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Stabilizing the Cytokines for Improved Immunological Memory Responses

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Abstract: **Generation, maintenance, and reactivation of Ag-specific T cell memory are the important considerations for selecting adjuvant for vaccine regimen. Strong $CD8^+$ T cell response is a critical requirement to combat various diseases e.g., HIV, TB, and Malaria. Reactivation of tumor-infiltrating $CD8^+$ T cells is essential for tumor regression. Cytokines have central roles in all these areas. They exhibit the potential for maintaining long-lasting T cell responses to pathogens by supporting the survival of $CD8^+$ T cells. In many experiments, it has been shown that cytokines successfully regress the tumor burden by activating tumor-infiltrating cells. But like many other immune mediators, these cytokines have unstable nature and clear rapidly from the body. Therefore, using them for therapeutic potential is challenging and highly expensive. The current study demonstrates a method for designing and producing modified or mutant cytokines to induce $CD8^+$ T cell proliferation, function, and longevity. Taken together, these data suggest that the modified cytokines are stable and might be suitable for further studies.**

Index Terms: **Cytokines, T cell memory, Mutation, Stability, Structure**

I. Introduction

Immunological memory is an important characteristic of the adaptive immune system that provides long-lived immunity against several pathogenic agents whom the host encountered before. Immunological memory is broadly governed by two cell types i.e., memory B cells and memory T cells. Achieving this property of the immune response is the ultimate goal of vaccination. As per our own experience, the γc-cytokines (mainly IL-2, IL-7, and IL-15) significantly control the differentiation of memory T cells. These cytokines control each checkpoint of T cell memory differentiation. For example, at the beginning of the T-cell response, the activation of dendritic cells and MHC-TCR ligation are under the influence of IL-15 (Interleukin 15). Similarly, the next stage of T-cell clonal expansion is controlled by the activities of IL-2 (Interleukin 2). We have seen that IL-15 also involve in this stage and improves the proliferation of antigen-specific T cells (M. Patidar et al., 2016).

Once the antigens are removed or their load declined from the host system, it is mandatory for the safety of the host, to regulate the expansion of these antigen-specific activated T cells. Therefore in the contraction phase, IL-2 induces autoimmune cell death (AICD) that results in massive cell death. The negative impact of this contraction phase is the loss of antigen-specific T cells occurs. And for the generation of memory T cells, it is required to rescue a few of the antigen-specific T cells from death. IL-7 (Interleukin 7) and IL-15 help in rescuing the T cells from AICD and enable memory T-cell generation. IL-7 promotes the survival of memory T cells (Schluns & Lefrancois, 2003). Apart from the role in deciding the fate of immunological memory, the cytokines have various types of anti-bacterial, anti-viral, anti-cancerous, and potent therapeutic values (M. Patidar et al., 2016). These characteristics of cytokines have made them highly attractive biologics for immunotherapy. However, the unstable nature vis-à-vis shorter half-lives of these molecules limit their therapeutic potentials and pose a great challenge to clinical use. This study is an attempt to address the underlying issues.

II. Methodology

A. Amino Acid Sequences

The amino acid sequences of various cytokines were obtained from the Uniprot database (13). Each sequence has a Uniprot id and a Protein Data Bank (PDB) (14, 15) entry code. The selected sequences were analyzed for their sequence similarity using the BLAST program (16). The protein names, Uniprot id, PDB codes, and the total number of amino acids of the proteins used in this study are listed in Table I.

Table I. Uniprot ID, PDB ID, and amino acid length of different proteins taken in the study

Cytokines	Human (*Homo sapiens*)			Mouse (*Mus musculus*)		
	Uniprot ID	PDB Code	Total Amino-Acids	Uniprot ID	PDB Code	Total Amino- acids
IL-2	P60568	1M47	153	P04351		169
IL-4	P05112	2B8U	153	P07750		140
IL-6	P05231	1P9M	212	P08505	2L3Y	211
IL-7	P13232	3DI2	177	P10168		154
IL-9	P15248		144	P15247		144
IL-10	P22301	2H24	178	P18893		178
IL-15	P40933	2Z3Q	162	P48346	2PSM	162
IL-19	Q9UHD0	1N1F	177	Q8CJ70		176
IL-20	Q9NYY1	4DOH	176	Q9JKV9		176

B. Structure Prediction and Visualization

After getting the right sequences, the structure was prepared from the primary amino acid sequence by threading-based approach by various tools including the Swiss model (Arnold, Bordoli, Kopp, & Schwede, 2006) and I-TASSER (Zhang, 2008). Whenever needed the modeled structure was downloaded from the protein database as pdb.

C. Structure improvements (When needed) and Refinement

MaxSprout server was used to reconstruct the Protein backbone and side-chain co-ordinates from C(alpha) trace (Holm & Sander, 1991). MolProbity server was used to add Hydrogens by the "Add hydrogens" option of (V. B. Chen et al., 2010). Chiron server was used to remove the steric clashes by the "clash removal" option (Hospital et al., 2012). Kobamin, 3Drefine, CABS-flex, and Galaxy servers were used for energy minimization (Rodrigues, Levitt, & Chopra, 2012) and for molecular simulation (dynamics) Modrefiner and MDWeb (Xu & Zhang, 2011) were used.

D. Site- Directed Mutagenesis

The selected mutations into cytokine genes were introduced. The selections of amino-acids used for substitution were based on literature and amino-acid substitution tools. SDM server was used for the prediction of novel sites for the mutation (Pandurangan, Ochoa-Montaño, Ascher, & Blundell, 2017). The mutant structures were generated by submitting the mutated sequences to I-TASSER and Swiss model servers (Arnold et al., 2006; Zhang, 2008). And also the mutations were introduced by the Pymol tool (The PyMOL Molecular Graphics System). Further, the CUPSAT server was used for the stability prediction of mutant proteins (Parthiban, Gromiha, & Schomburg, 2006).

E. Structural Assessment

Quality assessment of models is a pre-requisite to test the suitability of models for the subsequent experiments (Manoj, Naveen, & Dalai, 2017; Manoj Patidar, Yadav, & Dalai, 2018). Physico-chemical analysis, cysteine classification, and prediction of disulfide connectivity were determined by ExPASy tool ProtParam and DiANNA web-server (Fariselli & Casadio, 2001; Gasteiger et al., 2003). Rampage and Molprobity servers were used for the generation of the Ramachandran map (Lovell et al., 2003). To check any conformational changes in structures QMEAN, QMEAN Z-score, RMSD values, and TM Score were also determined (Benkert, Kunzli, & Schwede, 2009; Maiti, Van Domselaar, Zhang, & Wishart, 2004). Superpose server was used for the super-imposition of wild and mutant structures (Maiti et al., 2004).

F. Protein- Protein Docking

ZDOCK (R. Chen, Li, & Weng, 2003), PatchDock, and FireDock (Duhovny, Nussinov, & Wolfson, 2002; Schneidman-Duhovny, Inbar, Nussinov, & Wolfson, 2005) servers were used for docking of generated mutants with their respective receptors. The docked structures were visualized by the UCSF Chimera software package (Pettersen et al., 2004) and Accelrys Discovery Studio Visualizer. The intermolecular hydrogen bonds and the salt bridges were analyzed by the Chimera and Accelrys Discovery Studio Visualizer (Sanner, 1999).

III. Result and Discussion

A. Structural Insight

The structures of cytokines were systematically analyzed (Fig. 1) and it was found that these are tiny biologics with the molecular weight of ~14-20 KDa. And this is the reason for their fast clearance through renal excretion. Now it is not possible to enhance their molecular weight. Therefore, other strategies like mutagenesis should be employed. The approximate size of the cytokines gene is around 34 kb or more. The 4 a-helix bundle cytokines like IL-2 and IL-15 have tri receptor systems i.e., Receptor- α, -β, and –γ.

B. In silico Identification of Amino Acid Residues Involved in the Binding Sites

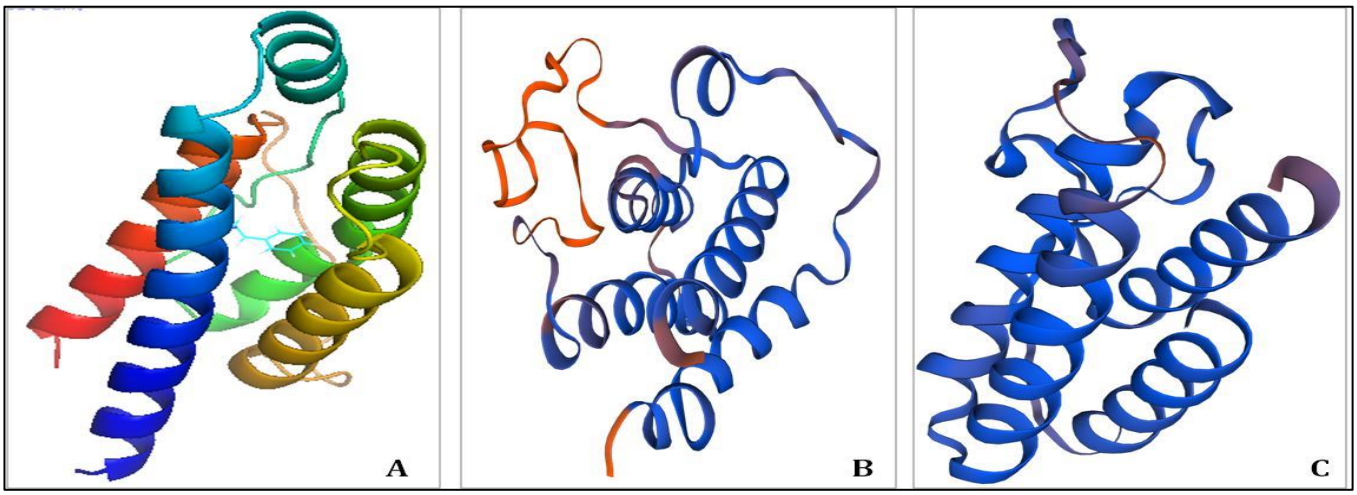

Fig. 1. Structure of various cytokines: IL-2 (A), IL-7 (B), and IL-15 (C)

The interactions of cytokines with their respective receptors were analyzed through PDB sum. For example, the IL-2 molecule has three receptors i.e. IL-2 Rα, IL-2 Rβ, and IL-2Rγ. IL-2, IL-2 Rα, IL-2 Rβ, and IL-2Rγ have 120, 122, 196, and 191 amino-acids respectively. The number of interface residues between IL-2 and IL-2 Rα is 15:17; the number of salt bridges is 2, the number of hydrogen bonds is 10 and 91 non-bonded contact formed between these two chains. Similarly, the interface residues between IL-2 and IL-2 Rβ, and IL-2 and IL-2 Rγ are analyzed (Fig. 2).

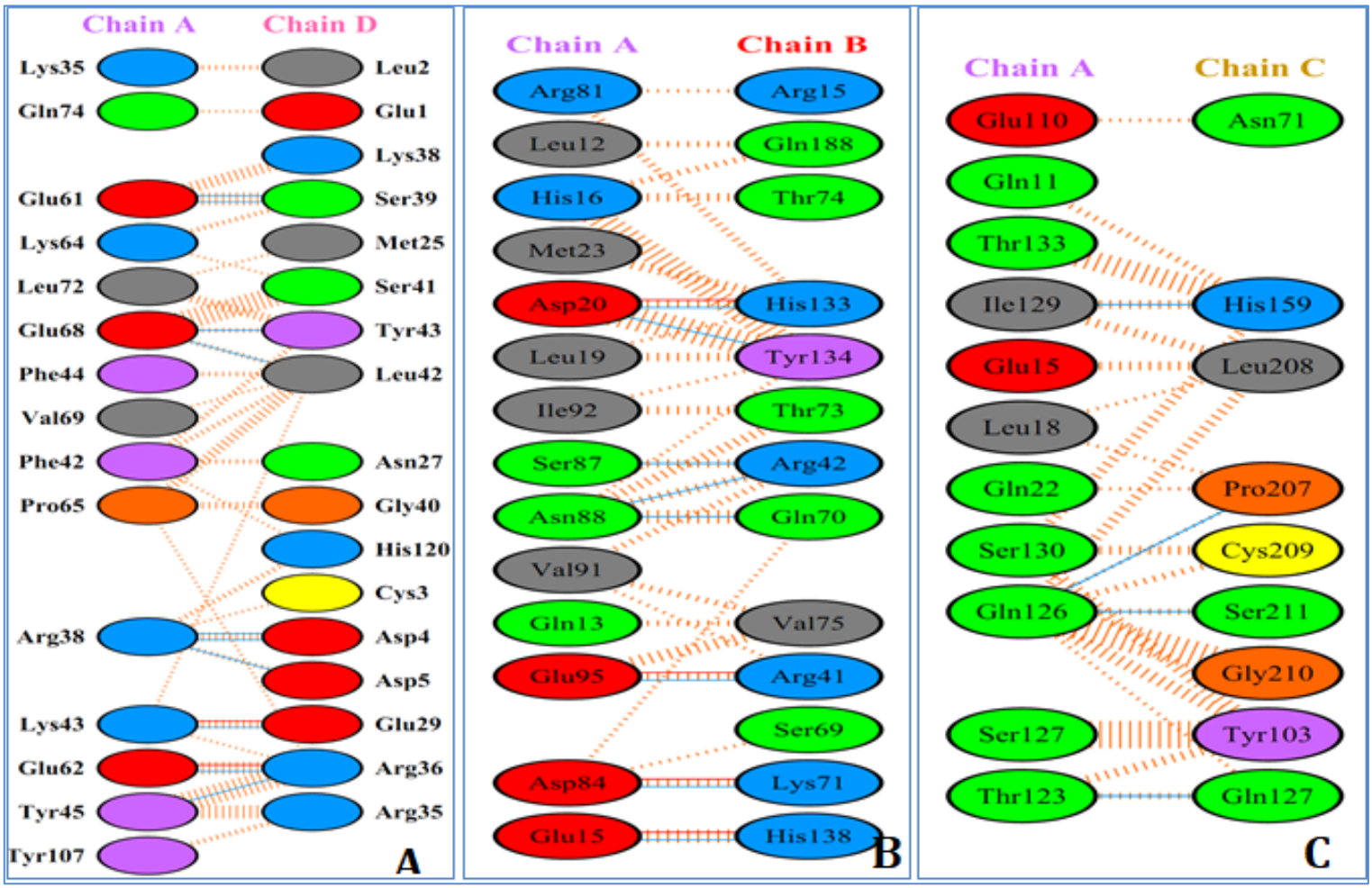

Fig. 2. The pattern of interactions between cytokine and its receptors. The figure depicts the interaction between IL-2 and IL-2 Rα (A), IL-2 and IL-2 Rβ (B), and IL-2 and IL-2 Rγ (C).

C. Selection of Amino Acids for Mutations

Some mutations were introduced into the binding/interacting sites of the ligand-receptor and some residues far away from the receptor and antibody interacting surface were also being selected. We have previously reported that the mutant recombinant cytokines have a higher affinity for their receptors and also improved stability (M. Patidar et al., 2016). This study also utilized SDM server for the calculation of the free energy difference to find the stability of the mutant proteins. The CUPSAT data also suggested the stability of mutants.

D. In silico Site- Directed Mutagenesis into Cytokine Gene

The selected mutations were introduced in the respective regions of cytokines to increase their affinity towards the receptors and stability. The .pdb files for the mutant structures were generated by using the SDM server and visualize into the PyMOL tool (Fig. 3). SDM server suggested the introduced mutations would be more stable and non-disease associated. The pseudo $\Delta\Delta G$ values for the mutations were 1.63 and 0.70 suggesting the mutations are stabilizing in nature.

E. Validation of the Mutant Strutures

The mutant structures were validated by superimposing the wild-type structures over mutant structures, RMSD value, and Ramachandran plot analysis. The analysis suggests that mutations are allowable and mutant structures are favorable (Fig. 4).

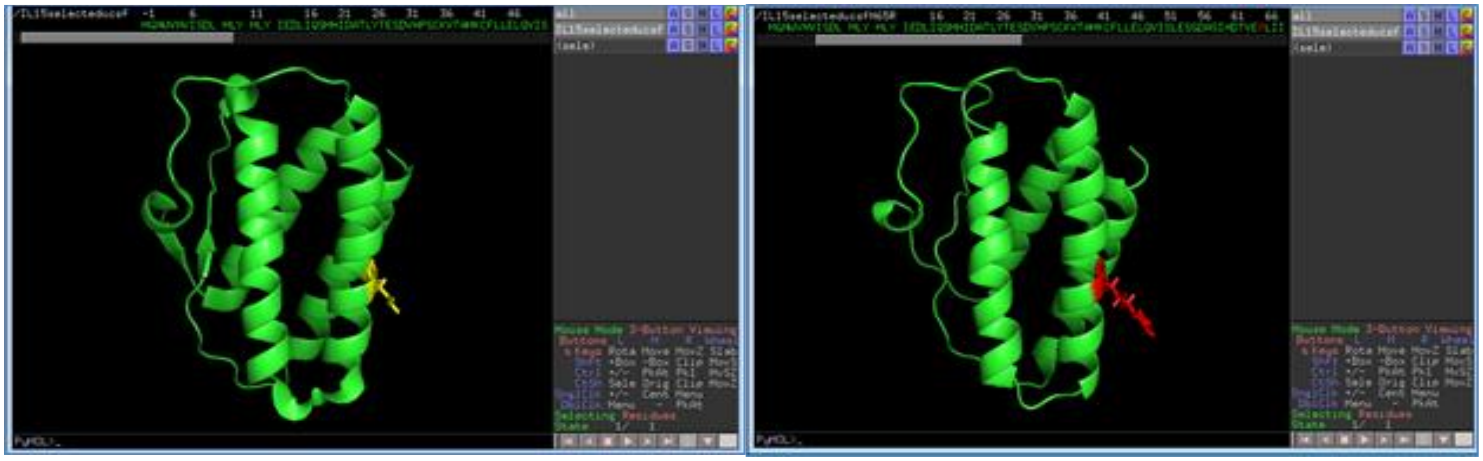

Fig. 3. Wild vs. mutant cytokine: Yellow stick structure depicts N65 molecule in wild type cytokine (A), and the Red stick shows N65R mutation (B).

F. Docking Studies

The protein-protein docking was performed *in silico* to predict the binding/interaction of ligands with receptors. Hydrogen bonds and salt bridges determine the interaction of designed proteins with their targets and the same were calculated. The results suggest that mutant structures have higher affinities to

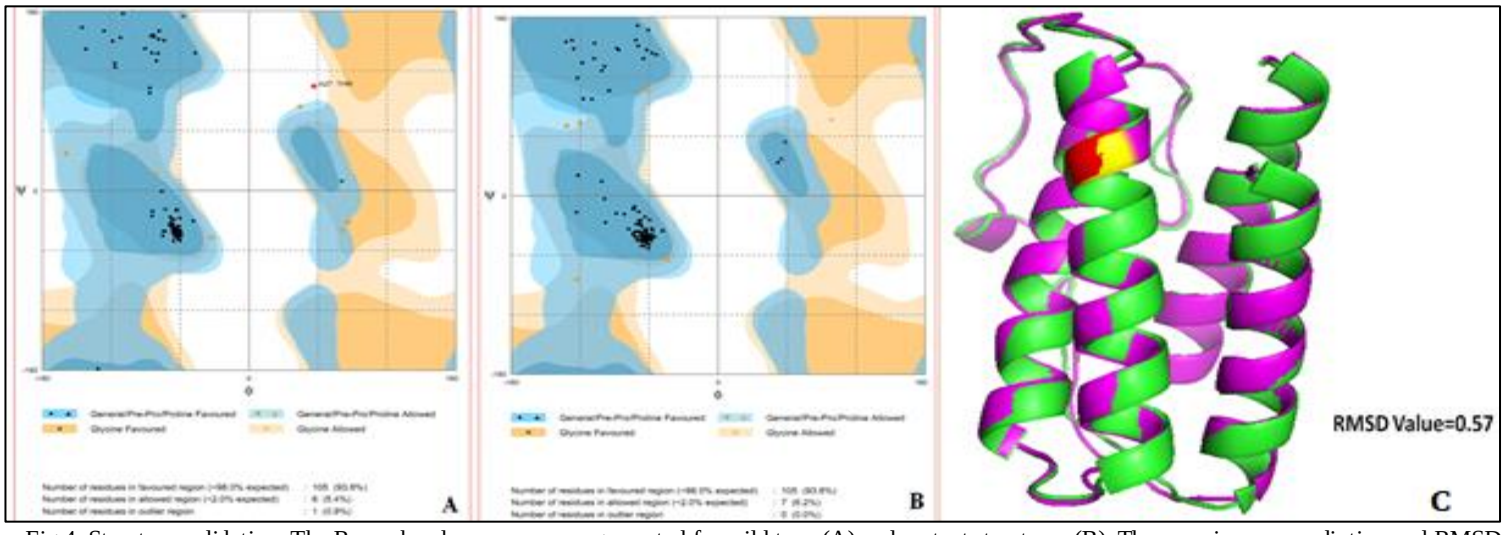

Fig.4. Structure validation: The Ramachandran maps were generated for wild type (A) and mutant structures (B). The superimpose prediction and RMSD value depict that mutation does not bring in any conformational changes (C). Wild-type cytokine in green, mutant cytokine in pink, and mutation is shown in the red/yellow color.

bind with their respective receptors (Fig. 5).

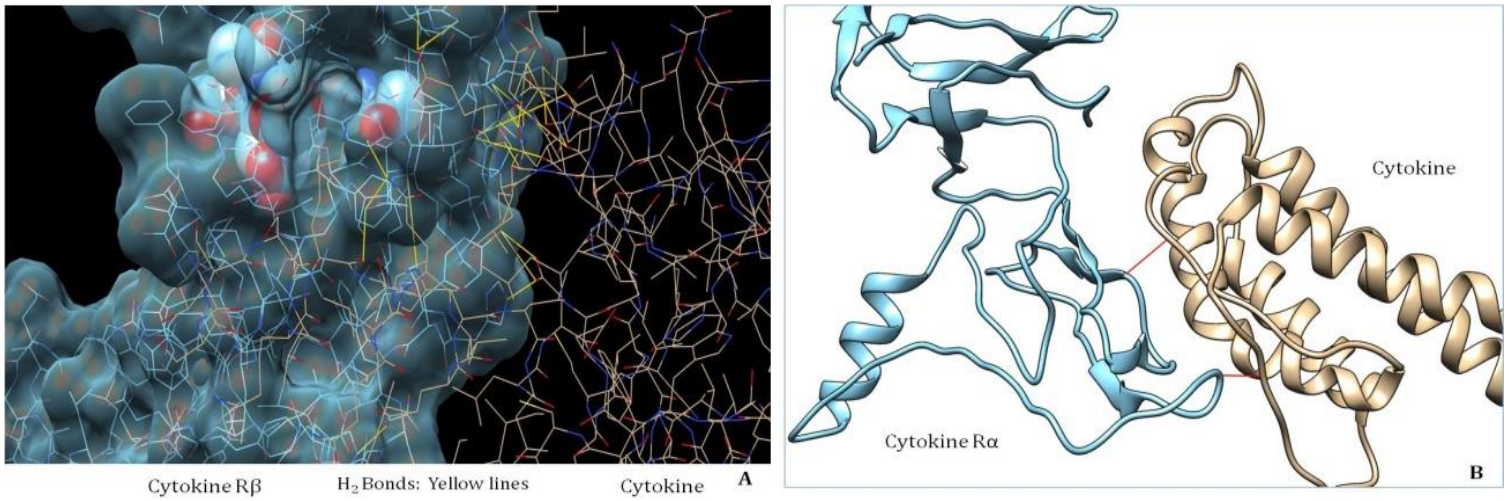

Fig.5. Docking studies: the docked structure shows the presence of hydrogen bonds, Yellow (A), and Red (B).

CONCLUSION

The use of cytokines as adjuvants in vaccine regimens has been shown to improve the efficacy of vaccines (M. Patidar et al., 2016). Cytokines like IL-2 and IL-15 show potent anti-tumor activities and are found to be efficient in solid tumor regression. Cytokines show promising results in the treatments of Malaria, HIV, and TB that are serious threats to human health (M. Patidar et al., 2016). The various therapeutic properties of cytokines have attracted researchers worldwide to explore them. However, their unstable nature and shorter half-life limit their therapeutic use. The use of unstable cytokines requires a higher amount with multiple infusions to achieve the desired therapeutic response. These might increase the chances of side effects, cost, and limitations for the large populations. This study is an attempt to use site-directed mutagenesis to address these challenges. It was found that by mutating the cytokines their stability, as well as efficacy, can be improved.

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