscript. All authors declare no Potential Conflicts of Interest.

Authors' contributions

F.S: Laid out the main idea and participated in the design of the study, conducted coordination, and revised the manuscript. K.M, R.Gh, Z.R, Gh.A and H.M: participated in the data collection and analysis and drafted the manuscript. All authors read and approved the final manuscript.

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