Altered Peptide Ligand (APL) Therapeutic Vaccine against Autoimmune Disease: Rheumatoid Arthritis

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ALP	Altered Peptide Ligand
RA	Rheumatoid Arthritis
IFN-γ	Interferon gamma
PAD	Peptidyl Arginine Deiminase
SEs	Shared Epitopes
IL	Interleukin
TNF-α	Tumor Necrosis Factor-Alpha
TGF-β	Transforming Growth Factor Beta
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
DMARDs	Disease-Modifying Antirheumatic Drugs
MTX	Methotrexate
FH2	Dihydrofolic Acid
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complexes
HLA	Human Leukocyte Antigen
ACPAs	Anti-Citrullinated Protein Antibodies
Th1	T helper 1
TCR	T-cell receptors
NCBI	National Center for Biotechnology Information
IEDB	Immune Epitope Database
MD	Molecular Dynamics
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
BLAST	Basic Local Alignment Search Tool
PDB	Protein Data Bank

Abstract

Rheumatoid Arthritis (RA) is a prevalent autoimmune disease, affecting millions worldwide. Its treatment is often costly, placing a significant economic burden on patients and healthcare systems. Current therapies primarily rely on immunosuppression, which can have drawbacks, such as increased susceptibility to infections and long-term medication dependency. The development of targeted APL vaccines represents a promising avenue for more effective and safer RA management, offering hope for improved patient outcomes and reduced treatment costs in the future.

In this research, an Altered Peptide Ligand (APL) therapeutic vaccine against Rheumatoid Arthritis (RA) was designed through *in silico* methods. The Vimentin protein sequence, sourced from NCBI, served as the starting point. A specific B cell epitope, "STRTYSLGSALRPSTSRSLY," which exhibited strong binding with both HLADRB1 and HLADRB4 receptors, was the focus. However, it demonstrated high immunogenicity and IFN-γ production, coupled with reduced IL10 and IL4 levels. To enhance its regulatory response while reducing inflammation, a double substitution was performed. At positions 1 and 3, S was replaced by E, and R was substituted with E, respectively. Remarkably, these alterations did not compromise binding to HLADRB1. Furthermore, the peptide was linked to Alpha-Melanocyte Stimulating Hormone through an EAAAK linker. The resultant sequence, "SYSMEHFRWGKPVEAAAKETETYSLGSALRPSTSRSLY," exhibited reduced IFN-γ production and increased IL10 and IL4 levels. This innovative peptide is proposed as a potential APL vaccine candidate against RA, underscoring the efficacy of *in silico* methodologies in therapeutic vaccine design.

Chapter1

1. Introduction

1.1 Introduction:

Rheumatoid arthritis (RA), an autoimmune disorder is characterized by inflammation in the joints. Immune system mistakenly targets healthy joint tissues, leading to persistent pain, tenderness, and joint damage over time(Padyukov 2022). Signs and symptoms of RA include extreme tiredness that does not go away even after resting. Patient might feel their muscles and joints burning, aching, and being sore. It is common for them to experience weakness in their hand, arm, and leg muscles. Swollen glands can be found in various parts of the body, including armpits, throat, and groin areas(Alam, Jantan *et al.* 2017).

Development of RA is caused by combination of different genetic, epigenetic, and environmental factors. However, genetic predisposition is a significant contributor, while the onset and progression of the disease is caused by various environmental factors. Factors such as cigarette smoke, exposure to dust, and the microbiome, which constitutes an "internal" environment, have been shown to be particularly influential.(Scherer, Häupl *et a*l. 2020) Similar to many autoimmune disorders in humans, RA susceptibility and intensity result from the involvement of multiple genes. The most extensive genetic correlation is observed in connection with HLA-DRB1 genes, particularly the HLA-DR4 variants. These variants encompass commonly found molecules such as HLA DRB1*04:01, *04:04, and *01: 01. Citrullination is a natural process involve in case of RA where Arginine (amino acid present in composition of proteins of cartilage in joints) changes into citrulline, and it's controlled by enzymes called peptidyl arginine deiminases (PAD). This activity increases during inflammation, stress, and apoptosis, leading to more diverse epitopes after protein exposure. This Citrullinated process allow immune system to release anti citrullinated protein anti bodies (ACPA) and inflammatory cytokines against these proteins. In people with rheumatoid arthritis (RA), the joints' tissue shows Citrullinated proteins and Peptidyl Arginine Deiminases (PAD) enzymes from inflammatory cells, which are important factors in causing the disease(Scally, Petersen *et*

al. 2013). The presence of anti citrullinated protein antibody (ACPA) has become a widely accepted method for diagnosing and predicting this disease due to its high accuracy (specificity >97%) in clinical settings. ACPA arises from an abnormal immune response to various Citrullinated proteins, such as fibronectin, α-enolase, fibrin, vimentin, type II collagen, and histones. These proteins are distributed throughout the body. Genetic and environmental factors both influence the production of ACPA. Among the genetic factors, the most potent risk determinant for ACPA-positive RA is located in the genes that code for HLA-DR, specifically HLA-DR1 and HLA-DR4, which are also referred to as "shared epitopes" (SEs)(Guo, Wang *et al*. 2018).

The emergence of autoimmune processes that initiate RA is thought to result from an uneven equilibrium between regulatory T cells (Tregs) and CD4+ effector T cells, with a prevalence of the former. Tregs derived from the peripheral blood of individuals with RA have been observed to possess a diminished capacity to control the production of proinflammatory cytokines by effector T cells and monocytes. This impairment has been linked to various factors, including epigenetic modifications and the influence of TNF on Tregs, among other mechanisms (Schinnerling, Aguillón *et al*. 2017). T-cells migrate into the synovial joint and elevate the concentration of pro-inflammatory cytokines like interferon-γ and IL-2, which consequently leads to the degradation of synovial cartilage and bone tissue(Akahoshi-Ikeda, Yoshizawa *et al*. 2016). Cytokines serve as protein messengers that facilitate communication between cells through specific receptor molecules on their surfaces. The secretion of certain cytokines into the bloodstream has also been observed in various inflammatory conditions, including RA, often indicating the severity and outlook of the disease. Cytokines are categorized into proinflammatory types [such as interferon-γ (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukins (IL)-1, IL-2, IL-6, IL-8, IL-12, and IL-18], anti-inflammatory types (IL-4, IL-10), and regulatory types [such as transforming growth factor beta (TGF-β)]. As per the established classification in the study of RA, IL-10, IL4 and TGF-β fall into the category of regulatory cytokines. The equilibrium between pro-inflammatory and anti-inflammatory cytokines in RA governs the level and scope of inflammation, thereby influencing diverse clinical outcomes(Singh, Khan *et al*. 2014). T helper cells can be categorized into two main subsets, primarily based on the cytokines they generate. Th1 cells release IFN-γ, TNF-α, and IL-2,

while IL-4, IL-13, and IL-10 are secreted by Th2 cells. The excessive production of cytokines and growth factors stemming from the inflamed synovial tissue could cause underlying mechanisms of RA. Notably, the subdued inflammation in early-stage RA is driven by cytokines. Specifically, TNF-α, IL-2, and IFN-γ have been proposed as pivotal contributors to the progression of RA, influencing both synovial inflammation and chondrocyte activation(Singh, Khan *et al*. 2014).

An estimated 0.1% to 2.0% of the world's population is affected by this condition. Despite recent progress in treatments, there's no known cure for RA. Rheumatoid Arthritis is a complex condition influenced by both genetics and the environment. On a larger scale, Australia has the highest reported RA prevalence worldwide (2%), based on data from a survey conducted in 2014-2015. In specific communities, like the Pima and Chippewa Indians, RA rates are much higher at 5.3% and 6.8% respectively. On the other hand, population living in rural areas of South Africa (0.0026%) and Nigeria (0%) have reported very low occurrences of RA. The differences in reported prevalence rates stem from various factors like how cases are identified, where people live, their socioeconomic status, and their exposure to genetic and environmental elements. Knowing the true prevalence of RA is crucial for understanding the impact it has on healthcare and the economy. This information guides healthcare policies and the allocation of resources. Analyzing existing data on RA prevalence through systematic reviews and meta-analysis can provide valuable insights for planning both now and in the future(Almutairi, Nossent *et al*. 2021). Historical data suggests that the occurrence of RA in Pakistan has been inconsistent. Prevalence rates for RA have shown regional variations, with reports ranging from 0.142% in the south to 1% in the north of the country. In a recent study conducted at a specialized medical facility in Karachi, located in the southern region, the prevalence of RA was found to be 12.9% among patients who sought care at the hospital's rheumatology clinic, out of a total of 4900 patients. This finding indicates a significant rise in the burden of the disease in this region, previously recorded at 0.142%. Moreover, the study highlighted that RA was more prevalent among females(Naqvi, Hassali *et al*. 2017).Global studies indicate an occurrence of RA, ranging from 0.5% to 1%. The prevalence of RA varies by region, with higher rates seen in polar and torrid countries. Hunter and his team found that between 2004 and 2014, the prevalence of RA in the US population was around 0.53% to 0.55%. The condition was

more frequent in females, with an estimated prevalence of 0.73% to 0.78% among them. In the USA, the prevalence of RA seemed to rise from 2004 to 2014, is affecting approximately 1.28 to 1.36 million people by 2014. In Canada, prevalence was 0.9%, while in Japan, it ranged from 0.6% to 1%. A nationwide study in the UK People noted 0.67% prevalence of RA. In the broader European context, the prevalence of RA was reported as 0.38% (0.24%-0.57%) for females and 0.14% (0.0%-0.22%) for males. In People living in Western Europe specifically, it was 0.63% (0.55%-0.75%) for females and 0.24% (0.21%-0.28%) for males. In a systematic review, Naqvi and his colleagues did a research and reported a prevalence of 0.142% for RA in Pakistan(Naqvi, Hassali *et al*. 2020).

Depending upon this prevalence of RA some primary and second line treatments are available but these are not fully applicable and satisfactory with drawbacks. The primary aim of initial treatment is to alleviate pain and reduce inflammation. Nonsteroidal anti-inflammatory drugs (NSAIDs) are swift-acting medications used for this purpose, which encompass acetylsalicylate (Aspirin), ibuprofen (Advil and Motrin), and etodolac (Iodine). Aspirin, when administered at high doses, effectively combats inflammation in cases of RA by blocking prostaglandin production. It's one of the earliest NSAIDs employed for joint pain management. However, higher doses of aspirin can lead to adverse effects like ringing in the ears, hearing loss, and stomach intolerance {Roubille, 2015}.

In addition to aspirin, there are newer NSAIDs available that are just as effective and require fewer daily doses. NSAIDs function by inhibiting cycle-oxygenase, preventing the creation of prostacyclin, prostaglandins, and thromboxane’s. Common side effects are nausea, ulcers, abdominal pain, and gastrointestinal (GI) bleeding. These symptoms can be eradicated by taking the medication with food, antacids, misoprostol (Cytotec) or proton pump inhibitors. An even more recent addition to the NSAID category is celecoxib (Celebrex), a selective Cox-2 inhibitor that carries a lower risk of gastrointestinal side effects(Bullock, Rizvi *et al*. 2019).

Corticosteroids are stronger type of anti-inflammatory drugs compared to NSAIDs, but they do carry more significant side effects. Due to these potential risks, corticosteroids are typically prescribed for a brief duration and at low doses, specifically during episodes of heightened inflammation or RA flare-ups. In cases where inflammation is localized, such

as in specific joints, corticosteroids can be administered through injections directly into the affected area to manage the symptoms effectively(Combe, Landewe *et al*. 2017).

The main objective of second-line treatment is to slow down or halting the progression of joint damage and deformities. These treatments are categorized as slow acting because they require weeks or months to demonstrate their effectiveness. Additionally, these medications, known as disease-modifying antirheumatic drugs (DMARDs) which can reduce the risk of lymphoma development, which is sometimes linked to RA {Bullock, 2019}.

Methotrexate (MTX) is the main second-line drug, often referred to as an anchor drug. It resembles folic acid and does compete with dihydrofolic acid (FH2) to bind to the enzyme that converts FH2 to folinic acid (FH4). This disruption impairs the metabolism of pyrimidine and purine, as well as the synthesis of polyamines and amino acids. MTX is an immunosuppressive drug which necessitates regular blood tests due to potential side effects like liver issues, cirrhosis, and bone marrow decline. Taking folic acid alongside MTX can help mitigate these risks. MTX is an effective DMARD with fewer side effects compared to other options, and it allows for flexible dosage adjustments {Brown, 2016}.

While there is strong evidence supporting the use of conventional synthetic DMARDs over MTX alone, combining biological and synthetic DMARDs are seen to be more effective than MTX, albeit with many side effects and higher costs {Katturajan, 2021}.

Hydroxychloroquine (Plaque nil) is an antimalarial drug which can be used for long-term RA treatment. It works by reducing the release of proinflammatory cytokines from monocytes. Common side effects involve the skin, gastrointestinal tract, and central nervous system. High doses can affect the eyes, so patients on this medication should regularly consult an ophthalmologist {Rempenault, 2020}.

Sulfasalazine (Azulfidine), primarily used to treat irritable bowel disease, is also a DMARD that, with combination with anti-inflammatory drugs, can be used for RA treatment. Although the exact mechanism of its action against RA is not fully understood, it's believed that the reduced form of the drug, sulphapyridine, may decrease amount of secretion of interleukin (IL)-8 and monocyte chemoattractant protein (MCP). While

generally well-tolerated, this drug can lead to central nervous system and gastrointestinal symptoms as well as rash. It should be avoided by individuals allergic to sulfa and salicylate compounds, as it contains these components(Bullock, Rizvi *et al*. 2019).

Individuals with rheumatoid arthritis (RA) face a higher susceptibility to infections compared to healthy individuals. This vulnerability is attributed to a complex interplay of factors, including compromised immune function, coexisting health conditions, disease activity, and the effects of immunosuppression. The emergence of targeted therapies, such as tumor necrosis factor inhibitor (TNFi) drugs, tocilizumab (TCZ), rituximab (RTX), abatacept (ABA), and more recent tofacitinib (TOF), has revolutionized the management of RA. Inspite of this there's a notable concern among healthcare professionals and patients regarding the high risk of infections linked with these treatments {Oray, 2016}.

Depending upon these side effects which are above mentioned in primary and secondary line treatments for R.A. Vaccine is the best option to avoid these side effects {Friedman, 2016}.

Guidelines provided by reputable organizations like the British Organization for Rheumatology, American College of Rheumatology and European League Against Rheumatism (EULAR) stress the importance of getting vaccinated against preventable diseases, which includes pneumococcal and influenza infections. Research literature elaborate and supports the safety of vaccinations in the context of autoimmune diseases. Notably, the Swedish Epidemiological Investigation of Rheumatoid Arthritis study found no any elevated risk of developing RA using routine vaccinations(Subesinghe, Bechman *et al*. 2018).

Vaccines have a vital role in managing the levels of fatalities and illnesses. They not only stop the commencement of diverse diseases but also create a route for their elimination, thus reducing their harmful impact(Sunita, Sajid *et al*. 2020). The process of formulating vaccines is intricate, but with the progression of bioinformatics, the task of vaccine design and pharmaceutical development might become more easy(Sieber, Kiesswetter *et al*. 2018). Over the last two decades, numerous computational tools have been devised to facilitate the advancement of immunotherapy and the discovery of peptide-based drugs. Consequently, there is a significant importance in creating innovative treatments,

encompassing prophylactic vaccines, Therapeutic vaccines and computational resources, to combat various diseases such as malaria, HIV-AIDS, tuberculosis and autoimmune diseases such as Sclerosis, Allergy, Diabetes and Rheumatoid Arthritis(Hotez, Molyneux *et al*. 2006).

In recent years, the field of immunotherapy has witnessed remarkable progress in the development of novel therapeutic approaches for various autoimmune disorders, including rheumatoid arthritis (RA). Among these innovative strategies, the utilization of altered peptide ligands (APLs) as therapeutic vaccines has garnered substantial attention. Rheumatoid arthritis, a chronic and debilitating autoimmune disease, is characterized by the dysregulation of the immune system, leading to persistent inflammation and subsequent joint damage. Traditional treatment options, while offering some relief, often come with limitations and potential side effects. As a result, the exploration of alternative therapies that can modulate the immune response in a more targeted and precise manner has become a critical area of research {Zhang, 2018}.

The emergence of computational tools and in silico methods has revolutionized the landscape of drug discovery and vaccine design. These tools offer a unique opportunity to expedite the process of identifying potential therapeutic candidates and optimizing their properties before proceeding to experimental validation. In the context of autoimmune diseases like RA, where the delicate balance between regulatory and inflammatory responses is disrupted, the rational design of therapeutic interventions holds immense promise {Usmani, 2018}.

The central focus of this research revolves around harnessing the potential of *in silico* methods to design an APL therapeutic vaccine tailored specifically for RA. This design involves the incorporation of autoantigen vimentin, a protein closely linked to RA pathogenesis due to its Citrullinated forms being prevalent in affected joints {Raffin, 2018}. The underlying principle of this approach lies in the ability to finely tune the immune response by altering the presentation of antigens to immune cells. By strategically modifying the peptide ligands that interact with major histocompatibility complexes (MHC), it becomes feasible to promote a heightened regulatory response against Citrullinated proteins while concurrently dampening the inflammatory reactions that

contribute to disease progression. This research endeavor seeks to address several key questions. Can computational simulations accurately predict the interactions between APLs targeting Citrullinated vimentin and MHC molecules, providing insights into their binding affinities and structural stability? How can these predictions guide the selection of candidate APLs for further experimentation? Moreover, how can the therapeutic potential of these designed APLs be assessed in preclinical models of rheumatoid arthritis that mimic the complex immune dysregulation and joint pathology observed in patients?

By addressing these questions, this study aspires to bridge the gap between computational insights and practical therapeutic applications, thereby advancing the field of immunotherapy for autoimmune conditions. Through the application of *in- Silico* methods to design an altered peptide ligand therapeutic vaccine that incorporates Citrullinated vimentin epitopes, this research aims to offer new avenues for achieving a balanced regulatory response while mitigating the inflammatory processes that underlie the pathogenesis of rheumatoid arthritis. Ultimately, the success of this approach could pave the way for a more targeted and effective immunotherapy that addresses the multifaceted aspects of RA and enhances the quality of life for affected individuals.

1.2 Objectives:

The objectives of this study which were achieved by using *in silico* tools included:

- To evaluate most immunogenic epitope from Citrullinated proteins involved in RA pathogenesis.
- To construct and evaluate the structural characteristics of APL based vaccine.
- To study the ability to modulate the immune response by using the APL vaccine construct.

The development of vaccine will be helpful in the treatment of RA. However, it will also be the successful alternate to the conventional and expensive drugs for RA.

Chapter2

2. Literature Review:

2.1 Rheumatoid Arthritis:

Rheumatoid arthritis (RA) is a common autoimmune condition known for causing long-lasting joint inflammation. This inflammation can lead to damage in the cartilage, bones, and surrounding areas, which affects how well the joints work. RA not only causes lasting disability and health problems but also results in persistent pain that greatly affects a person's everyday life. This impact isn't just personal; it also creates significant costs for society(Meier, Frerix *et al*. 2013).

2.2 Autoantigens and Pathogenesis of RA:

In the intricate puzzle of Rheumatoid Arthritis (RA) development, epigenetic factors emerge as key players, initiating a chain reaction within the human body. These factors set in motion the activation of Human Leukocyte Antigen (HLA) genes, setting the scene for a crucial process. This process involves the citrullination of proteins found predominantly in the cartilage—autoantigens such as vimentin and fibrinogen. This modification, triggered by epigenetic changes, sparks the formation of Anti-Citrullinated Protein Antibodies (ACPAs), which mark a turning point in the course of the disease. As ACPAs make their presence felt, they amplify the inflammatory response, overshadowing the regulatory mechanisms responsible for maintaining immune equilibrium. Consequently, a surge of cytokines is unleashed, igniting an intense wave of inflammation that takes the forefront in the pathogenesis of RA(Darrah and Andrade 2018).

This intricate interplay—where epigenetic factors influence HLA genes, triggering protein citrullination and subsequent immune responses—creates the complex landscape of Rheumatoid Arthritis. Insights into the roles of autoantigens like vimentin and fibrinogen, coupled with the impact of epigenetic changes, shed light on potential avenues for therapeutic strategies. This deeper understanding not only unravels the mechanisms driving

RA but also holds promise for targeted interventions that could bring relief to those grappling with this challenging autoimmune condition(Karami, Aslani *et al*. 2019).

2.3 Role of Interleukins in RA:

Mateen S, *et al.* elaborated that in the early stages of Rheumatoid Arthritis (RA), we witness the activation of both T and B cells, marking a critical phase in the disease's progression. Cytokines, signaling molecules in the immune system, assume a pivotal role in the pathophysiology of RA. Notably, pro-inflammatory cytokines like TNFα, IL-1, and IL-17 fuel inflammation while simultaneously promoting the breakdown of bone and cartilage. This intricate balance between pro-inflammatory and anti-inflammatory cytokines becomes disrupted, leading to a complex web of immune complications affecting multiple body systems. Furthermore, a decline in the population of regulatory T cells (Tregs) emerges as another key player in the disease's pathophysiology. These factors collectively contribute to the multifaceted nature of RA and its impact on the immune system(Mateen, Zafar *et al*. 2016).

Activated CD4+ T cells trigger a range of immune responses. Traditionally, T helper 1 (Th1) cells have been associated with the regulation of cellular immunity, while Th2 cells have been linked to the control of humoral immunity(Arend 2001). Initially, autoimmune conditions like rheumatoid arthritis were primarily attributed to Th1-mediated responses. However, the landscape shifted with the revelation of the Th17 subset. Th17 cells, responsible for generating cytokines like IL-17, IL-21, and IL-22, are now recognized as having a pivotal role in autoimmune disorders(Shen, Zhang *et al*. 2015). Another category of T cells, known as Regulatory T cells (Treg cells), are acknowledged for their protective function against bacterial and fungal infections, as well as their role in suppressing autoimmune responses. These Treg cells express specific markers like fork head box P3 (FoxP3), CD4, and CD25, and they produce essential cytokines such as TGFβ and IL-10. The balance between Th17 and Treg cells, often referred to as the Th17/Treg balance, plays a pivotal role in influencing the course of various inflammatory and autoimmune conditions(Honorati, Neri *et al*. 2006).

2.4 Role of B cells in Rheumatoid Arthritis:

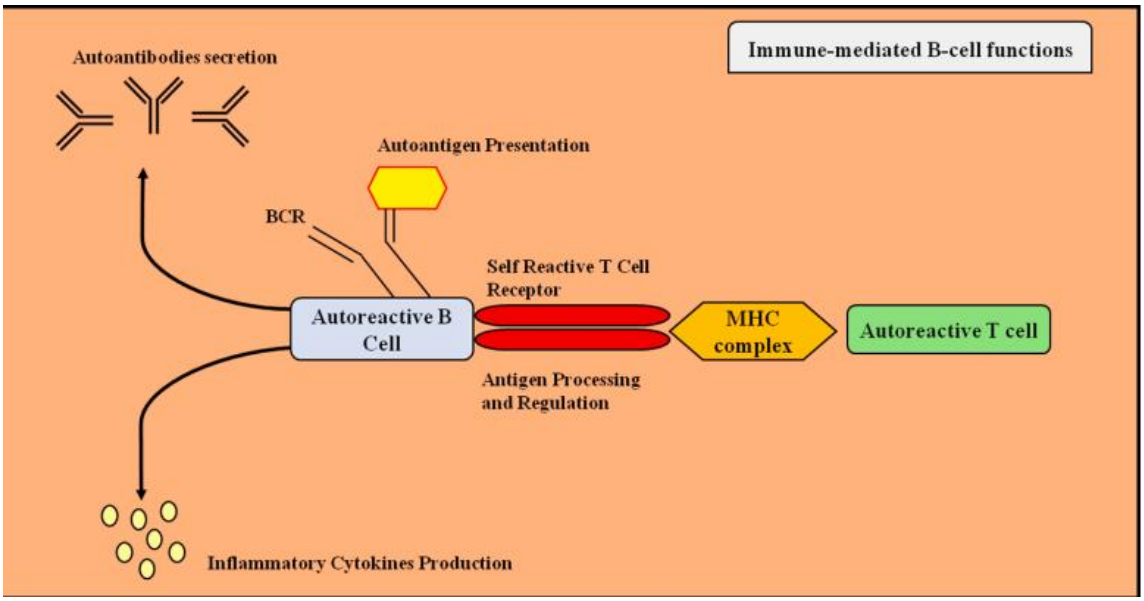

Fig 2.1: Regulation of B-cells: Autoreactive T cells produce multiple inflammatory cytokines which promote the differentiation of B cells into plasma cells and memory B cells. These B cells then produce antibodies that bind to antigens and neutralize them or target them for destruction by other immune cells (Singh, Behl *et al*. 2021).

Multiple B cell activities can contribute to the development of autoimmune disorders, including:

- The production of autoantibodies
- The presentation of autoantigens
- The release of inflammatory cytokines
- The processing and control of antigens (Singh, Behl *et al*. 2021)

In the context of RA, B cells secrete autoantibodies, particularly rheumatoid factor (RF) and anti-Citrullinated protein antibodies (ACPAs), through a complex process involving the recognition of autoantigens, such as Citrullinated proteins, by B cell receptors (BCRs)(Harbers, Crocker *et al*. 2007). In RA, certain Citrullinated proteins, like Citrullinated vimentin, are incorrectly modified within the body. B cells possess BCRs that can recognize these Citrullinated proteins as foreign or altered. When BCRs on B cells encounter Citrullinated proteins, they become activated. This activation triggers a series of intracellular signaling events that lead to B cell proliferation and differentiation. Activated

B cells differentiate into plasma cells, specialized cells responsible for antibody production. These plasma cells produce autoantibodies, including RF and ACPAs. The autoantibodies, specifically RF and ACPAs, are released into the bloodstream. They circulate throughout the body and can target various tissues, including the synovium in joints. Once in the synovium, these autoantibodies can interact with immune cells, such as macrophages and neutrophils, leading to the release of pro-inflammatory cytokines. This creates a chronic inflammatory environment within the joints, contributing to the characteristic symptoms of RA(Isaacs, Cohen *et al*. 2013).

2.5 Role of T cells in RA:

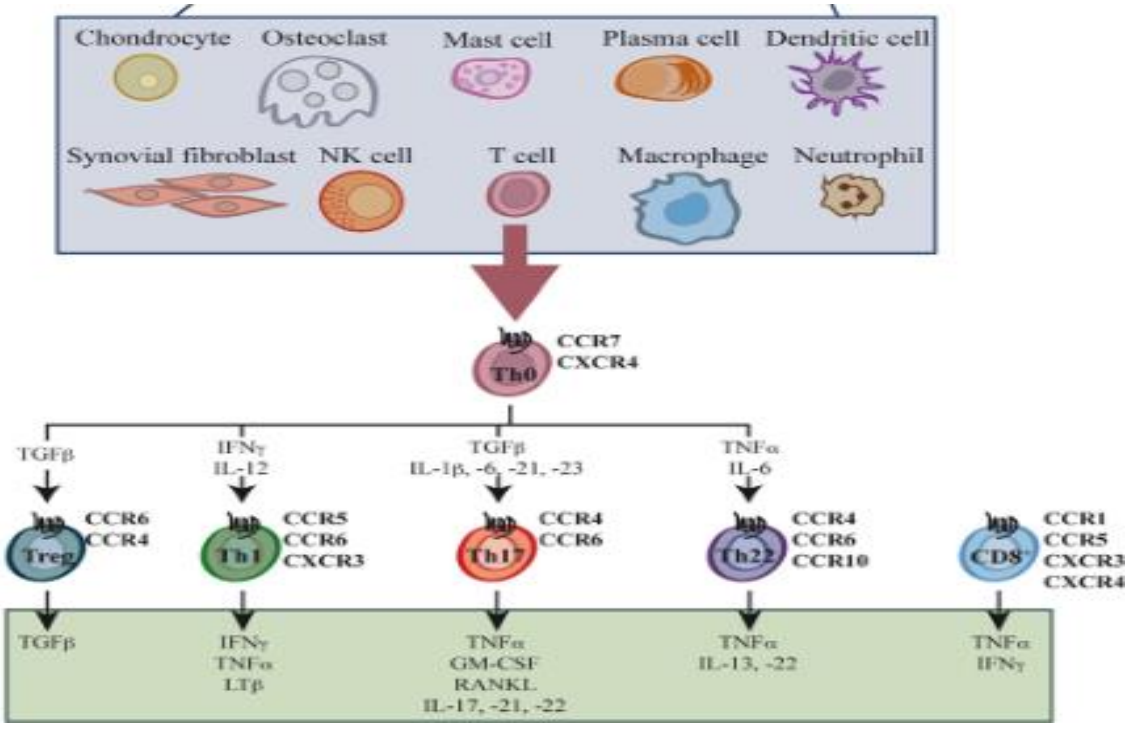

Fig 2.2: Role of T-cell in body protection: Autoreactive T cells, activated by factors like inflammation and infection, recognize and respond to self-antigens. They produce cytokines that promote B cell differentiation and antibody production. B cells, white blood cells, produce antibodies, which can play a role in protective and pathological immune responses. Antibodies can protect the body from infections, on the other hand, antibodies can also attack the body's own tissues, leading to autoimmune diseases.

Figure 2.2 represented that Interactions between environmental factors and susceptibility genes play a pivotal role in disrupting the immune system's tolerance to self-proteins that have undergone post-autoimmune response. This response, involving dendritic cells (DCs), T cells, and B cells, is co-stimulation-dependent and primarily unfolds in the lymph nodes, although it also extends to the inflamed joints. Within the lymph nodes and the affected joints, adaptive and innate immune cells are drawn to the scene, where immune pathways converge, contributing to both tissue remodeling and damage. This intricate process is further fueled by positive feedback loops that involve interactions between various immune cells, synovial fibroblasts, chondrocytes, and osteoclasts, along with the molecular byproducts of tissue damage. These dynamics collectively propel the chronic phase of Rheumatoid Arthritis (RA) pathogenesis. Notably, the synovial tissue sees an influx of highly activated memory CD4+ and CD8+ T cells, which are induced through cytokine-driven differentiation of naïve cells. This infiltration of T cells, historically associated with Th1 responses, now emphasizes the significance of Th17 cells in RA pathogenesis. Additionally, evidence suggests that Th22 cells also contribute to the complex landscape of RA development. Furthermore, the function of regulatory T cells (Tregs) becomes compromised, and effector cells develop resistance to suppression, further disrupting the delicate immune balance within inflamed joints. The accompanying figure illustrates the distinctive chemokine receptor expression patterns and the primary secreted cytokines associated with each T cell subtype, highlighting the intricate immune interactions at play in the pathogenesis of RA(Mellado, Martínez-Muñoz *et al*. 2015).

Throughout the progression of Rheumatoid Arthritis (RA), there's a notable recruitment of T cells and various immune cells to the synovial tissue. Here, they engage in the substantial production of proinflammatory cytokines and establish interactions with synovial fibroblasts and macrophages, all of which significantly contribute to the development of the disease. These immune cells encompass both CD4+ and CD8+ T cells, predominantly in an activated state. While RA was traditionally characterized as a Th1-mediated disorder, contemporary evidence underscores the clear involvement of Th17, Th22, and Treg cells. However, it's worth noting that whether these represent entirely separate subpopulations or reflect plasticity and diversity within the Th17 lineage remains an area of ongoing research. Each of these distinct cell subsets plays a role at various stages in the RA disease process,

participating in the intricate web of cell-to-cell interactions that govern the initiation and progression of RA. Their contributions encompass the release of inflammatory mediators, induction of cell proliferation, and promotion of angiogenesis, all contributing to the complex pathogenesis of RA(McInnes and Schett 2011).

2.6 Immunotherapy for RA and its Drawbacks:

The treatments available for rheumatoid arthritis (RA) include medications like anti-inflammatory drugs, corticosteroids, and disease-modifying drugs. These medicines work by calming down the overactive immune system that causes RA symptoms, which helps relieve pain. But, there's a downside – weakening the immune system in this way can make a person more prone to getting infections(Bullock, Rizvi *et al*. 2019).

2.6.1 NSAIDs:

Aspirin can be quite useful for reducing inflammation in rheumatoid arthritis (RA) when taken in high doses. It works by blocking prostaglandins, which play a role in causing inflammation. Aspirin has been around for a long time and is one of the earliest non-steroidal anti-inflammatory drugs (NSAIDs) used to manage joint pain in RA. However, when you take aspirin in high amounts, it can lead to some unwanted effects like ringing in the ears (tinnitus), hearing problems, and stomach discomfort. Moreover, these medications have the advantage of needing less frequent daily dosing. NSAIDs function by blocking cyclooxygenase enzymes, which in turn prevents the production of substances like prostaglandins, prostacyclin, and thromboxane that contribute to inflammation. There are other NSAIDs that are newer on the market than aspirin and just as effective. However, they can come with typical side effects such as nausea, stomach discomfort, ulcers, and the risk of gastrointestinal (GI) bleeding(Ong, Lirk *et al*. 2007).

2.6.2 Corticosteroids:

Corticosteroids are a more powerful anti-inflammatory option compared to NSAIDs, but they bring along a higher risk of side effects. Consequently, they are typically prescribed for a brief duration and at lower doses, specifically during periods of RA exacerbations or

flares. In certain cases, corticosteroid injections directly into the affected joints can be employed to address localized inflammation and its symptoms(Combe, Landewe *et al*. 2017). Their mechanism of action involves inhibiting the release of phospholipids and reducing the activities of eosinophils, which in turn leads to a reduction in inflammation. However, these potent medications come with potential side effects, including decreased bone density, weight gain, the risk of developing diabetes, and immune system suppression(Liu, Ahmet *et al*. 2013).

2.6.3 DMARDs:

The primary objective of disease-modifying antirheumatic drugs (DMARDs) is to achieve remission by impeding or halting the advancement of joint damage and deformities. These drugs are classified as slow-acting because their therapeutic effects typically take several weeks to months to become noticeable. Furthermore, it's worth noting that DMARDs can also lower the likelihood of developing lymphoma, a condition sometimes linked to rheumatoid arthritis (RA)(Smolen, Landewé *et al*. 2010).

2.6.4Methotrexate (MTX):

Methotrexate (MTX) is a type of DMAARDs which serves as the initial second-line medication, often referred to as an anchor drug, in the treatment of certain conditions. It acts as an analogue to folic acid and competitively hinders the binding of dihydrofolic acid (FH2) to the enzyme responsible for converting FH2 into folinic acid (FH4). When FH4 is in short supply, it disrupts the metabolism of purine and pyrimidine, impairs the synthesis of amino acids, and inhibits polyamine production. TX is classified as an immunosuppressive drug and necessitates regular blood tests due to its potential side effects, including liver complications, cirrhosis, and deterioration of bone marrow function(Tian and Cronstein 2007).

2.6.5 Hydroxychloroquine:

Hydroxychloroquine, also known as Plaque nil, is an antimalarial medication that can be employed for the extended management of rheumatoid arthritis (RA). This drug operates by reducing the release of proinflammatory cytokines derived from monocytes. Typical

side effects encompass issues in the gastrointestinal (GI) tract, skin, and central nervous system. Notably, the eyes can be vulnerable to adverse effects, especially when high doses of the medication are used. Therefore, individuals on this treatment regimen need to schedule regular consultations with an ophthalmologist to monitor their eye health(Silva, Mariz *et al*. 2013).

2.6.6 Sulfasalazine:

Sulfasalazine, also known as Azulfidine, is a disease-modifying antirheumatic drug (DMARD) primarily employed for the treatment of irritable bowel disease. When used in conjunction with anti-inflammatory medications, it can also be an option for managing rheumatoid arthritis (RA).

The precise mechanism of how this drug operates in RA treatment remains unidentified. However, it is conjectured that sulfa pyridine, a metabolite produced after the drug is administered, may potentially reduce the secretion of interleukin (IL)-8 and monocyte chemoattractant protein (MCP), both of which are involved in inflammation.

While sulfasalazine is generally well-tolerated, it can produce gastrointestinal and central nervous system symptoms, along with the possibility of causing skin rashes. Notably, individuals with sulfa allergies or sensitivities to salicylate compounds should avoid this medication, as it contains both of these elements(Volin, Harlow *et al*. 1999).

2.7 RA and Vaccine Treatment:

Given the limitations and potential side effects associated with the current treatments for rheumatoid arthritis (RA), some researchers have explored alternative approaches, including vaccines, as potential solutions. One promising avenue of investigation is the development of APL (antigen-presenting cell-targeted peptide) vaccines. These vaccines aim to modulate the immune response by selectively targeting specific cells involved in RA pathogenesis. By focusing on the root causes of the disease, such as abnormal immune responses, APL vaccines have the potential to offer a more precise and effective treatment option with fewer systemic side effects. However, it's important to note that vaccine development and testing are complex processes, and more research is needed to determine

the safety and efficacy of APL vaccines for RA. Nevertheless, the pursuit of innovative therapies like APL vaccines underscores the ongoing commitment to improving the quality of life for individuals living with RA(Subesinghe, Bechman *et al*. 2018).

2.7.1 APL Vaccine and RA:

Rosenthal K.S *et al*. narrated that Altered peptide ligand vaccines employ peptides where specific amino acid residues within an antigen are replaced with different ones. This alteration aims to change the antigen's ability to stimulate the immune system, adjust its electric charge, enhance its stability, or lower the risk of unwanted reactions. In the end, the modified peptide should still effectively provoke the intended immune response so the APL vaccine is the best option to treat RA to avoid side effects without suppressing the immune system(Rosenthal, Mikecz *et al*. 2015).

Correale J, *et al*. did research to check the role of APL against Multiple Sclerosis and stated that autoimmune diseases, such as multiple sclerosis (MS), are characterized by the immune system mistakenly targeting the body's own tissues. In the quest for more effective treatments for these conditions, altered peptide ligand (APL) vaccines have emerged as a promising strategy. APL vaccines involve modifying specific amino acid residues within native disease-related peptides critical for interaction with T-cell receptors (TCRs). These modifications are designed to harness the immune system's response to combat the autoimmune process. In experimental models of autoimmune diseases like MS, APLs have shown the potential to induce immune responses that can protect against or even reverse the disease. The idea behind APL vaccination is to redirect the immune response away from harmful autoreactive T cells towards a more regulatory and tolerogenic response. This therapeutic approach holds great promise because it aims to mitigate the autoimmune response at its core, addressing the root cause of the disease rather than merely managing its symptoms(Correale, Farez *et al*. 2008).

In 2005 *Larche* and his coworkers research about the role of APL against Allergic diseases and stated that Allergic and autoimmune diseases can cause long-lasting health problems, and current treatments often only manage symptoms without fixing the underlying immune issues. Therapeutic vaccines, like altered peptide ligand (APL) vaccines, offer a way to

specifically target and improve these immune responses. By learning from natural processes and immune desensitization methods, we can create effective therapies. One approach is using vaccines with synthetic peptides to target problem-causing T cells, like APL vaccines, which have shown promise. Future work should focus on choosing the right antigens and peptides, optimizing dosages and delivery methods, and finding ways to control immune responses. This strategy, exemplified by APL vaccines, aims for lasting improvements in allergic and autoimmune diseases by directly addressing their immune-related causes(Larche and Wraith 2005).

Barberá *et al.* focused on finding ways to induce immune tolerance using antigen-specific therapies, with the mechanisms, including apoptosis and regulatory T-cells (Tregs). APL-1 is a modified peptide derived from a new CD4+ T-cell target found in the human heat-shock protein of 60 KDa, an autoantigen associated with RA development. Studies have shown that APL can generate CD4+ CD25highFoxp3+ Tregs in various systems. In this investigation, we explored APL-1's ability to trigger apoptosis in peripheral blood mononuclear cells (PBMCs) obtained from RA patients, classified as either active or inactive based on their disease activity score (DAS28). APL reduced the viability of PBMCs from active patients but not from inactive ones. We confirmed this effect through DNA fragmentation tests and by observing typical cellular changes indicative of apoptosis. Specifically, APL-1 targeted activated CD4+ CD25+ T-cells, not resting CD4+ CD25- T-cells. Moreover, the CD4+ T-cell responses to APL-1 were reliant on presentation through the HLA-DR molecule. In summary, APL is a promising CD4+ T-cell epitope with potential to modulate inflammatory immune responses in PBMCs from RA patients. It achieves this by promoting the development of CD4+ CD25highFoxp3+ Tregs and inducing apoptosis in activated CD4+ T-cells. These findings suggest that further research into APL as a potential therapeutic option for RA treatment is warranted, emphasizing its significance in the context of APL-based therapies(Barberá, Lorenzo *et al*. 2013).

Mayer's *et al.* aimed to find peptides that could change the immune response to type II collagen (CII) within the context of HLA-DR. By suppressing the immune reaction to CII, they sought to better understand its role in causing disease. They created synthetic analog peptides with deliberate changes in specific positions to disrupt the DR1-restricted immune

response. When these analog peptides were used to treat collagen-induced arthritis in DR1 transgenic mice, they discovered one particular analog peptide, CII 256–276 (N263, D266), that reduced T-cell responses in laboratory tests. Their findings indicate that CII 256–276 (N263, D266) is a powerful inhibitor of the DR-mediated immune response to CII and that its effects involve interleukin-4. This suggests that an analog peptide of CII, which can be recognized by T-cells in the context of the human major histocompatibility complex, could hold promise as a potential therapy for autoimmune arthritis. In the context of altered peptide ligand (APL) therapies, these results underscore the potential therapeutic significance of such analog peptides for autoimmune diseases like arthritis(Myers, Sakurai *et al*. 2004).

Upon reviewing this initial research on autoimmune diseases like RA and the effectiveness of APL vaccines against them, it becomes evident that the APL vaccine represents a promising treatment option. This choice appears preferable to existing treatments and addresses limitations found in previous research.

Chapter3

3. Materials and Methods

3.1 Selection of Target Protein:

Vimentin protein was selected as a target because Vimentin is a protein that plays a role in the pathogenesis of RA. In RA, the immune system mistakenly attacks the synovium, the lining of the membranes that surround the joints. Vimentin is one of the autoantigens involved in RA, meaning that it can trigger an autoimmune response in individuals with RA after citrullination (Vossenaar, Deprés *et al*. 2004).

3.2 Proteome Retrieval:

The retrieval of protein sequences was conducted using the National Center for Biotechnology Information (NCBI) database, accessible at (https://www.ncbi.nlm.nih.gov/). NCBI is a national database where we can find information about genes, proteins and medical research(O'Leary, Wright *et al*. 2016). All the sequences of Vimentin protein present on the NCBI were retrieved.

3.3 Development of Consensus Sequence:

Sequences obtained from NCBI was analyzed by performing a BLAST search. All of these sequences displayed a 100% similarity, so there was no need to create a consensus sequence. Consequently, one of the vimentin sequences obtained was selected for further analysis.

3.3 Prediction of B Cell Epitopes:

The selected vimentin sequence was then employed to predict B cell epitopes. Online tool, ABCpred (http://crdd.osdd.net/raghava/abcpred/), was utilized for this purpose. ABCpred specifically focuses on predicting the linear continuous epitope sequences recognized by B cells(Saha, Raghava *et al*. 2006).

The vimentin sequence was uploaded on this software application. In the software ABCpred, the threshold level was set at 0.51. Only the epitopes with prediction scores exceeding their respective threshold levels were chosen for further analysis.

3.4 Prediction of T Cell Epitopes:

Vimentin sequence was further uploaded to HLApred software to predict T cell epitopes. For this task, the online prediction tool HLApred (http://crdd.osdd.net/raghava/hlapred/ref.html) was utilized. HLApred is an online tool designed to predict the HLA binding regions within a provided antigenic query sequence. It can predict binding regions for both class I and class II HLA molecules(Brusic, Rudy *et al*. 1994).

The query sequences were uploaded to the online HLApred server, and both HLA classes were chosen for predicting binding regions within our query sequence. A threshold level of 3% was selected to include the larger number of epitopes. Epitopes displaying high scores and strong binding affinities were then singled out for further analysis.

3.5 Selection of B-cell Epitopes:

Only B-cell Epitopes ranked from 1 to 23 were selected for further analysis.

3.6 Prediction of IFN-

The prediction of IFN-γ epitopes was performed using the IFNepitope online server (http://crdd.osdd.net/raghava/ifnepitope/). This particular online tool is specialized for the prediction of epitopes that can induce the production of IFN-γ(Dhanda, Vir *et al*. 2013).

The B-cell epitopes that were previously identified and prioritized were submitted to the IFNepitope server. Only those epitopes that demonstrated the capability to induce an IFN-γ response were chosen for further consideration.

3.7 Immunogenic Potential:

To assess the immunogenic potential of the epitopes, the MHC1 immunogenicity score was determined using the Immune Epitope Database (IEDB) server, which can be accessed at http://tools.iedb.org/immunogenicity/(Vita, Mahajan *et al*. 2019).

The epitopes that had been previously chosen were uploaded to the server, and their immunogenic potential was assessed. Any epitopes that exhibited negative immunogenic values were subsequently eliminated from consideration.

3.8 Antigenic Potential:

The antigenicity potential of the epitopes was determined using the Scratch Protein Predictor tool, accessible at https://scratch.proteomics.ics.uci.edu/(Cheng, Randall *et al*. 2005). Epitopes with scores above the threshold of 0.5 were included, while those below the threshold and considered non-antigenic by the tool were excluded from further consideration.

3.9 Allergen Prediction:

To assess the allergenicity of the epitopes, two online tools, AllergenFP (https://ddg-pharmfac.net/AllergenFP/) and Allertop (https://www.ddg-pharmfac.net/AllerTOP/), were employed(Dimitrov, Flower *et al*. 2013). Only the epitopes that were predicted as non-allergen by these tools were retained. Consequently, the selected epitopes exhibited IFNγ producing, positive immunogenicity scores, high antigenicity scores, and were non-allergen in nature. These attributes are paramount for an effective vaccine as they trigger robust immune responses, enhance recognition by the immune system, and ensure safety, making them key components in RA.

3.10 3D Structure Modeling of the B-cell epitopes:

The three-dimensional modeling of the chosen B-cell epitopes was conducted using the trRosetta tool, which can be accessed at http://yanglab.nankai.edu.cn/trRosetta/. trRosetta is an online tool utilized for the prediction of 3D structures of proteins(Du, Su *et al*. 2021).

3.11 Molecular Docking of B-cell epitopes with Receptors:

A series of docking experiments was conducted, where we individually docked selected B-cell epitopes with the receptors HLADRB1 and HLADRB4 using the ClusPro software (accessible at https://cluspro.bu.edu/)(Kozakov, Hall *et al*. 2017). It was observed that, similar to numerous autoimmune disorders in humans, the susceptibility and severity of rheumatoid arthritis (RA) is influenced by the involvement of multiple genes. Notably, the most substantial genetic correlation is associated with the HLA-DRB1 genes, with a particular focus on the HLA-DR4 variants. These variants encompass commonly found molecules, including HLA DRB1*04:01, *04:04, and *01:01. HLADRB1 genes can make someone more likely to get Rheumatoid Arthritis (RA), while HLADRB4 genes affect how the immune system responds in RA. Together, these genes play a role in who gets the disease and how severe it can be. (Louthrenoo, Kasitanon *et al*. 2015).

3.12 Selection of B-cell epitope for alteration:

To develop a therapeutic vaccine for the treatment of Rheumatoid Arthritis (RA), the most favorable binding B-cell epitopes was identified with HLADRB1 and HLADRB4 receptors, based on their lowest energy scores. These selected epitopes served as the foundation for amino acid substitutions, with the aim of constructing a peptide suitable for therapeutic use against RA. The alteration of the selected peptide was carried out to achieve a lower score for IFN-γ and higher scores for IL-10 and IL-4, with the objective of designing an APL vaccine for RA that emphasizes regulatory responses over inflammatory ones. This strategic shift is grounded in the recognition that in RA, an overactive immune response characterized by excessive IFN-γ production can contribute to tissue damage and inflammation. By prioritizing the induction of IL-10 and IL-4, which are associated with regulatory and anti-inflammatory responses, the APL vaccine is aimed at restoring immune balance. This concept aligns with the goal of developing a therapeutic intervention that not only targets the underlying causes of RA but also mitigates the autoimmune-driven inflammation, ultimately improving patient outcomes and quality of life.

3.13 Docking Interactions:

For in-depth analysis of the selected docked clusters, the PDBsum server was employed, which can be accessed at https://www.ebi.ac.uk/pdbsum/online. This server allowed to perform a comprehensive examination of the docking results and obtain valuable insights into the interactions and structural aspects of the complexes(Porollo, Adamczak *et al*. 2004).

3.14 Alteration of The Selected B-cell epitopes:

Selected B-cell epitopes were altered by replacing one or two amino acids that had the strongest bonds with the receptor. This modification was carried out using the Discovery Studio software, which can be accessed at https://discover.3ds.com/discovery-studio-visualizer-download. Discovery Studio is a powerful software tool widely used in molecular modeling and drug discovery. It enables researchers to perform complex molecular modifications, such as substituting amino acids in proteins or altering chemical structures in small molecules. The software provides a user-friendly interface and a suite of tools for visualizing and analyzing molecular structures, making it a valuable resource for designing and optimizing compounds for various biomedical applications, including drug development and protein engineering(Li and Biotechnology 2004).

3.15 Ramachandran Plot Analysis:

The Ramachandran plot analysis is a fundamental tool for assessing the validity of 3D protein structures and vaccine constructs. It primarily focuses on the torsion angles phi (φ) and psi (ψ) of amino acids within a given protein. This plot provides valuable insights into the conformational quality of a protein or construct by revealing which torsion angles are permissible and likely. Analyzing the Ramachandran plot aids in determining the overall quality and stability of a protein's structure, helping researchers ensure the accuracy and reliability of their models(Sheik, Sundararajan *et al*. 2002).

The Pdb-formatted structure of modified peptide were submitted to the RAMPAGE server, available at http://mordred.bioc.cam.ac.uk/~rapper/rampage.php, to conduct a Ramachandran analysis. This analysis exhibits the conformational quality and validity of

the altered peptide's structure, helping ensure its suitability for further applications, particularly in therapeutic contexts.

3.16 Docking of Altered Peptide:

A subsequent round of docking experiments was performed by using the ClusPro software (accessible at https://cluspro.bu.edu/) to assess the binding interactions of the altered peptides with the receptors. This step aimed to determine if the modified peptides exhibited binding to a different location than the previous interactions. Such changes in binding sites can potentially yield a desired therapeutic response against the disease, enhancing the effectiveness of the peptide in targeting and treating the condition.

3.17 M. D Simulations of docked complex:

To evaluate the stability of the docked complex, Molecular Dynamics (MD) simulations was conducted using the GROMACS software, which can be accessed at https://www.gromacs.org/. The simulations were carried out over a duration of 50 nanoseconds (ns). During these simulations, Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) analysis were performed. These analysis provided valuable insights into the structural stability and flexibility of the altered peptide within the complex, helping us assess its suitability for therapeutic purposes. GROMACS is a widely used and powerful molecular dynamics simulation software designed for the study of bio molecular systems. It enables researchers to simulate the behavior of molecules at an atomic level over time, providing insights into their structural dynamics and interactions. GROMACS is particularly valuable in fields like computational chemistry and structural biology, where understanding molecular behavior is crucial for drug discovery, protein folding, and other biophysical studies. Its versatility, efficiency, and extensive analysis tools make it an essential tool for researchers exploring complex bio molecular systems(Uchôa, Jorge *et al*. 2004).

Chapter4

4. Results

4.1 Proteome Retrieval:

A total of 16 sequences of Vimentin protein were retrieved from NCBI. Their accession number with the sequences are given in the table 4.1.

Table4.1: Sequences of Vimentin protein with their accession numbers

>NP_003371.2 MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYAS SPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQ ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE
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>KAI4075365.1 MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYAS SPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQ ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE
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>KAI2555179.1

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>KAI2555178.1

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>AAH66956.1

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DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
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>AAH30573.1

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DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE

YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

>AAH00163.2
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ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
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>ALQ33846.1
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SPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQ
ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
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>EAW86216.1
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DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
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YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
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>EAW86215.1
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ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
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>ACA06102.1
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DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
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>ACA06101.1

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ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
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>BAD96322.1

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ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

>BAD96227.1

MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYAS
SPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQ
ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ
NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

>BAD96202.1

MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYAS
SPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQ
ELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQV
DQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNA
SLARLDLERKMESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTA
ALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTE
YRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQ

NMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSLNLR
ETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

4.2 Sequence Alignment Results:

In the course of this study, a comprehensive sequence alignment analysis was conducted on a set of 16 protein sequences of interest. Notably, all 16 sequences exhibited an extraordinary level of similarity, with a 100% sequence identity. This exceptional level of homogeneity underscores the conservation of these sequences and suggests a strong functional or evolutionary relationship among them. The results of this sequence alignment provide a foundation for further investigations into the functional roles and implications of these highly conserved proteins in our research.

Here is the sequence alignment descriptive table:

Table 4.2: The sequence alignment of the vimentin protein, revealing the high degree of conservation with different proteins

Description	Max Score	Total Score	Query Cover	E value	%age identity	Acc. Length	Accession
BAD96227.1	941	941	1	0	100	466	Query_117518
BAD96322.1	941	941	1	0	100	466	Query_117517
ACA06101.1	941	941	1	0	100	466	Query_117516
ACA06102.1	941	941	1	0	100	466	Query_117515
EAW86215.1	941	941	1	0	100	466	Query_117514
EAW86216.1	941	941	1	0	100	466	Query_117513
ALQ33846.1	941	941	1	0	100	466	Query_117512
AAH00163.2	941	941	1	0	100	466	Query_117511
AAH30573.1	941	941	1	0	100	466	Query_117510
AAH66956.1	941	941	1	0	100	466	Query_117509
KAI4075362.1	941	941	1	0	100	466	Query_117506
KAI4075365.1	941	941	1	0	100	466	Query_117505
NP_003371.2	941	941	1	0	100	466	Query_117503
BAD96202.1	941	941	1	0	99.79	466	Query_117519
KAI2555178.1	940	940	1	0	99.79	466	Query_117508
KAI2555179.1	940	940	1	0	99.79	466	Query_117507
AAA61279.1	938	938	1	0	99.57	466	Query_117504

4.2.1 Graphic Summary of Alignment:

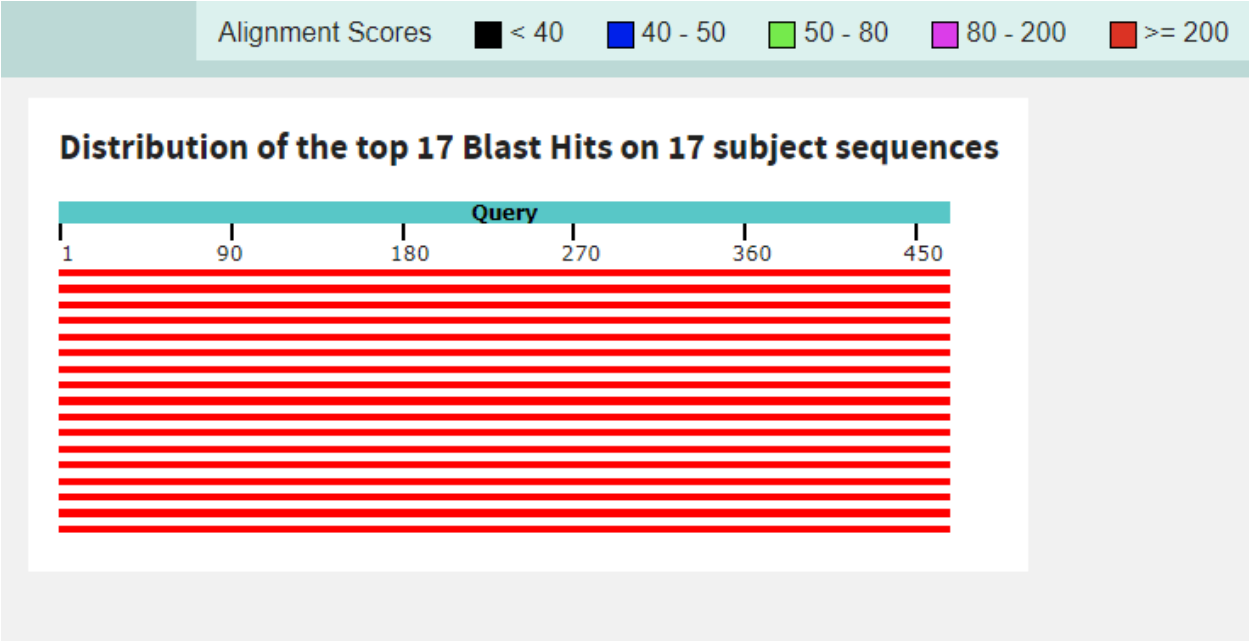


Fig 4.1: Graphic Summary of Alignment: Vimentin protein was BLAST to determine its Homology with other proteins present in database and 17 Hits were found with higher similarity index.

The results from the NCBI BLAST analysis revealed a graphical summary showcasing an exceptional finding. All 16 examined protein sequences exhibited an absolute 100% similarity. This graphical representation visually underscores the profound likeness among these sequences, emphasizing their shared evolutionary importance and potential functional roles in research. This compelling visual aid bolsters the reliability of sequence alignment results and underscores the significance of discovery in this study. These sequences are hundred percent so only one sequence was further selected for study.

4.3 Predicted B-cell epitopes:

ABCpred predicted 42 linear B-cell epitopes for Vimentin protein.

Table 4.3: B cell epitopes predicted by ABCpred.

Rank	Sequence	Start Position	Score
1	EYRRQVQSLTCEVDALKGTN	318	0.87
	LLAELEQLKGQGKSRLGDLY	131	0.87
2	STRTYSLGSALRPSTSRSLY	34	0.83

	TNDKARVEVERDNLAEDIMR	165	0.83
	RMFGGPGTASRPSSSRSYVT	13	0.83
3	LLQDSVDFSLADAINTEFKN	79	0.81
	SSPGGVYATRSSAVRLRSSV	55	0.81
	LLIKTVETRDGQVINETSQH	442	0.81
	QELQAQIQEQHVQIDVDVSK	243	0.81
4	QELQAQIQEQHVQIDVDVSK	426	0.80
	FANYIDKVRFLEQQNKILLA	114	0.80
5	ISLPLPNFSSLNLRETNLDS	411	0.78
	NVKMALDIEIATYRKLLEGE	388	0.78
	HLREYQDLLNVKMALDIEIA	379	0.78
	SRPSSSRSYVTTSTRTYSLG	22	0.78
6	QMREMEENFAVEAANYQDTI	343	0.77
7	LKKLHEEEIQELQAQIQEQH	234	0.76
8	QAKQESTEYRRQVQSLTCEV	311	0.75
	SEAANRNNDALRQAKQESTE	299	0.75
9	NYQDTIGRLQDEIQNMKEEM	357	0.73
10	LARLDLERKVESLQEEIAFL	215	0.70
	GDLYEEEMRELRRQVDQLTN	147	0.70
11	VDTHSKRTLLIKTVETRDGQ	434	0.69
	EIATYRKLLEGEESRISLPL	396	0.69
12	QNMKEEMARHLREYQDLLNV	370	0.68
	VDVSKPDLTAALRDVRQQYE	258	0.68
	LQSFRQDVDNASLARLDLER	203	0.68
13	FKNTRTNEKVELQELNDRFA	96	0.67
	SSAVRLRSSVPGVRLLQDSV	65	0.67
14	YESVAAKNLQEAEEWYKSKF	276	0.66
	EKLQEEMLQREEAENTLQSF	187	0.66
	EVERDNLAEDIMRLREKLQE	172	0.66
15	CEVDALKGTNESLERQMREM	328	0.65
16	QREEAENTLQSFRQDVDNAS	195	0.64
17	EDIMRLREKLQEEMLQREEA	180	0.63
	LEQQNKILLAELEQLKGQGK	124	0.63
18	KVESLQEEIAFLKKLHEEEI	223	0.62
19	GSALRPSTSRSLYASSPGGV	41	0.61
20	LKGQGKSRLGDLYEEEMREL	138	0.58
21	AALRDVRQQYESVAAKNLQE	267	0.57
22	VELQELNDRFANYIDKVRFL	105	0.55
23	EQHVQIDVDVSKPDLTAALR	251	0.52
*threshold = 0.5			

4.4 Predicted T-cell epitopes:

T-cell epitopes were predicted and derived from B-cell epitopes using HLApred. Furthermore, MHCpred was utilized to predict two classes of major histocompatibility complex (MHC) binders: MHCI binders and MHCII binders. This comprehensive analysis allowed to identify potential antigenic regions that may be recognized by both cytotoxic T cells (MHCI binders) and helper T cells (MHCII binders), contributing to a more comprehensive understanding of the immune response against the target antigens. Predicted MHCI and MHCII binders are shown in table 4.4

Table4.4: Predicted MHC1 and MHCII Binders T-cells

B-cell epitopes	-cell	MHCII binder T-cell
EYRRQVQSLTCEVDALKGTN	CEVDALKGT	CEVDALKGT
	EVDALKGTN	EVDALKGTN
	EYRRQVQSL	EYRRQVQSL
	LTCEVDALK	
		QSLTCEVDA
	QVQSLTCEV	QVQSLTCEV
	RQVQSLTCE	RQVQSLTCE
	RRQVQSLTC	RRQVQSLTC
	SLTCEVDAL	SLTCEVDAL
		TCEVDALKG
	VQSLTCEVD	
	YRRQVQSLT	YRRQVQSLT
LLAELEQLKGQGKSRLGDLY	**AELEQLKGQ**	
	ELEQLKGQG	ELEQLKGQG
	EQLKGQGKS	
	GKSRLGDLY	
	GQGKSRLGD	
	KGQGKSRLG	
	LEQLKGQGK	LAELEQLKG
	LKGQGKSRL	
	LLAELEQLK	
	QGKSRLGDL	
	QLKGQGKSR	QGKSRLGDL
		QLKGQGKSR
	ALRPSTSRS	ALRPSTSRS
STRTYSLGSALRPSTSRSLY	**GSALRPSTS**	
		LGSALRPST
	LRPSTSRSL	LRPSTSRSL
	RPSTSRSLY	RPSTSRSLY
	RTYSLGSAL	RTYSLGSAL
	SALRPSTSR	SALRPSTSR
	SLGSALRPS	SLGSALRPS
	STRTYSLGS	STRTYSLGS
	TRTYSLGSA	TRTYSLGSA
	TYSLGSALR	TYSLGSALR
		YSLGSALRP
	ARVEVERDN	

TNDKARVEVERDNLAEDIMR	**DKARVEVER**	
	DNLAEDIMR	DNLAEDIMR
	ERDNLAEDI	ERDNLAEDI
	EVERDNLAE	EVERDNLAE
	KARVEVERD	KARVEVERD
		NDKARVEVE
	RDNLAEDIM	
	RVEVERDNL	RVEVERDNL
		TNDKARVEV
	VERDNLAED	
	VEVERDNLA	VEVERDNLA
RMFGGPGTASRPSSSRSYVT	ASRPSSSRS	ASRPSSSRS
		FGGPGTASR
	GGPGTASRP	GGPGTASRP
	GPGTASRPS	
	GTASRPSSS	
	MFGGPGTAS	
		PGTASRPSS
	PSSSRSYVT	PSSSRSYVT
	RMFGGPGTA	
	RPSSSRSYV	RPSSSRSYV
	SRPSSSRSY	SRPSSSRSY
	TASRPSSSR	TASRPSSSR
LLQDSVDFSLADAINTEFKN	**ADAINTEFK**	
	DAINTEFKN	DAINTEFKN
	DFSLADAIN	DFSLADAIN
	DSVDFSLAD	DSVDFSLAD
	FSLADAINT	FSLADAINT
		LADAINTEF
	LQDSVDFSL	LLQDSVDFS
	QDSVDFSLA	QDSVDFSLA
	SLADAINTE	SLADAINTE
	SVDFSLADA	SVDFSLADA
	VDFSLADAI	VDFSLADAI
SSPGGVYATRSSAVRLRSSV	ATRSSAVRL	ATRSSAVRL
	GGVYATRSS	
	GVYATRSSA	GVYATRSSA
		PGGVYATRS
		RSSAVRLRS
	SAVRLRSSV	SAVRLRSSV

	SPGGVYATR	
		SSAVRLRSS
	SSPGGVYAT	
	TRSSAVRLR	TRSSAVRLR
	VYATRSSAV	
	YATRSSAVR	YATRSSAVR
LLIKTVETRDGQVINETSQH	**DGQVINETS**	
	ETRDGQVIN	
	GQVINETSQ	
	IKTVETRDG	
	KTVETRDGQ	KTVETRDGQ
		LIKTVETRD
		LLIKTVETR
	QVINETSQH	QVINETSQH
	RDGQVINET	
	TRDGQVINE	TRDGQVINE
	TVETRDGQV	TVETRDGQV
	VETRDGQVI	
QELQAQIQEQHVQIDVDVSK	**AQIQEQHVQ**	
	ELQAQIQEQ	ELQAQIQEQ
	EQHVQIDVD	EQHVQIDVD
	HVQIDVDVS	HVQIDVDVS
	IQEQHVQID	
	LQAQIQEQH	
	QAQIQEQHV	QAQIQEQHV
	QELQAQIQE	QELQAQIQE
	QEQHVQIDV	QEQHVQIDV
	QHVQIDVDV	
	QIQEQHVQI	QIQEQHVQI
	VQIDVDVSK	
TNLDSLPLVDTHSKRTLLIK	DSLPLVDTH	DSLPLVDTH
	DTHSKRTLL	DTHSKRTLL
	HSKRTLLIK	HSKRTLLIK
		LDSLPLVDT
	LPLVDTHSK	
	LVDTHSKRT	LVDTHSKRT
		NLDSLPLVD
	PLVDTHSKR	PLVDTHSKR
	SLPLVDTHS	SLPLVDTHS
	THSKRTLLI	

	TNLDSLPLV	TNLDSLPLV
	VDTHSKRTL	
FANYIDKVRFLEQQNKILLA	**ANYIDKVRF**	
	DKVRFLEQQ	DKVRFLEQQ
	EQQNKILLA	EQQNKILLA
	FANYIDKVR	FANYIDKVR
	FLEQQNKIL	
		IDKVRFLEQ
	KVRFLEQQN	KVRFLEQQN
	LEQQNKILL	
	NYIDKVRFL	NYIDKVRFL
	RFLEQQNKI	RFLEQQNKI
	VRFLEQQNK	
	YIDKVRFLE	YIDKVRFLE
ISLPLPNFSSLNLRETNLDS	FSSLNLRET	FSSLNLRET
		ISLPLPNFS
	LNLRETNLD	LNLRETNLD
		LPLPNFSSL
	LPNFSSLNL	LPNFSSLNL
	NFSSLNLRE	NFSSLNLRE
		NLRETNLDS
	PLPNFSSLN	PLPNFSSLN
	PNFSSLNLR	PNFSSLNLR
	SLNLRETNL	SLNLRETNL
	SLPLPNFSS	SLPLPNFSS
	SSLNLRETN	
NVKMALDIEIATYRKLLEGE	**ALDIEIATY**	
	ATYRKLLEG	ATYRKLLEG
	DIEIATYRK	DIEIATYRK
	EIATYRKLL	EIATYRKLL
		IATYRKLLE
	IEIATYRKL	IEIATYRKL
	KMALDIEIA	
		LDIEIATYR
	MALDIEIAT	MALDIEIAT
	NVKMALDIE	NVKMALDIE
	TYRKLLEGE	TYRKLLEGE
	VKMALDIEI	
HLREYQDLLNVKMALDIEIA	DLLNVKMAL	DLLNVKMAL
	EYQDLLNVK	EYQDLLNVK

	HLREYQDLL	HLREYQDLL
	KMALDIEIA	
		LLNVKMALD
		LNVKMALDI
		LREYQDLLN
	NVKMALDIE	NVKMALDIE
	QDLLNVKMA	QDLLNVKMA
	REYQDLLNV	REYQDLLNV
	VKMALDIEI	
	YQDLLNVKM	YQDLLNVKM
SRPSSSRSYVTTSTRTYSLG	PSSSRSYVT	PSSSRSYVT
	RPSSSRSYV	RPSSSRSYV
	RSYVTTSTR	RSYVTTSTR
	SRPSSSRSY	SRPSSSRSY
	SRSYVTTST	
	SSRSYVTTS	
	SSSRSYVTT	
	SYVTTSTRT	SYVTTSTRT
	TSTRTYSLG	TSTRTYSLG
	TTSTRTYSL	TTSTRTYSL
	VTTSTRTYS	VTTSTRTYS
	YVTTSTRTY	YVTTSTRTY
QMREMEENFAVEAANYQDTI	**AVEAANYQD**	
	EAANYQDTI	
	EENFAVEAA	
	EMEENFAVE	EMEENFAVE
	ENFAVEAAN	
	FAVEAANYQ	FAVEAANYQ
	MEENFAVEA	MEENFAVEA
	MREMEENFA	MREMEENFA
		NFAVEAANY
	QMREMEENF	
	REMEENFAV	
	VEAANYQDT	
LKKLHEEEIQELQAQIQEQH	EEEIQELQA	EEEIQELQA
	EEIQELQAQ	EEIQELQAQ
	EIQELQAQI	EIQELQAQI
	ELQAQIQEQ	ELQAQIQEQ
	HEEEIQELQ	HEEEIQELQ
		IQELQAQIQ

	KKLHEEEIQ	
	KLHEEEIQE	KLHEEEIQE
	QELQAQIQE	QELQAQIQE
QAKQESTEYRRQVQSLTCEV	**AKQESTEYR**	
	ESTEYRRQV	ESTEYRRQV
	EYRRQVQSL	EYRRQVQSL
	KQESTEYRR	KQESTEYRR
		QAKQESTEY
	QESTEYRRQ	QESTEYRRQ
	QVQSLTCEV	QVQSLTCEV
	RQVQSLTCE	RQVQSLTCE
	RRQVQSLTC	RRQVQSLTC
	STEYRRQVQ	
	TEYRRQVQS	TEYRRQVQS
	YRRQVQSLT	YRRQVQSLT
SEAANRNNDALRQAKQESTE	**AANRNNDAL**	
	ALRQAKQES	ALRQAKQES
	ANRNNDALR	
	DALRQAKQE	DALRQAKQE
	EAANRNNDA	
		LRQAKQEST
	NDALRQAKQ	NDALRQAKQ
	NNDALRQAK	NNDALRQAK
	NRNNDALRQ	NRNNDALRQ
	RNNDALRQA	RNNDALRQA
	RQAKQESTE	
	SEAANRNND	SEAANRNND
NYQDTIGRLQDEIQNMKEEM	**DEIQNMKEE**	
	DTIGRLQDE	DTIGRLQDE
	EIQNMKEEM	EIQNMKEEM
	GRLQDEIQN	
	IGRLQDEIQ	
	LQDEIQNMK	LQDEIQNMK
	NYQDTIGRL	NYQDTIGRL
	QDEIQNMKE	
		QDTIGRLQD
	RLQDEIQNM	RLQDEIQNM
	TIGRLQDEI	TIGRLQDEI
	YQDTIGRLQ	YQDTIGRLQ
LARLDLERKVESLQEEIAFL	ARLDLERKV	ARLDLERKV

		DLERKVESL
	ERKVESLQE	ERKVESLQE
	ESLQEEIAF	
	KVESLQEEI	KVESLQEEI
		LARLDLERK
		LDLERKVES
	LERKVESLQ	
	RKVESLQEE	
		RLDLERKVE
	SLQEEIAFL	SLQEEIAFL
	VESLQEEIA	
GDLYEEEMRELRRQVDQLTN		**DLYEEEMRE**
		EEEMRELRR
	EEMRELRRQ	EEMRELRRQ
	ELRRQVDQL	ELRRQVDQL
	EMRELRRQV	EMRELRRQV
	GDLYEEEMR	
	LRRQVDQLT	
		LYEEEMREL
		MRELRRQVD
	RELRRQVDQ	RELRRQVDQ
	RRQVDQLTN	RRQVDQLTN
	YEEEMRELR	YEEEMRELR
VDTHSKRTLLIKTVETRDGQ	DTHSKRTLL	DTHSKRTLL
	HSKRTLLIK	HSKRTLLIK
	IKTVETRDG	
	KRTLLIKTV	KRTLLIKTV
	KTVETRDGQ	KTVETRDGQ
		LIKTVETRD
	RTLLIKTVE	RTLLIKTVE
	SKRTLLIKT	SKRTLLIKT
	THSKRTLLI	
		TLLIKTVET
	VDTHSKRTL	
EIATYRKLLEGEESRISLPL	ATYRKLLEG	ATYRKLLEG
	EESRISLPL	EESRISLPL
		EGEESRISL
	EIATYRKLL	EIATYRKLL
	GEESRISLP	GEESRISLP
		IATYRKLLE

		LLEGEESRI
	TYRKLLEGE	TYRKLLEGE
	YRKLLEGEE	YRKLLEGEE
QNMKEEMARHLREYQDLLNV	**ARHLREYQD**	
		EEMARHLRE
	HLREYQDLL	HLREYQDLL
	KEEMARHLR	KEEMARHLR
	MARHLREYQ	MARHLREYQ
	MKEEMARHL	MKEEMARHL
	NMKEEMARH	NMKEEMARH
	QNMKEEMAR	
	REYQDLLNV	REYQDLLNV
	RHLREYQDL	
VDVSKPDLTAALRDVRQQYE	ALRDVRQQY	ALRDVRQQY
		DLTAALRDV
	DVSKPDLTA	
	KPDLTAALR	KPDLTAALR
		LRDVRQQYE
		LTAALRDVR
		PDLTAALRD
	SKPDLTAAL	SKPDLTAAL
		VSKPDLTAA
LQSFRQDVDNASLARLDLER	ASLARLDLE	ASLARLDLE
	DNASLARLD	DNASLARLD
	DVDNASLAR	DVDNASLAR
	FRQDVDNAS	FRQDVDNAS
		LQSFRQDVD
		NASLARLDL
	QDVDNASLA	QDVDNASLA
	QSFRQDVDN	QSFRQDVDN
		RQDVDNASL
	SFRQDVDNA	SFRQDVDNA
	SLARLDLER	SLARLDLER
	VDNASLARL	VDNASLARL
FKNTRTNEKVELQELNDRFA	**EKVELQELN**	
	ELQELNDRF	ELQELNDRF
	FKNTRTNEK	FKNTRTNEK
	KNTRTNEKV	KNTRTNEKV
	KVELQELND	KVELQELND
		LQELNDRFA

	NEKVELQEL	NEKVELQEL
	NTRTNEKVE	NTRTNEKVE
	RTNEKVELQ	
	TNEKVELQE	
	TRTNEKVEL	TRTNEKVEL
	VELQELNDR	VELQELNDR
SSAVRLRSSVPGVRLLQDSV	AVRLRSSVP	AVRLRSSVP
	GVRLLQDSV	GVRLLQDSV
	LRSSVPGVR	
	PGVRLLQDS	PGVRLLQDS
		RSSVPGVRL
	SAVRLRSSV	SAVRLRSSV
		SSAVRLRSS
	SSVPGVRLL	SSVPGVRLL
	SVPGVRLLQ	SVPGVRLLQ
	VRLRSSVPG	
YESVAAKNLQEAEEWYKSKF	AAKNLQEAE	AAKNLQEAE
	AEEWYKSKF	
	AKNLQEAEE	
	EAEEWYKSK	
	ESVAAKNLQ	ESVAAKNLQ
	KNLQEAEEW	KNLQEAEEW
	LQEAEEWYK	
	QEAEEWYKS	QEAEEWYKS
	SVAAKNLQE	SVAAKNLQE
	VAAKNLQEA	
		NLQEAEEWY
	YESVAAKNL	YESVAAKNL
EKLQEEMLQREEAENTLQSF	EAENTLQSF	EAENTLQSF
	EEAENTLQS	EEAENTLQS
	EEMLQREEA	EEMLQREEA
	EKLQEEMLQ	
	KLQEEMLQR	KLQEEMLQR
		LQEEMLQRE
		LQREEAENT
	MLQREEAEN	MLQREEAEN
	QEEMLQREE	QEEMLQREE
	QREEAENTL	QREEAENTL
	REEAENTLQ	
EVERDNLAEDIMRLREKLQE		**AEDIMRLRE**

	DIMRLREKL	DIMRLREKL
	DNLAEDIMR	DNLAEDIMR
	EDIMRLREK	EDIMRLREK
	ERDNLAEDI	ERDNLAEDI
	EVERDNLAE	EVERDNLAE
	IMRLREKLQ	IMRLREKLQ
	LAEDIMRLR	LAEDIMRLR
	MRLREKLQE	MRLREKLQE
		NLAEDIMRL
	RDNLAEDIM	
	VERDNLAED	
CEVDALKGTNESLERQMREM	**ALKGTNESL**	
	CEVDALKGT	CEVDALKGT
		DALKGTNES
		ESLERQMRE
	EVDALKGTN	EVDALKGTN
	GTNESLERQ	
	KGTNESLER	KGTNESLER
	LKGTNESLE	
	NESLERQMR	NESLERQMR
	SLERQMREM	SLERQMREM
	TNESLERQM	TNESLERQM
	VDALKGTNE	
QREEAENTLQSFRQDVDNAS	AENTLQSFR	AENTLQSFR
	EAENTLQSF	EAENTLQSF
	EEAENTLQS	EEAENTLQS
	ENTLQSFRQ	ENTLQSFRQ
	FRQDVDNAS	FRQDVDNAS
		LQSFRQDVD
	NTLQSFRQD	NTLQSFRQD
	QREEAENTL	QREEAENTL
	QSFRQDVDN	QSFRQDVDN
	REEAENTLQ	
	SFRQDVDNA	SFRQDVDNA
		TLQSFRQDV
EDIMRLREKLQEEMLQREEA	DIMRLREKL	DIMRLREKL
	EDIMRLREK	EDIMRLREK
	EEMLQREEA	EEMLQREEA
	EKLQEEMLQ	
	IMRLREKLQ	IMRLREKLQ

	KLQEEMLQR	KLQEEMLQR
		LQEEMLQRE
	MRLREKLQE	MRLREKLQE
	QEEMLQREE	QEEMLQREE
	REKLQEEML	REKLQEEML
		RLREKLQEE
LEQQNKILLAELEQLKGQGK	**AELEQLKGQ**	
	ELEQLKGQG	ELEQLKGQG
	EQQNKILLA	EQQNKILLA
		ILLAELEQL
		KILLAELEQ
		LAELEQLKG
	LEQLKGQGK	
	LEQQNKILL	
	LLAELEQLK	
	NKILLAELE	NKILLAELE
	QNKILLAEL	QNKILLAEL
		QQNKILLAE
KVESLQEEIAFLKKLHEEEI	**AFLKKLHEE**	
	EEIAFLKKL	EEIAFLKKL
	EIAFLKKLH	EIAFLKKLH
	ESLQEEIAF	
	FLKKLHEEE	FLKKLHEEE
	IAFLKKLHE	IAFLKKLHE
	KVESLQEEI	KVESLQEEI
	LQEEIAFLK	LQEEIAFLK
	QEEIAFLKK	QEEIAFLKK
	SLQEEIAFL	SLQEEIAFL
	VESLQEEIA	
GSALRPSTSRSLYASSPGGV	ALRPSTSRS	ALRPSTSRS
	GSALRPSTS	
	LRPSTSRSL	LRPSTSRSL
		PSTSRSLYA
	RPSTSRSLY	RPSTSRSLY
		RSLYASSPG
	SALRPSTSR	SALRPSTSR
	SLYASSPGG	
	SRSLYASSP	SRSLYASSP
	STSRSLYAS	STSRSLYAS
	TSRSLYASS	TSRSLYASS

LKGQGKSRLGDLYEEEMREL		**DLYEEEMRE**
	GKSRLGDLY	
	KGQGKSRLG	
		LYEEEMREL
		QGKSRLGDL
	SRLGDLYEE	SRLGDLYEE
AALRDVRQQYESVAAKNLQE	AALRDVRQQ	AALRDVRQQ
	ALRDVRQQY	ALRDVRQQY
	DVRQQYESV	
	ESVAAKNLQ	ESVAAKNLQ
		LRDVRQQYE
	QQYESVAAK	QQYESVAAK
	QYESVAAKN	QYESVAAKN
	RDVRQQYES	RDVRQQYES
	RQQYESVAA	RQQYESVAA
	SVAAKNLQE	SVAAKNLQE
	VRQQYESVA	
	YESVAAKNL	YESVAAKNL
VELQELNDRFANYIDKVRFL	**ANYIDKVRF**	
	DRFANYIDK	
		ELNDRFANY
	ELQELNDRF	ELQELNDRF
	FANYIDKVR	FANYIDKVR
		LNDRFANYI
		LQELNDRFA
	NDRFANYID	
	NYIDKVRFL	NYIDKVRFL
	QELNDRFAN	QELNDRFAN
	RFANYIDKV	RFANYIDKV
	VELQELNDR	VELQELNDR
EQHVQIDVDVSKPDLTAALR	DVDVSKPDL	DVDVSKPDL
	DVSKPDLTA	
	EQHVQIDVD	EQHVQIDVD
	HVQIDVDVS	HVQIDVDVS
	IDVDVSKPD	IDVDVSKPD
	KPDLTAALR	KPDLTAALR
	QHVQIDVDV	
	QIDVDVSKP	QIDVDVSKP
	SKPDLTAAL	SKPDLTAAL
	VDVSKPDLT	

	VQIDVDVSK	
		VSKPDLTAA

4.5 Evaluation of B-cell epitopes:

The B-cell epitopes were subjected to an evaluation that considered their potential to trigger immune responses (immunogenicity), their ability to act as immune system targets (antigenicity), their allergenic properties, and their capacity to induce the production of IFN-γ, an important immune signaling molecule. This comprehensive assessment aimed to determine the suitability of these epitopes for various immunological applications. It provided valuable insights into their functional characteristics and potential applications in the context of immunotherapy and related research.

4.5.1. Immunogenic Potential

Immunogenic potential of B-cell epitope was determined using IEDB and results are shown in table 4.5

Table4.5: The B-cell epitopes with their predicted immunogenic Score

Sr No.	B-cell epitopes	Immunogenic Score
1	QMREMEENFAVEAANYQDTI	0.42371
2	TNDKARVEVERDNLAEDIMR	0.3752
3	VELQELNDRFANYIDKVRFL	0.26658
4	FKNTRTNEKVELQELNDRFA	0.23382
5	LARLDLERKVESLQEEIAFL	0.22904
6	GDLYEEEMRELRRQVDQLTN	0.20536
7	LKKLHEEEIQELQAQIQEQH	0.19622
8	QREEAENTLQSFRQDVDNAS	0.16733
9	NVKMALDIEIATYRKLLEGE	0.15466
10	LLQDSVDFSLADAINTEFKN	0.15347
11	LLIKTVETRDGQVINETSQH	0.12194
12	EVERDNLAEDIMRLREKLQE	0.11234
13	LQSFRQDVDNASLARLDLER	0.09272
14	KVESLQEEIAFLKKLHEEEI	0.04628
15	EIATYRKLLEGEESRISLPL	0.03979
16	NYQDTIGRLQDEIQNMKEEM	0.01052
17	HLREYQDLLNVKMALDIEIA	-0.00951
18	EKLQEEMLQREEAENTLQSF	-0.01212
19	VDTHSKRTLLIKTVETRDGQ	-0.03921
20	EQHVQIDVDVSKPDLTAALR	-0.07749

21	QELQAQIQEQHVQIDVDVSK	-0.084
22	ISLPLPNFSSLNLRETNLDS	-0.09555
23	FANYIDKVRFLEQQNKILLA	-0.10578
24	QNMKEEMARHLREYQDLLNV	-0.11128
25	LKGQGKSRLGDLYEEEMREL	-0.13448
26	SSPGGVYATRSSAVRLRSSV	-0.16546
27	CEVDALKGTNESLERQMREM	-0.1849
28	EDIMRLREKLQEEMLQREEA	-0.18748
29	YESVAAKNLQEAEEWYKSKF	-0.20178
30	SSAVRLRSSVPGVRLLQDSV	-0.23354
31	SEAANRNNDALRQAKQESTE	-0.23732
32	EYRRQVQSLTCEVDALKGTN	-0.26824
33	TNLDSLPLVDTHSKRTLLIK	-0.26862
34	VDVSKPDLTAALRDVRQQYE	-0.30623
35	AALRDVRQQYESVAAKNLQE	-0.30885
36	QAKQESTEYRRQVQSLTCEV	-0.32542
37	LEQQNKILLAELEQLKGQGK	-0.41982
38	LLAELEQLKGQGKSRLGDLY	-0.46761
39	RMFGGPGTASRPSSSRSYVT	-0.48593
40	STRTYSLGSALRPSTSRSLY	-0.56262
41	GSALRPSTSRSLYASSPGGV	-0.6145
42	SRPSSSRSYVTTSTRTYSLG	-0.67545

4.5.2 Antigenic Potential:

Antigenic Potential of B-cell epitopes was evaluated by using Scratch Protein Predictor and table 4.6 is showing the score of all B-cell epitopes.

Table4.6: The B-cell epitopes with their predicted Antigenic Potential

Sr. No	B-cell Epitopes	Score
1.	SEAANRNNDALRQAKQESTE	0.76294
2.	GSALRPSTSRSLYASSPGGV	0.663246
3.	LLIKTVETRDGQVINETSQH	0.611471
4.	EDIMRLREKLQEEMLQREEA	0.609354
5.	ISLPLPNFSSLNLRETNLDS	0.606826
6.	NYQDTIGRLQDEIQNMKEEM	0.589577
7.	NVKMALDIEIATYRKLLEGE	0.574369
8.	VDTHSKRTLLIKTVETRDGQ	0.566901
9.	TNDKARVEVERDNLAEDIMR	0.553327
10.	SSAVRLRSSVPGVRLLQDSV	0.535184
11.	SRPSSSRSYVTTSTRTYSLG	0.532904
12.	QAKQESTEYRRQVQSLTCEV	0.426207
13.	GDLYEEEMRELRRQVDQLTN	0.411992

14.	QELQAQIQEQHVQIDVDVSK	0.41083
15.	RMFGGPGTASRPSSSRSYVT	0.407922
16.	EVERDNLAEDIMRLREKLQE	0.396491
17.	EYRRQVQSLTCEVDALKGTN	0.383234
18.	CEVDALKGTNESLERQMREM	0.357733
19.	LKKLHEEEIQELQAQIQEQH	0.347494
20.	EQHVQIDVDVSKPDLTAALR	0.30726
21.	YESVAAKNLQEAEEWYKSKF	0.304838
22.	LKGQGKSRLGDLYEEEMREL	0.291275
23.	AALRDVRQQYESVAAKNLQE	0.287791
24.	EKLQEEMLQREEAENTLQSF	0.285451
25.	QNMKEEMARHLREYQDLLNV	0.24297
26.	QMREMEENFAVEAANYQDTI	0.237643
27.	VDVSKPDLTAALRDVRQQYE	0.229743
28.	TNLDSLPLVDTHSKRTLLIK	0.216048
29.	QREEAENTLQSFRQDVDNAS	0.184858
30.	LQSFRQDVDNASLARLDLER	0.17594
31.	HLREYQDLLNVKMALDIEIA	0.169119
32.	SSPGGVYATRSSAVRLRSSV	0.16643
33.	EIATYRKLLEGEESRISLPL	0.151428
34.	LEQQNKILLAELEQLKGQGK	0.128853
35.	FKNTRTNEKVELQELNDRFA	0.080477
36.	LARLDLERKVESLQEEIAFL	0.076229
37.	VELQELNDRFANYIDKVRFL	0.072656
38.	STRTYSLGSALRPSTSRSLY	0.070172
39.	LLAELEQLKGQGKSRLGDLY	0.069445
40.	LLQDSVDFSLADAINTEFKN	0.04976
41.	FANYIDKVRFLEQQNKILLA	0.03851
42.	KVESLQEEIAFLKKLHEEEI	0.027297

4.5.3 Allergenecity Prediction:

The allergenecity of the B-cell epitopes was also predicted and results are shown in table 4.7

Table4.7: The predicted allergenecity score of the B-cell epitopes

NO.	B-cell epitopes	Score
1.	LKGQGKSRLGDLYEEEMREL	0.7105146
2.	LLQDSVDFSLADAINTEFKN	0.93872172
3.	FANYIDKVRFLEQQNKILLA	0.87099758
4.	VELQELNDRFANYIDKVRFL	0.70346152
5.	FKNTRTNEKVELQELNDRFA	0.69589421
6.	NVKMALDIEIATYRKLLEGE	0.69135148
7.	QREEAENTLQSFRQDVDNAS	0.66081548

8.	LARLDLERKVESLQEEIAFL	0.37901814
9.	EYRRQVQSLTCEVDALKGTN	0.364533
10.	EVERDNLAEDIMRLREKLQE	0.33489606
10.	HLREYQDLLNVKMALDIEIA	0.31151937
12.	QNMKEEMARHLREYQDLLNV	0.25853576
13.	NYQDTIGRLQDEIQNMKEEM	0.19049003
14.	GDLYEEEMRELRRQVDQLTN	0.17752
15.	AALRDVRQQYESVAAKNLQE	0.169171
16.	EKLQEEMLQREEAENTLQSF	0.147460
17.	CEVDALKGTNESLERQMREM	0.099754
18.	QAKQESTEYRRQVQSLTCEV	0.098671
19.	KVESLQEEIAFLKKLHEEEI	0.040581
20.	EDIMRLREKLQEEMLQREEA	0.000553
21.	QMREMEENFAVEAANYQDTI	-0.030285
22.	LEQQNKILLAELEQLKGQGK	-0.0648598
23.	SEAANRNNDALRQAKQESTE	-0.0664382
24.	LQSFRQDVDNASLARLDLER	-0.0940687
25.	YESVAAKNLQEAEEWYKSKF	-0.1064406
26.	TNDKARVEVERDNLAEDIMR	-0.1081661
27.	EIATYRKLLEGEESRISLPL	-0.1323189
28.	LKKLHEEEIQELQAQIQEQH	-0.1489009
29.	QELQAQIQEQHVQIDVDVSK	-0.1550364
30.	LLAELEQLKGQGKSRLGDLY	-0.1688428
31.	ISLPLPNFSSLNLRETNLDS	-0.1703889
32.	STRTYSLGSALRPSTSRSLY	-0.2089816
33.	SRPSSSRSYVTTSTRTYSLG	-0.2382153
34.	SSAVRLRSSVPGVRLLQDSV	-0.3033231
35.	GSALRPSTSRSLYASSPGGV	-0.3088009
36.	SSPGGVYATRSSAVRLRSSV	-0.3095818
37.	LLIKTVETRDGQVINETSQH	-0.3773067
38.	EQHVQIDVDVSKPDLTAALR	-0.4123263
39.	VDVSKPDLTAALRDVRQQYE	-0.4142024
40.	RMFGGPGTASRPSSSRSYVT	-0.4156408
41.	TNLDSLPLVDTHSKRTLLIK	-0.5521154
42.	VDTHSKRTLLIKTVETRDGQ	-0.7749992

4.5.4 Predicted IFN- Inducing epitopes:

The scores of B-cell epitopes capable of inducing IFN-γ production were predicted using the IFNepitope software. Subsequently, those epitopes that did not demonstrate the ability to induce IFN-γ production were excluded from consideration. This step was taken to narrow the selection to epitopes with this specific immunological characteristic, thereby

enhancing their potential relevance in the modulation of immune responses and therapeutic applications.

Table4.8: The IFN producing B-cell epitopes with its scores

Sr. No	B-cell epitopes	Score
1.	LLQDSVDFSLADAINTEFKN	4
2.	LEQQNKILLAELEQLKGQGK	4
3.	EDIMRLREKLQEEMLQREEA	2
4.	STRTYSLGSALRPSTSRSLY	1.972057
5.	GSALRPSTSRSLYASSPGGV	1.637243
6.	QMREMEENFAVEAANYQDTI	1.518895
7.	TNLDSLPLVDTHSKRTLLIK	1.466845
8.	SSAVRLRSSVPGVRLLQDSV	1.442498
9.	EIATYRKLLEGEESRISLPL	1.420712
10.	SRPSSSRSYVTTSTRTYSLG	1.351747
11.	ISLPLPNFSSLNLRETNLDS	1.318252
12.	QREEAENTLQSFRQDVDNAS	1.044403
13.	VELQELNDRFANYIDKVRFL	1.040962
14.	QNMKEEMARHLREYQDLLNV	1.000919
15.	CEVDALKGTNESLERQMREM	1
16.	EKLQEEMLQREEAENTLQSF	0.966425
17.	LARLDLERKVESLQEEIAFL	0.951389
18.	NYQDTIGRLQDEIQNMKEEM	0.944337
19.	FKNTRTNEKVELQELNDRFA	0.908216
20.	RMFGGPGTASRPSSSRSYVT	0.899409
21.	LKKLHEEEIQELQAQIQEQH	0.766762
22.	AALRDVRQQYESVAAKNLQE	0.690791
23.	HLREYQDLLNVKMALDIEIA	0.639767
24.	YESVAAKNLQEAEEWYKSKF	0.610298
25.	VDVSKPDLTAALRDVRQQYE	0.609928
26.	QELQAQIQEQHVQIDVDVSK	0.585565
27.	EVERDNLAEDIMRLREKLQE	0.522238
28.	LLIKTVETRDGQVINETSQH	0.444074
29.	QAKQESTEYRRQVQSLTCEV	0.405978
30.	SEAANRNNDALRQAKQESTE	0.396271
31.	LKGQGKSRLGDLYEEEMREL	0.338296
32.	LLAELEQLKGQGKSRLGDLY	0.337392
33.	EYRRQVQSLTCEVDALKGTN	0.148029

4.6 Selected B-cell epitopes:

From the initial group of 42 B-cell epitopes,28 were selected for advanced docking studies with HLADRB4 and HLADRB1 receptors. These 28 were selected because these immunogenic, antigenic, non-allergens and IFN gamma producing. Docked with these receptors because these receptors play key roles in immune responses, including those related to rheumatoid arthritis (RA). The goal was to pinpoint the epitopes with the strongest binding interactions. These epitopes, known for their ability to produce IFN-γ, as well as their antigenic and immunogenic properties, were chosen for their potential in further research. Selected B-cell epitopes are shown in table 4.9.

This investigation not only helps to understand immune responses in conditions like RA but also opens doors to possible new treatments based on these epitopes.

Table4.9: The selected B-cell epitopes with their predicted scores

Sr N0.	B-cell epitopes	IFN-Score	Antigenicity Score	Immunogenic score	Allergenic Score
1.	GSALRPSTSRSLYASSPGGV	1.6372434	0.663246	0.6145	Negative
2.	ISLPLPNFSSLNLRETNLDS	1.318252	0.606826	0.09555	Negative
3.	EIATYRKLLEGEESRISLPL	1.4207121	0.151428	0.03979	Negative
4.	STRTYSLGSALRPSTSRSLY	1.9720565	0.070172	0.56262	Negative
5.	SRPSSSRSYVTTSTRTYSLG	1.351747	0.532904	0.67545	Negative
6.	VELQELNDRFANYIDKVRFL	1.0409618	0.072656	0.26658	Negative
7.	QELQAQIQEQHVQIDVDVSK	0.58556536	0.41083	0.08468	Negative
8.	LLIKTVETRDGQVINETSQH	0.44407371	0.611471	0.12194	Negative
9.	QNMKEEMARHLREYQDLLNV	1.0009186	0.24297	0.11128	Negative
10.	YESVAAKNLQEAEEWYKSKF	0.61029789	0.304838	0.20178	Negative
11.	HLREYQDLLNVKMALDIEIA	0.63976685	0.169119	0.00951	Negative
12.	QAKQESTEYRRQVQSLTCEV	0.40597822	0.426207	0.32542	Negative
13.	FKNTRTNEKVELQELNDRFA	0.90821579	0.080477	0.23382	Negative
14.	EDIMRLREKLQEEMLQREEA	1.9956789	0.609354	0.18748	Negative
15.	QMREMEENFAVEAANYQDTI	1.5188949	0.237643	0.42371	Negative
16.	AALRDVRQQYESVAAKNLQE	0.69079123	0.287791	0.30885	Negative
17.	SSAVRLRSSVPGVRLLQDSV	1.4424984	0.535184	0.23354	Negative
18.	LARLDLERKVESLQEEIAFL	0.951389	0.076229	0.22904	Negative
19.	NYQDTIGRLQDEIQNMKEEM	0.9443366	0.589577	0.01052	Negative
20.	EVERDNLAEDIMRLREKLQE	0.52223767	0.396491	0.11234	Negative
21.	EYRRQVQSLTCEVDALKGTN	0.14802944	0.383234	0.26824	Negative

22.	LKKLHEEEIQELQAQIQEQH	0.76676225	0.347494	0.19622	Negative
23.	LKGQGKSRLGDLYEEEMREL	0.33829551	0.291275	0.13448	Negative
24.	CEVDALKGTNESLERQMREM	1	0.357733	0.1849	Negative
25.	LEQQNKILLAELEQLKGQGK	1.22456789	0.128853	0.41982	Negative
26.	LLAELEQLKGQGKSRLGDLY	0.33739224	0.069445	0.46761	Negative
27.	EKLQEEMLQREEAENTLQSF	0.96642477	0.285451	0.01212	Negative
28.	SEAANRNNDALRQAKQESTE	0.39627149	0.76294	0.23732	Negative

4.7 Prediction of 3D structures of B-cell epitopes:

The 3D structural models of the chosen 28 B-cell epitopes were generated utilizing the Tr-Rosetta software. This step was undertaken to facilitate the subsequent docking analysis, where these epitopes were paired with the receptors HLADRB1 and HLADRB4. The primary objective was to assess the binding interactions between these epitopes and the receptors.

The ability to predict the 3D structures of these epitopes is instrumental in understanding their structural features and how they may interact with specific receptors, such as HLADRB1 and HLADRB4. This analysis contributes to a more comprehensive comprehension of the molecular aspects underlying immune responses and provides valuable insights for potential therapeutic applications.

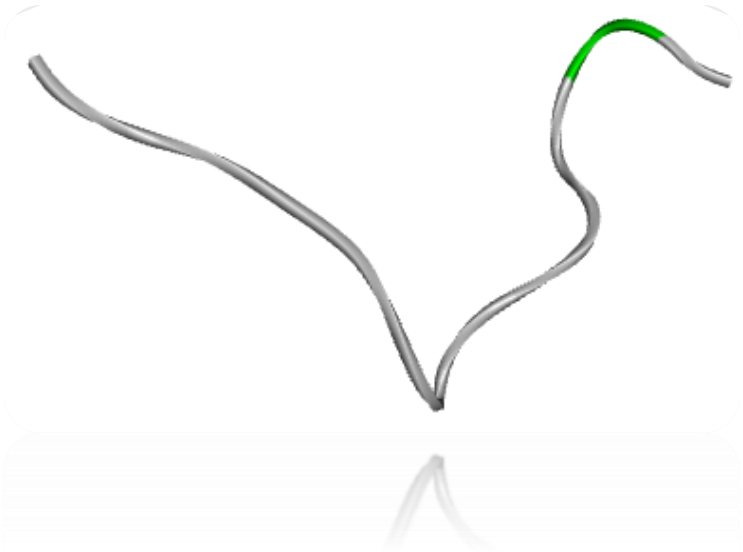


Fig 4.2: Predicted 3D structure of GSALRPSTSRSLYASSPGGV epitope: This epitope was selected for further study because it showed the highest affinity for the HLADRB1 and HLADRB4 receptors.

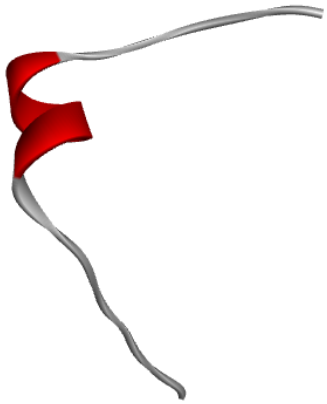


Fig 4.3: Predicted 3D Structure of STRTYSLGSALRPSTSRSLY Epitope: This epitope was selected for further study because it showed the highest affinity for the HLADRB1 and HLADRB4 receptors.

Figures 4.2 and 4.3 present the 3D structural representations (in PDB format) of two specific epitopes: GSALRPSTSRSLYASSPGGV and STRTYSLGSALRPSTSRSLY, with corresponding T.M scores of 0.147 and 0.22, respectively. These selected epitopes were chosen to advance in the research pipeline after undergoing docking simulations with HLADRB1 and HLADRB4 receptors.

These visual representations of the epitopes' 3D structures in Figures 4.2 and 4.3 provide a clear view of their spatial conformation, allowing us to appreciate their unique shapes and characteristics. The T.M scores associated with each epitope reflect the quality of the structural predictions. The epitopes were finalized for further investigation based on their structural attributes and their compatibility with HLADRB1 and HLADRB4 receptors, promising potential insights into their immunological and therapeutic roles.

4.8 Docking of B-cell epitopes with HLADRB1 and HLADRB4 Receptors:

The docking analysis of all 28 B-cell epitopes with their respective receptors was conducted using the Cluspro server. Each epitope was individually docked with both HLADRB1 and HLADRB4 receptors. The results of this analysis revealed a strong binding affinity between the epitopes and both receptors. To assess binding affinity more precisely, the lowest energy and optimal conformation were considered.

To achieve this, the 3D crystal structures of HLADRB1 and HLADRB4 were obtained from the RCSB PDB database, with PDB structure IDs 1AQD and 2SEB, respectively. Initially acquired in complex forms with other molecules, these structures were subsequently separated and refined using the Discovery Studio software.

This meticulous process ensured that well-defined receptor structures were obtained for further analysis, guaranteeing that subsequent investigations were conducted with the highest level of structural precision and reliability.

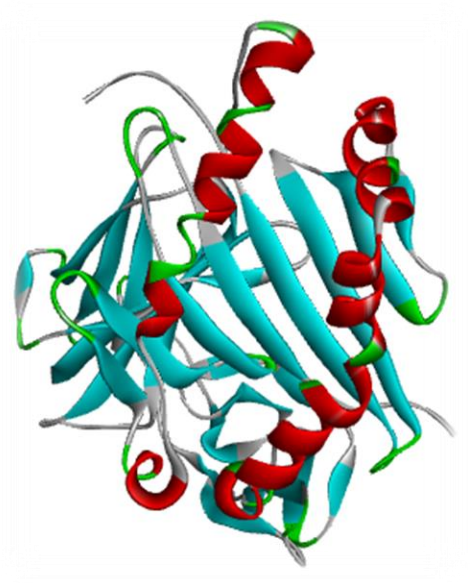


Fig4.4: Predicted 3D structure of HLADRB4 receptor

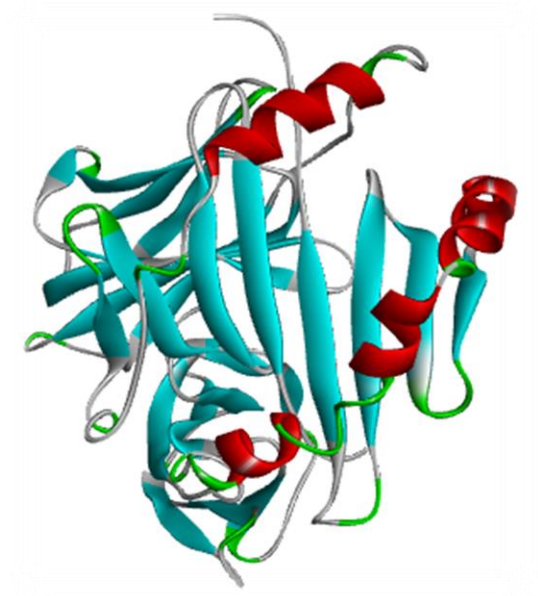


Fig4.5: Predicted 3D structure of HLADRB1 Receptor

Table4.10: The predicted lowest energies of docked complex of B-cell epitopes and HLADRB1

Sr. No	B-cell epitopes	Lowest energy
1.	STRTYSLGSALRPSTSRSLY	-1029.4

2.	GSALRPSTSRSLYASSPGGV	-959.6
3.	ISLPLPNFSSLNLRETNLDS	-957.3
4.	EIATYRKLLEGEESRISLPL	-941.9
5.	SRPSSSRSYVTTSTRTYSLG	-921.4
6.	YESVAAKNLQEAEEWYKSKF	-862.8
7.	VELQELNDRFANYIDKVRFL	-862.1
8.	HLREYQDLLNVKMALDIEIA	-849.3
9.	LLIKTVETRDGQVINETSQH	-808.7
10.	SSAVRLRSSVPGVRLLQDSV	-804
11.	QAKQESTEYRRQVQSLTCEV	-798.1
12.	EDIMRLREKLQEEMLQREEA	-770.1
13.	FKNTRTNEKVELQELNDRFA	-765.5
14.	LKKLHEEEIQELQAQIQEQH	-760.8
15.	QMREMEENFAVEAANYQDTI	-755.1
16.	LKGQGKSRLGDLYEEEMREL	-750.6
17.	EVERDNLAEDIMRLREKLQE	-749.4
18.	LARLDLERKVESLQEEIAFL	-734.3
19.	QNMKEEMARHLREYQDLLNV	-729.4
20.	NYQDTIGRLQDEIQNMKEEM	-720.5
21.	AALRDVRQQYESVAAKNLQE	-714
22.	QELQAQIQEQHVQIDVDVSK	-704
23.	CEVDALKGTNESLERQMREM	-699.7
24.	LEQQNKILLAELEQLKGQGK	-697
25.	LLAELEQLKGQGKSRLGDLY	-681.1
26.	EYRRQVQSLTCEVDALKGTN	-679.6
27.	EKLQEEMLQREEAENTLQSF	-625.4
28.	SEAANRNNDALRQAKQESTE	-519.3

Table4.11: The predicted lowest energies of docked complex of B-cell epitopes with HLADRB4

Sr. No	**B-cell epitopes**	**Lowest energies**
1.	GSALRPSTSRSLYASSPGGV	-1010
2.	ISLPLPNFSSLNLRETNLDS	-1000.5
3.	EIATYRKLLEGEESRISLPL	-945.5
4.	STRTYSLGSALRPSTSRSLY	-940.2
5.	SRPSSSRSYVTTSTRTYSLG	-937.1
6.	VELQELNDRFANYIDKVRFL	-863.1
7.	QELQAQIQEQHVQIDVDVSK	-850.2
8.	LLIKTVETRDGQVINETSQH	-819.2
9.	QNMKEEMARHLREYQDLLNV	-797.1
10.	YESVAAKNLQEAEEWYKSKF	-792.7
11.	HLREYQDLLNVKMALDIEIA	-783.4
12.	QAKQESTEYRRQVQSLTCEV	-746.3

13.	FKNTRTNEKVELQELNDRFA	-736.7
14.	EDIMRLREKLQEEMLQREEA	-730.3
15.	QMREMEENFAVEAANYQDTI	-719
16.	AALRDVRQQYESVAAKNLQE	-709.8
17.	SSAVRLRSSVPGVRLLQDSV	-709.3
18.	LARLDLERKVESLQEEIAFL	-699
19.	NYQDTIGRLQDEIQNMKEEM	-694.6
20.	EVERDNLAEDIMRLREKLQE	-688.4
21.	EYRRQVQSLTCEVDALKGTN	-671.6
22.	LKKLHEEEIQELQAQIQEQH	-664.5
23.	LKGQGKSRLGDLYEEEMREL	-653.3
24.	CEVDALKGTNESLERQMREM	-651.2
25.	LEQQNKILLAELEQLKGQGK	-640
26.	LLAELEQLKGQGKSRLGDLY	-624.8
27.	EKLQEEMLQREEAENTLQSF	-593.5
28.	SEAANRNNDALRQAKQESTE	-487.9

Tables 4.10 and 4.11 provide data indicating that the epitopes STRTYSLGSALRPSTSRSLY and GSALRPSTSRSLYASSPGGV exhibit the lowest energy values. This suggests exceptionally strong binding interactions with their respective receptors, implying their potential to effectively stimulate the immune system. As a result, these two epitopes were identified as promising candidates and were chosen for further in-depth analysis.

The selection of these epitopes based on their favorable binding energies underscores their significance in potential immunological applications and therapeutic investigations.

These structures depict the complex formations when the epitopes bind with their respective receptors. These predictions were generated using the ClusPro software, providing valuable insights into the molecular interactions and binding modes between the epitopes and the receptors. These structural models are essential for understanding the specific details of epitope-receptor interactions and their potential implications in immunological and therapeutic contexts.

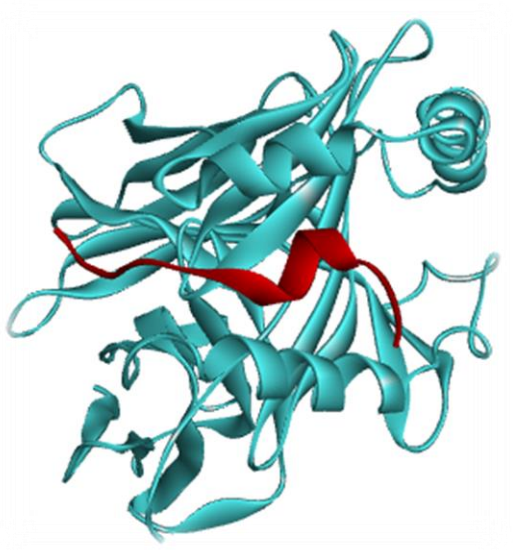


Fig4.6: Molecular model of a docked complex of the HLADRB1 and STRTYSLGSALRPSTSRSLY epitope

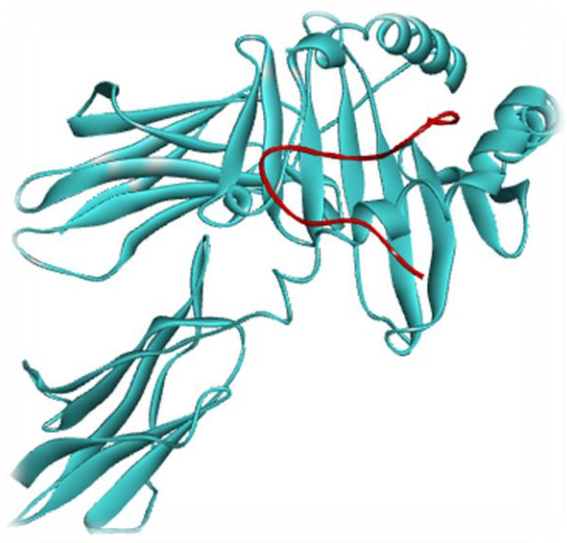


Fig4.7: Molecular model of a docked complex of the HLADRB1 and GSALRPSTSRSLYASSPGGV epitope

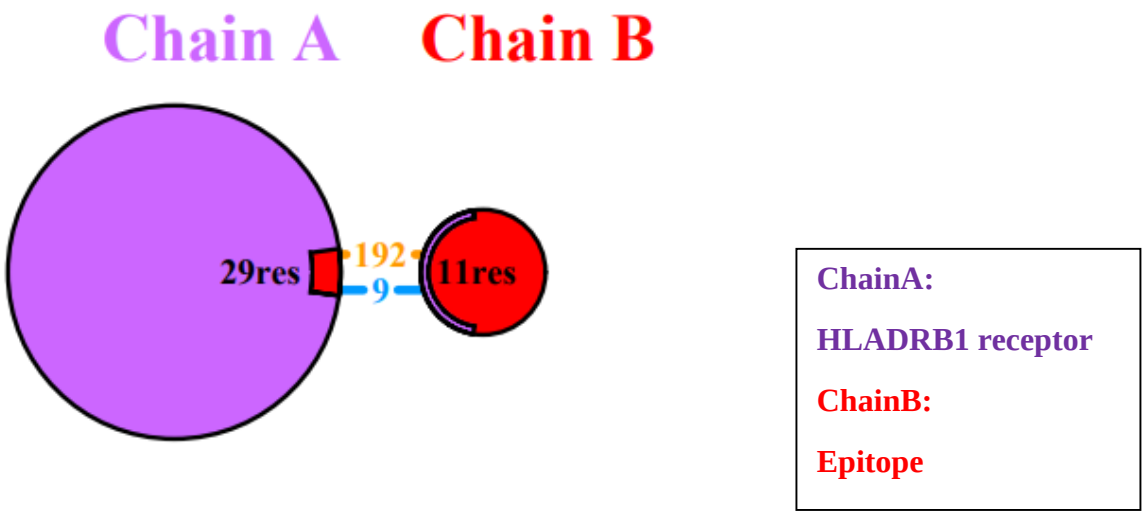
Figures 4.6 and 4.7 provide a clear representation of the docked complexes formed between the epitope STRTYSLGSALRPSTSRSLY and the HLADRB1 receptor, as well as between the epitope GSALRPSTSRSLYASSPGGV and the HLADRB4 receptor. These visual depictions offer valuable insights into the structural arrangements and intermolecular interactions between the epitopes and their respective receptors. Such

detailed information is crucial for understanding the specific binding mechanisms and potential functional implications in immunological and therapeutic contexts.

4.9 Docking Analysis of epitopes with Receptors:

A comprehensive analysis of the selected docked cluster was conducted utilizing the PDBsum online server. This analysis provided us with a detailed understanding of the individual residues involved and the intermolecular forces present within the docked cluster. Such insights are instrumental in unraveling the intricate molecular interactions and structural aspects critical for research investigation

(a)



Interface statistics

Chain	No. of interface residues	Interface area (Å²)	No. of salt bridges	No. of disulphide bonds	No. of hydrogen bonds	No. of non-bonded contacts
A	29	795	-	-	9	192
B	11	1116				

Fig4.8 (a): Docking of HLADRB1 receptor and STRTYSLGSALRPSTSRSLY epitope: results reveals key interactive residues.

(b)

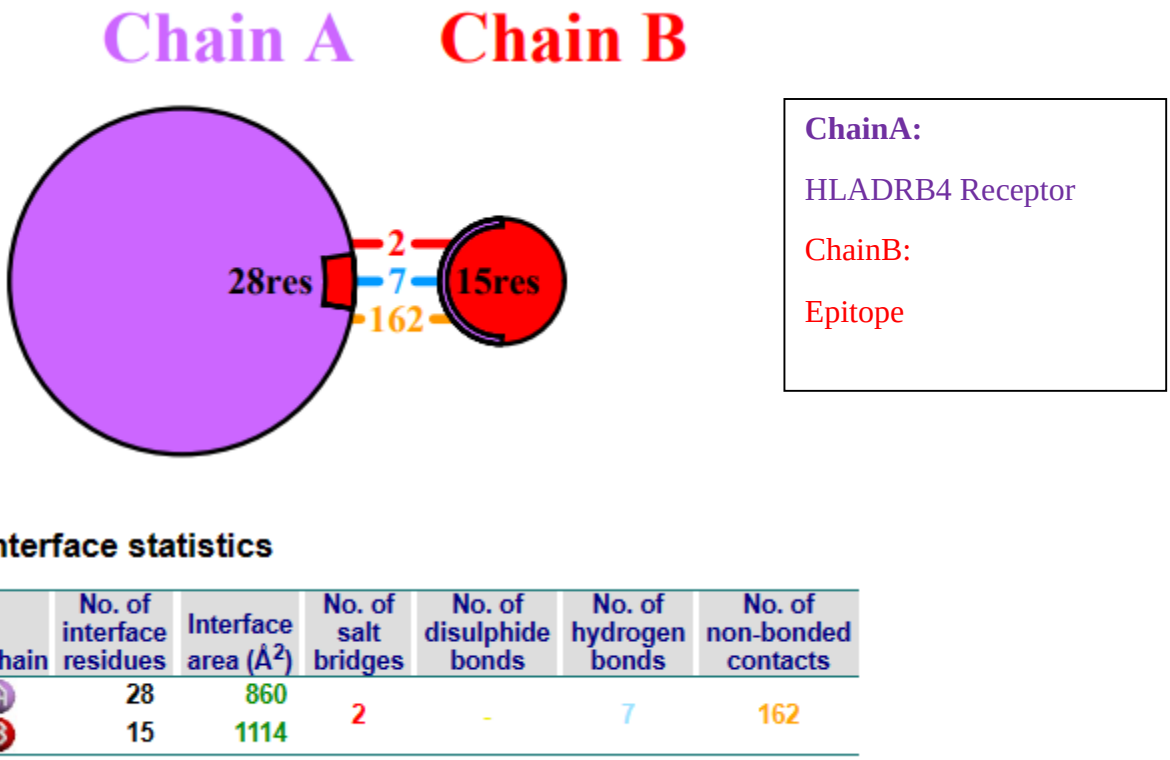


Fig4.8 (b) Docking of HLADRB1 receptor and GSALRPSTSRSLYASSPGGV epitope: results reveals key interacting residues.

4.10 Analysis of Residues Interaction in Docking:

The detailed analysis of the selected docked cluster was further conducted using the PDBsum online server. This allowed us to gain a thorough insight into the interacting residues and the intermolecular forces present within the docked cluster.

(a)

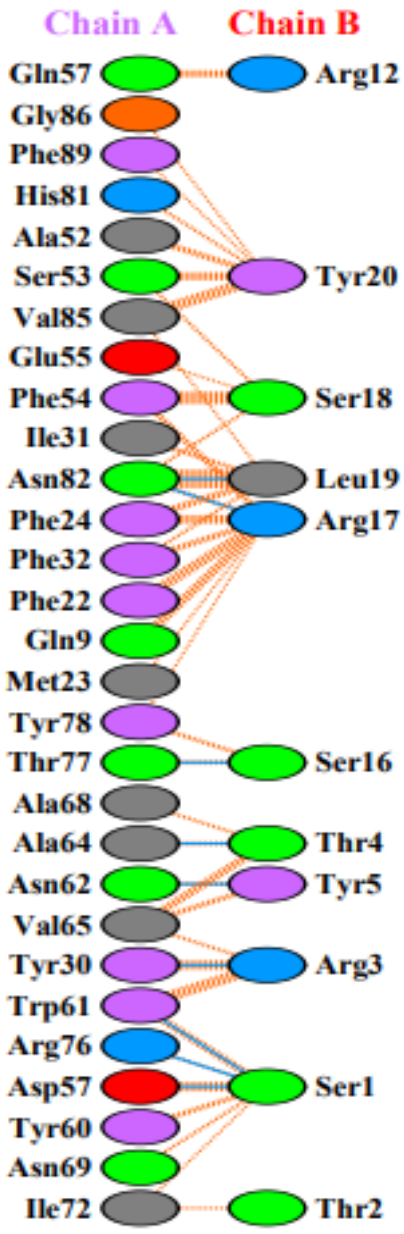


Fig 4.9(a) Protein-protein interaction between HLADRB1 and STRTYSLGSALRPSTSRSLY

(b)

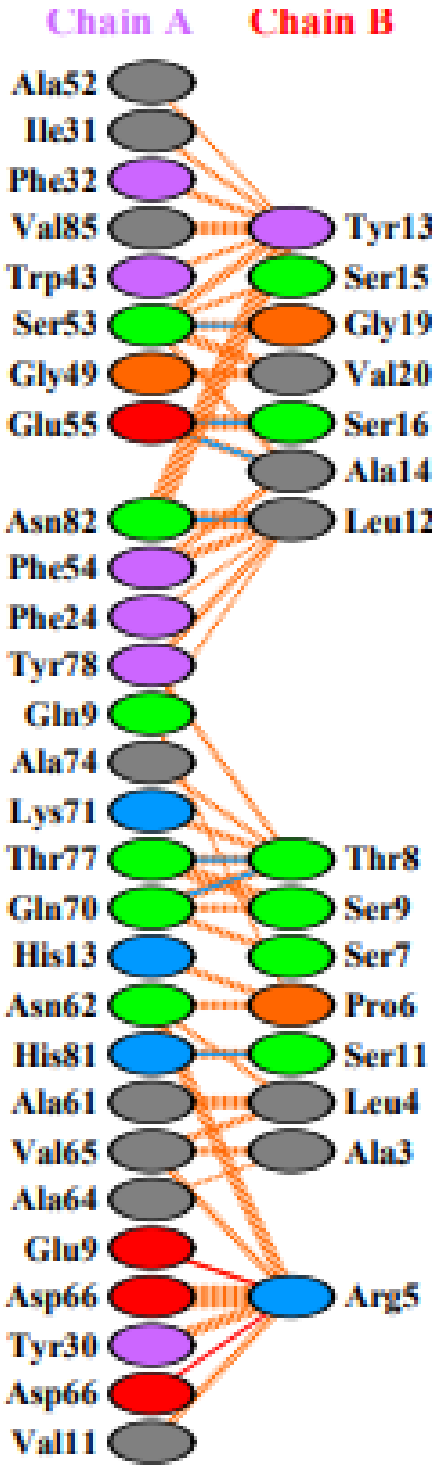


Fig 4.9 (b) Protein-protein interaction between HLADRB4 and GSALRPSTSRSLYASSPGGV

4.11 Peptide Alteration and Structural Analysis:

The process of peptide alteration for potential therapeutic vaccine development is explored. The peptide was strategically altered to enhance its functionality, with a specific focus on its binding interactions with receptors. Notably, it was observed that despite these modifications, the peptide maintains its ability to bind to the receptor. Furthermore, an alteration in the cytokine profile was detected, with the modified peptide exhibiting a significant increase in interleukin-10 (IL-10) and interleukin-4 (IL-4) production, while the production of interferon-γ (IFN-γ) was notably reduced compared to the original peptide. These findings contribute to an understanding of the peptide's potential as an antigenic therapeutic agent.

Arginine (R) at position5 was substituted with glutamine (Q) in the epitope GSALRPSTSRSLYASSPGGV represents a strategic modification aimed at enhancing the binding affinity with the HLADRB4 receptor. The decision to make this substitution was based on the observation that arginine (R) had strong interactions with seven residues of the receptor. By replacing it with glutamine (Q), we aimed to optimize the epitope's binding capacity while maintaining its overall structural integrity. This substitution strategy was designed to improve the epitope's compatibility with the receptor, ultimately enhancing its potential as an immunogenic agent and contributing to a more effective immune response.

In this altered epitope, STRTYSLGSALRPSTSRSLY, a substitution was made at position 1 where serine (S) was replaced with glutamic acid (E), and at position 3, arginine (R) was substituted with glutamic acid as well. Despite these modifications, the peptide still retains its binding affinity with the HLADRB1 receptor. Importantly, these changes resulted in the desired immune response characterized by reduced IFN-γ production and increased levels of IL-10 and IL-4. This demonstrates the potential of such alterations in fine-tuning immune responses for specific therapeutic applications.

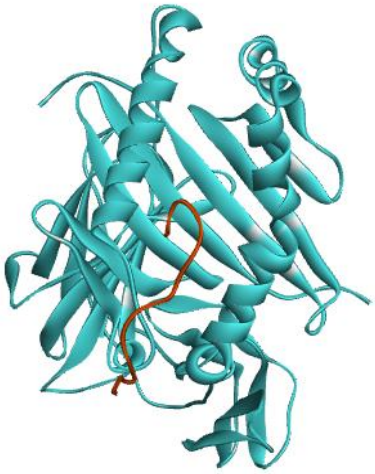


Fig4.10: Docking of Altered peptide and HLADRB4 receptor: Altered GSALQPSTSRSLYASSPGGV retains its binding affinity to respective receptor

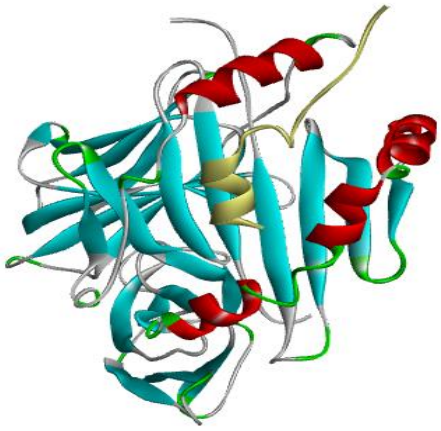


Fig4.11 Docking of Altered peptide and the HLADRB1 receptor: Altered peptide ETETYSLGSALRPSTSRSLY, retains its binding affinity.

Following the prediction of IL-10, IL-4, and IFN-γ responses for the altered peptide STRTYSLGSALRPSTSRSLY, which displayed favorable results with reduced IFN-γ production and increased IL-10 and IL-4 levels, an adjuvant known as alpha-melanocyte-stimulating hormone was strategically linked to this peptide. This linkage was achieved through the incorporation of a linker sequence, EAAAK. Subsequently, we evaluated the immune response, and the results demonstrated an even more promising outcome.

The adjuvant, alpha-melanocyte-stimulating hormone, played a pivotal role in enhancing the immunomodulatory properties of the peptide. This combination not only resulted in a heightened immune response but also exhibited a more favorable cytokine profile. These findings hold significant potential for advancing our understanding of peptide-adjuvant combinations and their potential applications in immunotherapy and vaccine development.

4.12 Molecular Dynamic Simulation Analysis:

To assess the stability of the molecular system, separate molecular dynamics (MD) simulations were conducted. Initially, simulations were performed on the receptor alone, followed by simulations of the docked complexes involving both the altered peptide and the original peptide. The objective was to scrutinize whether the altered peptide exhibited any distinct behavior compared to the original peptide. Remarkably, the results indicated that both peptides yielded similar outcomes.

To gain a deeper understanding of the system's stability and structural dynamics, root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) analyses were carried out. These analyses were conducted over a duration of 50 nanoseconds using the GROMACS software. The consistent behavior observed in both the altered and original peptides during the MD simulations underscores the reliability and stability of the molecular system, providing valuable insights for the subsequent phases of the research. In terms of stability altered peptide was same with original but with modulated immune response which was the purpose of research

In terms of stability, the altered peptide exhibited a behavior consistent with the original peptide during the molecular dynamics simulations. However, the significant difference lay in the altered peptide's ability to modulate the immune response, which was the primary objective of this research. While the structural stability remained comparable, the alterations made to the peptide successfully achieved the desired effect of influencing the immune response, showcasing the research's successful outcome.

Initially, a molecular dynamics (MD) simulation was conducted exclusively on the HLADRB1 receptor to assess its structural stability. This analysis aimed to confirm the stability of the receptor structure obtained from the RCSB PDB database.

The results of the root-mean-square deviation (RMSD) analysis indicated that the receptor's stability began to manifest at around 0.07 nanometers and reached a stable state at 1 nanometer. Notably, the deviations of residues within the range of 0.1 to 0.27 nanometers were minimal, underscoring the exceptionally high stability of the receptor's structure.

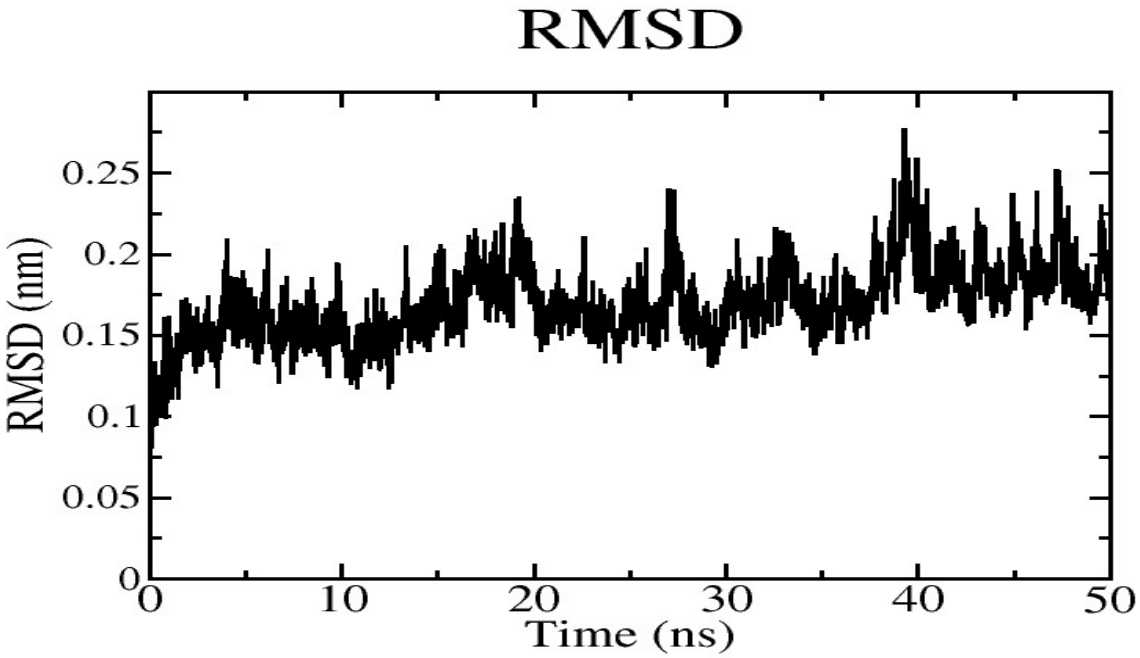


Fig4.12: Root mean square deviation (RMSD) plots for HLADRB1molecular dynamics simulation

Following the initial analysis, the structural fluctuations of the trajectories were further examined by analyzing the root-mean-square fluctuation (RMSF) plots. These RMSF plots provided additional confirmation of the stabilized nature of the HLADRB1 receptor. They revealed that the mobility of C-alpha atoms exhibited deviations within the narrow range of 0.056 to 0.27 nanometers throughout the 50 nanosecond simulation period. These findings further affirm the receptor's structural stability over the course of the simulation.

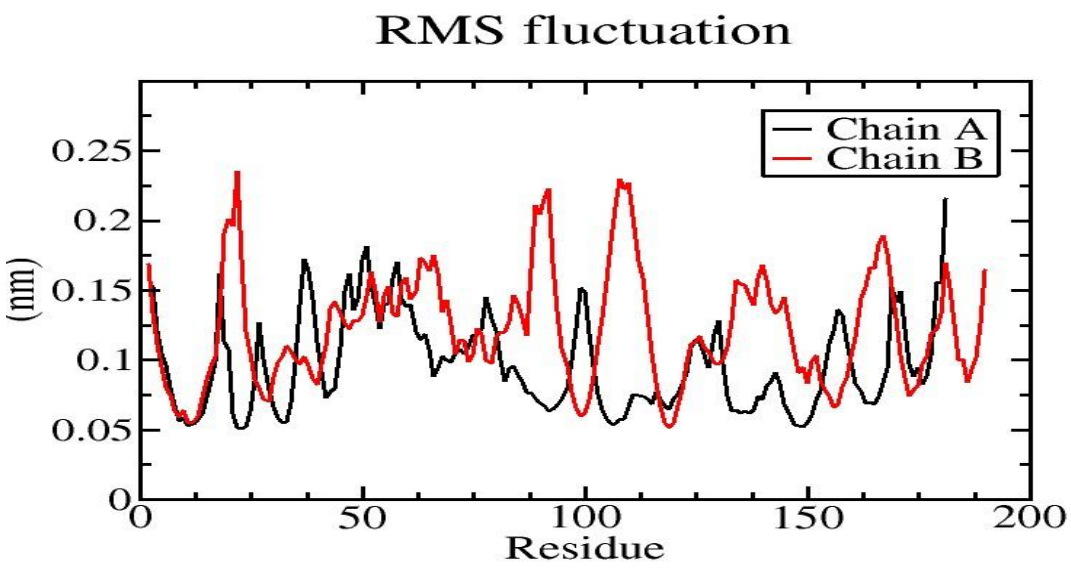


Fig4.13: Root-mean-square fluctuation (RMSF) plots of HLADRB1 using molecular dynamics simulation

A parallel molecular dynamics (MD) simulation was exclusively performed on the HLADRB4 receptor to evaluate its structural stability. This analysis served the purpose of validating the receptor's structural integrity as obtained from the RCSB PDB database.

The outcomes of the root-mean-square deviation (RMSD) analysis indicated that the receptor's stability commenced at approximately 0.13 nanometers and reached a stable state at 2 nanometers. It's noteworthy that deviations within the range of 0.2 to 0.36 nanometers were minimal for the residues. This reinforces the notion of the receptor's exceptionally robust structural stability throughout the simulation.

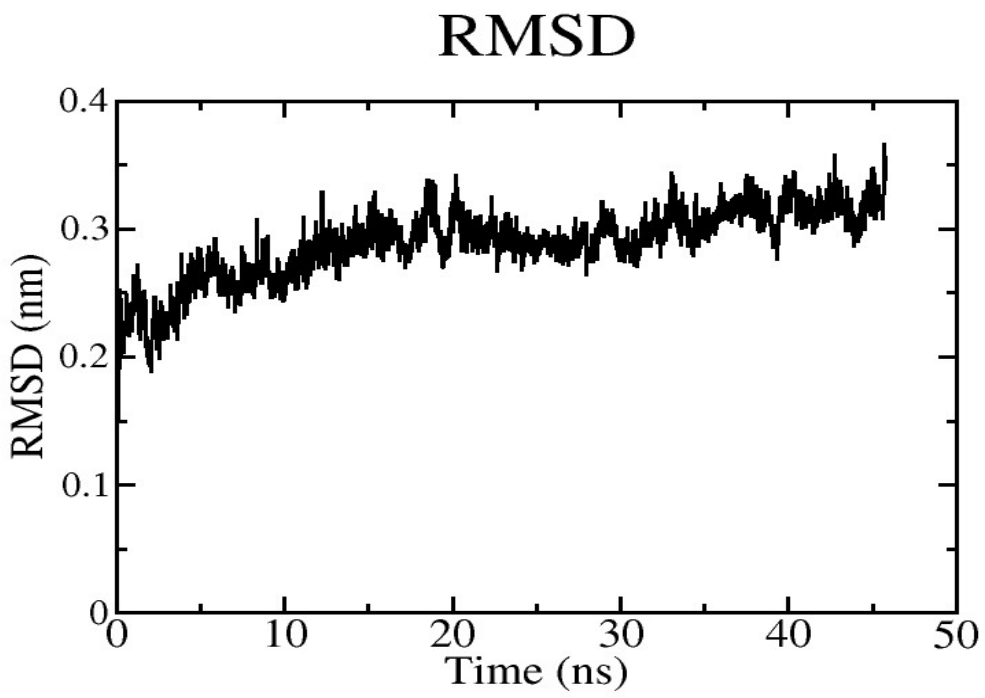


Fig4.14: Root mean square deviation (RMSD) plots of HLADRB4 through molecular dynamics simulation

Subsequent to the initial analysis, a closer investigation into structural fluctuations was conducted by analyzing root-mean-square fluctuation (RMSF) plots. These RMSF plots served as an additional validation of the firmly established stability of the HLADRB4 receptor. The results unveiled that the mobility of C-alpha atoms demonstrated minimal deviations, confined within the range of 0.1 to 0.66 nanometers during the entire 50 nanosecond simulation period. These observations serve as robust confirmation of the receptor's structural stability throughout the duration of the simulation.

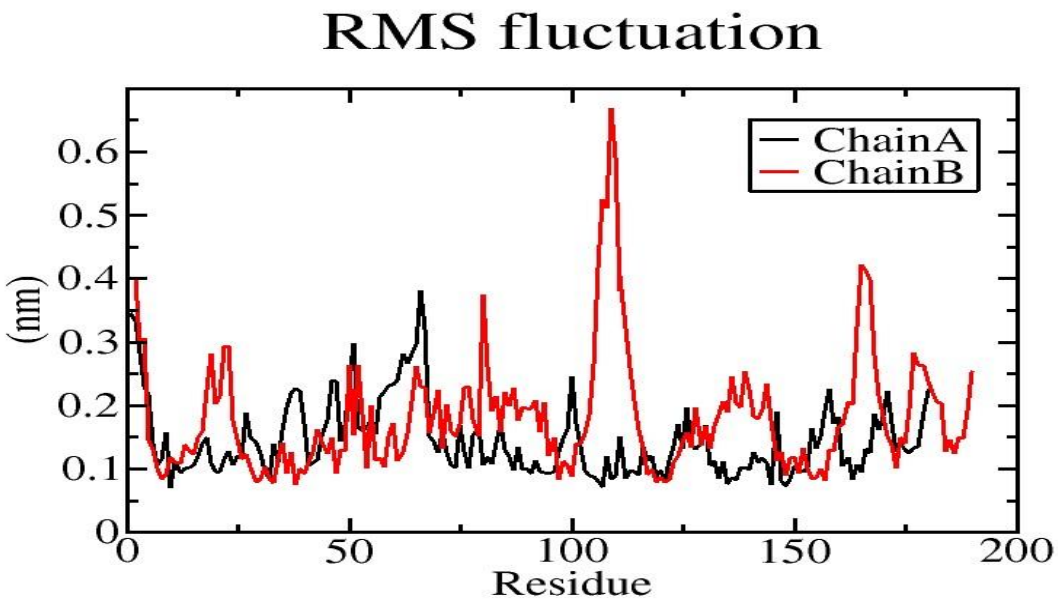


Fig4.15: Root-mean-square fluctuation (RMSF) plots of HLADRB4 through molecular dynamics simulation

After conducting a thorough assessment of the receptor's stability, the molecular dynamics (MD) simulation of the docked complex involving HLADRB1 and the original epitope STRTYSLGSALRPSTSRSLY was executed. The results, as revealed by the root-mean-square deviation (RMSD) analysis, indicated that the complex began to exhibit stability at approximately 0.2 nanometers. Throughout the simulation, it is demonstrated that minimal fluctuations within the range of 0.2 to 0.45 nanometers, further confirming its overall stability.

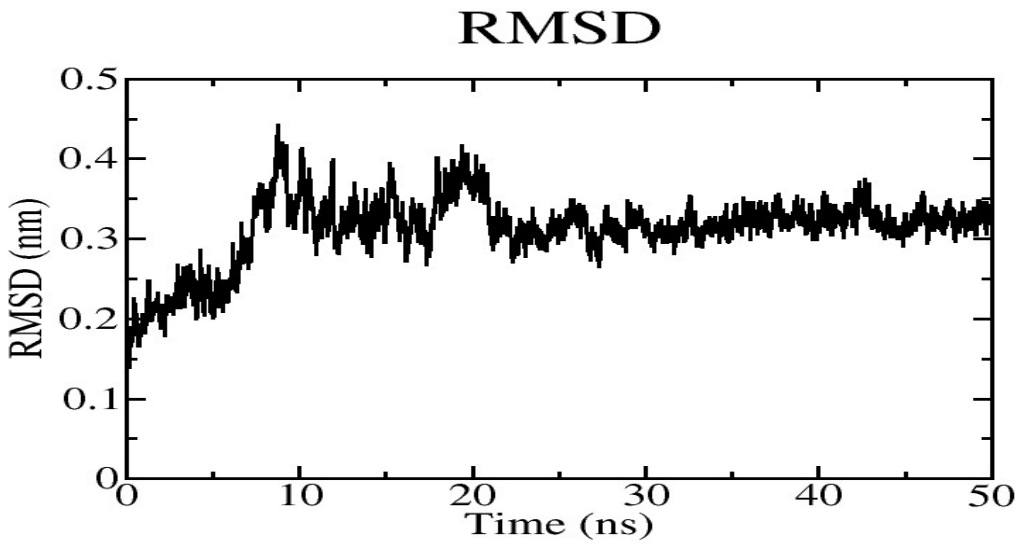


Fig4.16: Root mean square deviation (RMSD) plots of HLADRB1 receptor and Peptide STRTYSLGSALRPSTSRSLY through molecular dynamics simulation analysis

The RMSF analysis, which illustrates the fluctuation of residues, revealed that for the receptor, fluctuations occurred within the range of 0.09 to 0.30 nanometers, while for the ligand, they extended from 0.09 to 1 nanometer. These findings strongly suggest that the overall structure of the complex remains stable throughout the simulation.

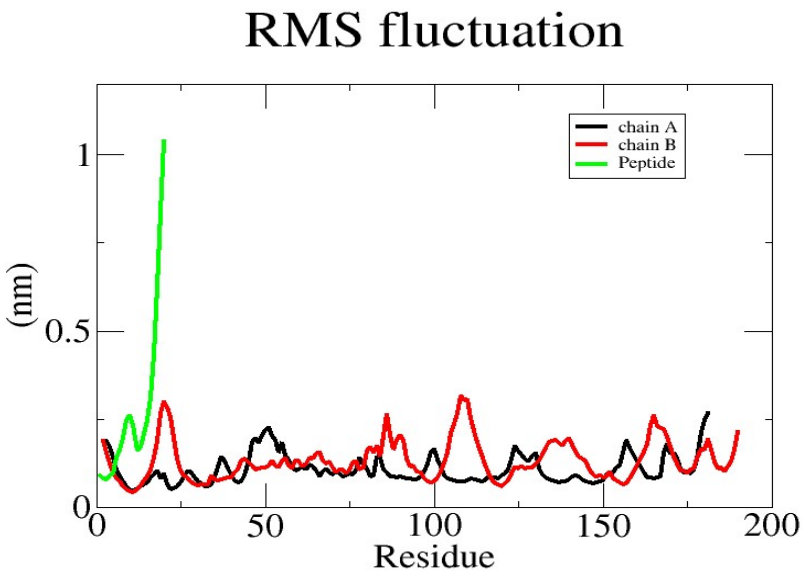


Fig4.17: Root-mean-square fluctuation (RMSF) plots of HLADRB1 and Peptide STRTYSLGSALRPSTSRSLY through molecular dynamics simulation analysis

The MD simulation conducted for the HLADRB4 receptor and the peptide GSALRPSTSRSLYASSPGGV also included RMSD analysis. This analysis demonstrated that the fluctuation during the simulation remained within the range of 0.2 to 0.45 nanometers. These results provide strong evidence indicating the stability of the complex throughout the simulation period.

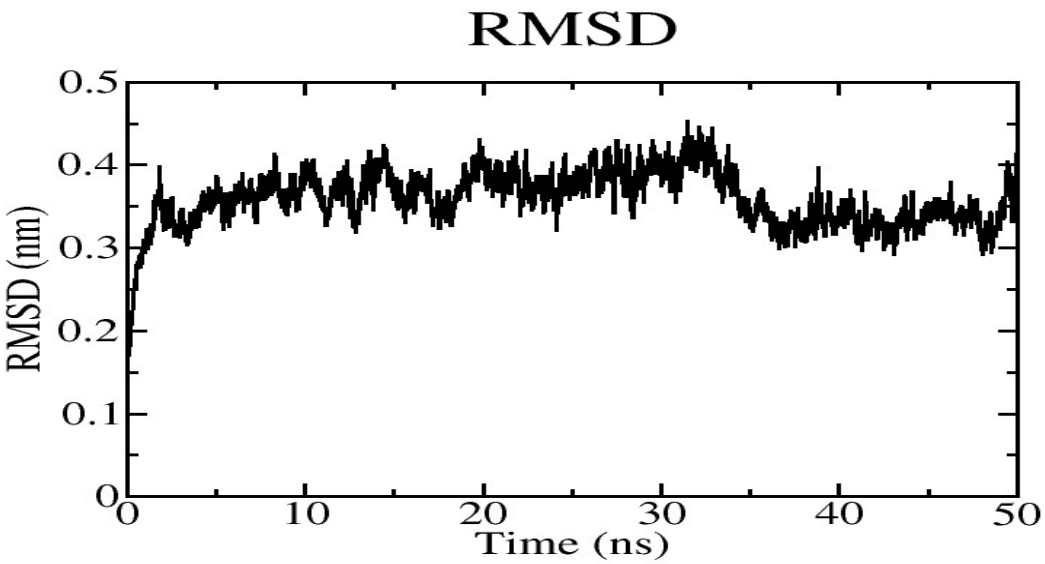


Fig4.18: Root mean square deviation (RMSD) plots of HLADRB1 receptor and Peptide GSALRPSTSRSLYASSPGGV through molecular dynamics simulation analysis

The RMSF analysis, portraying residue fluctuation, indicated that for the receptor, fluctuations fell within the range of 0.27 to 0.63 nanometers, while for the ligand, they spanned from 0.095 to 0.97 nanometers. These results provide robust evidence supporting the overall structural stability of the complex throughout the simulation.

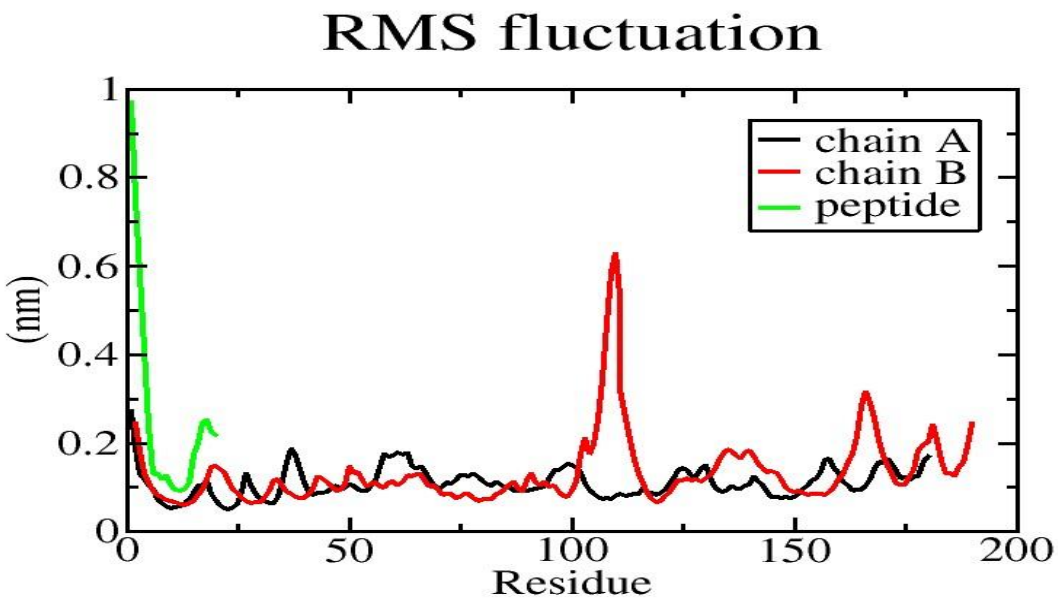


Fig4.19: Root-mean-square fluctuation (RMSF) plots of HLADRB4 and Peptide GSALRPSTSRSLYASSPGGV through molecular dynamics simulation analysis

Subsequent to these simulations, another molecular dynamics (MD) simulation was conducted for the docked complex involving HLADRB1 and the altered peptide ETETYSLGSALRPSTSRSLY. The primary objective was to assess whether the stability of this complex differed from that of the original peptide complex.

The MD simulation performed for the HLADRB1 receptor in conjunction with the altered peptide ETETYSLGSALRPSTSRSLY incorporated RMSD analysis. This analysis consistently revealed that the fluctuation throughout the simulation stayed within the narrow range of 0.1 to 0.30 nanometers. These outcomes offer compelling evidence supporting the sustained stability of the complex throughout the entire simulation duration.

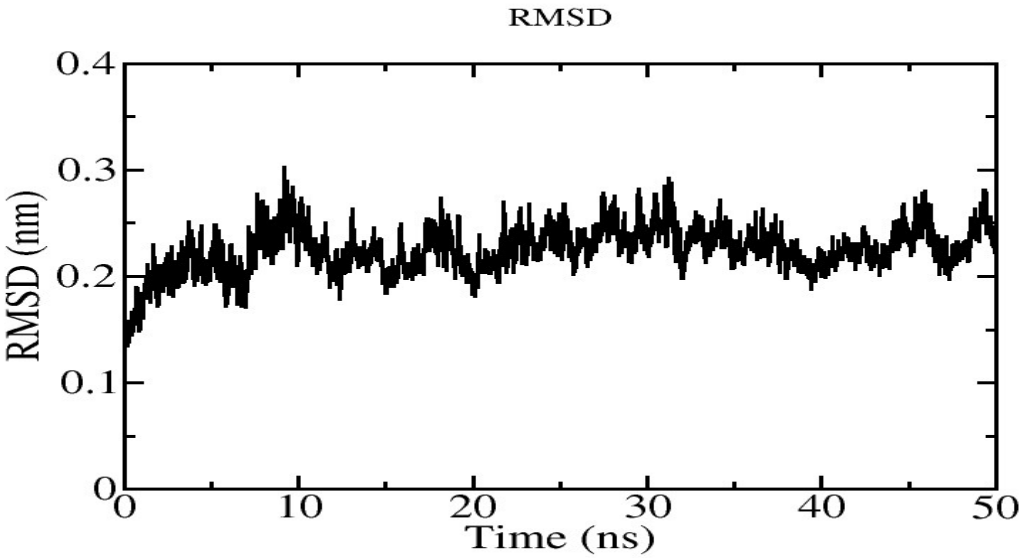


Fig4.20: Root mean square deviation (RMSD) plots of HLADRB1 receptor and Altered peptide ETETYSLGSALRPSTSRSLY through molecular dynamics simulation analysis

The RMSF analysis, which depicts residue fluctuation, demonstrated that fluctuations within the receptor ranged from 0.04 to 0.31 nanometers, while for the ligand, they extended from 0.23 to 0.36 nanometers. These findings furnish strong evidence affirming the sustained structural stability of the complex throughout the simulation period.

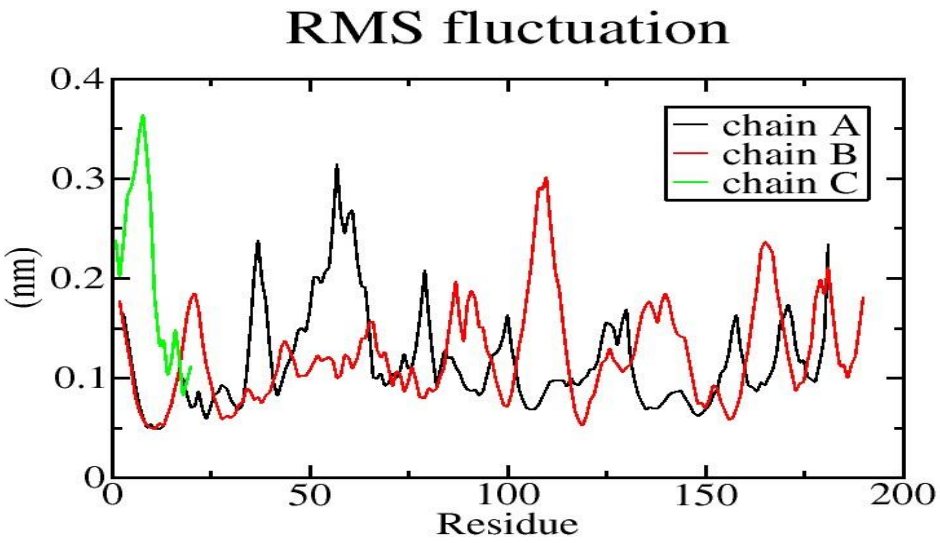


Fig4.21: Root-mean-square fluctuation (RMSF) plots of HLADRB4 and Altered Peptide ETETYSLGSALRPSTSRSLY through molecular dynamics simulation analysis

These results indicate that the altered peptide closely resembles the original one in terms of stability during the simulations. This similarity suggests that the altered peptide holds promise as a strong candidate for an Altered Peptide Ligand (APL) vaccine. Its structural stability and the desired immune response it elicits make it a compelling candidate for further investigation and potential therapeutic applications.

4.13 Comparative Analysis of Cytokine Responses:

In this study, the impact of amino acid substitution in the best binding peptide to HLADRB4 was investigated, resulting in the transformation of the original sequence GSALRPSTSRSLYASSPGGV into the altered sequence GSALQPSTSRSLYASSPGGV. Cytokine level scoring revealed noteworthy differences between the original and altered peptides. The original peptide demonstrated IL-10, IL-4, and IFN-γ scores of 0.336, 0.1, and 1.6031, respectively. Conversely, the altered peptide exhibited altered cytokine responses with scores of 0.53 for IL-10, 0.63 for IL-4, and 1 for IFN-γ. These findings highlight the potential of amino acid substitution in modulating cytokine profiles and may have implications in the design of therapeutic interventions.

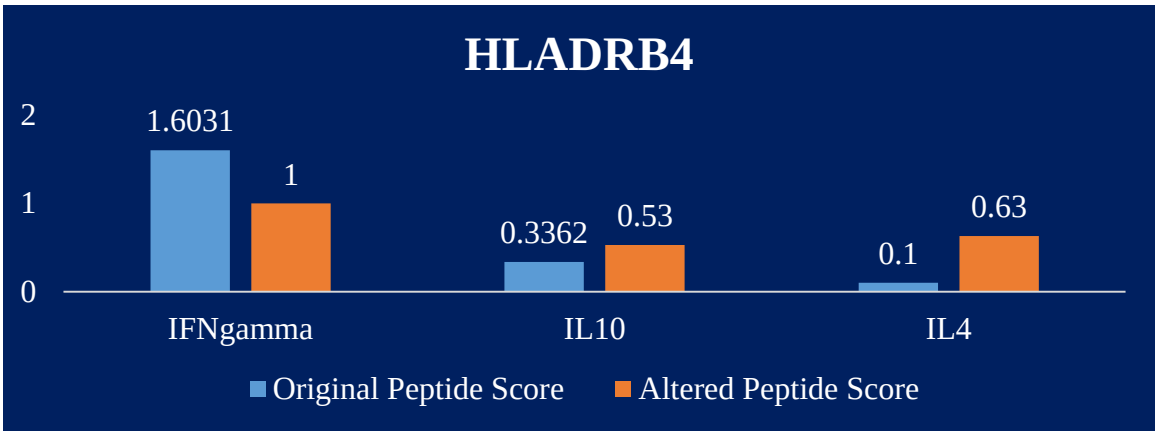


Fig4.22: The cytokine scores for both the original peptide and the altered peptide. It provides a visual representation of the differences in IL-10, IL-4, and IFN-γ scores between the two peptides.

Similarly, the best binding peptide to the HLADRB1 receptor, STRTYSLGSALRPSTSRSLY, initially exhibited cytokine level scores of 0.5623 for IL-10, 0 for IL-4, and 2.5644 for IFN-γ. To explore the impact of amino acid substitutions, the peptide was sequentially modified.

First, a single substitution at position 3 resulted in the peptide STETYSLGSALRPSTSRSLY. This alteration led to a decrease in IFN-γ levels and an increase in IL-10 and IL-4 scores, with values of 0.7817, 0.1, and 1.7715, respectively.

Further refinement involved a substitution at position 1, yielding the peptide ETETYSLGSALRPSTSRSLY. This subsequent alteration resulted in a further reduction in IFN-γ levels and an elevation of IL-10 and IL-4 scores, measuring 0.7833, 0.34, and 1.4783, respectively.

Subsequently, the alpha-melanocyte stimulating hormone was linked to the altered peptide through the EAAAK linker. This final modification resulted in a notable reduction in IFN-γ levels and a significant increase in IL-10 and IL-4 scores, registering values of1, 0.9321, 1.3, respectively. These sequential modifications underscore the potential of amino acid substitutions and the addition of adjuvants in modulating cytokine profiles, with implications for therapeutic development.

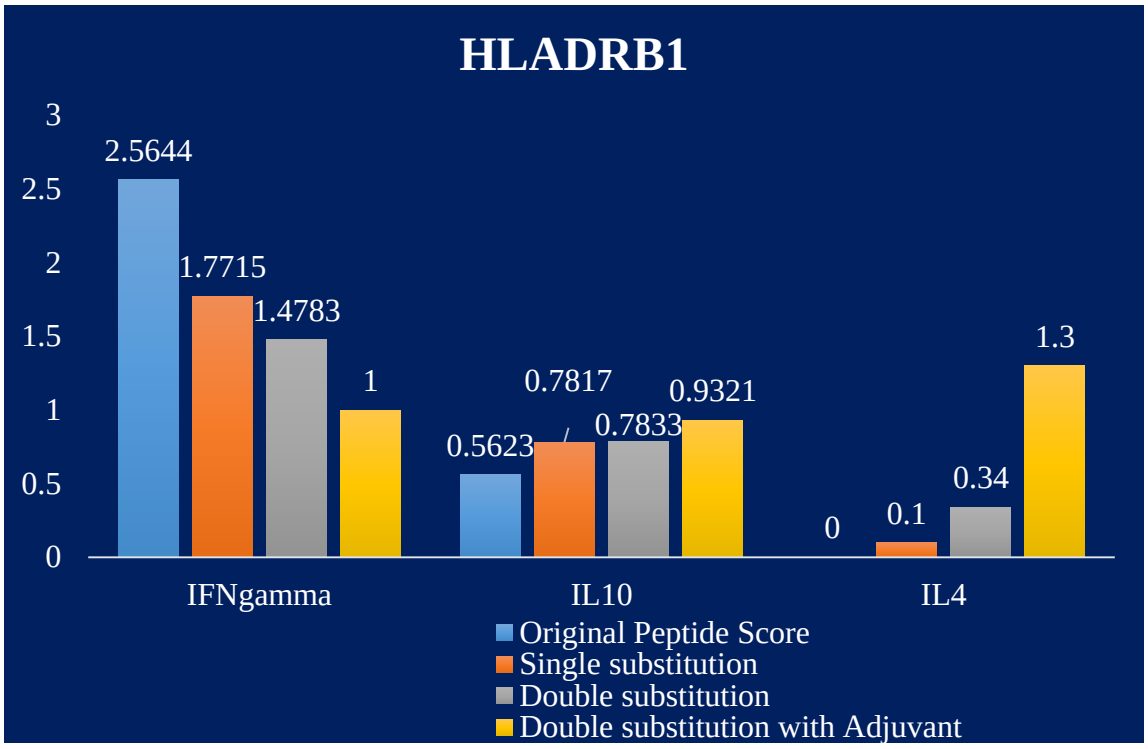


Fig4.23: The cytokine scores for both the original peptide and the altered peptide with single substitution, double substitution and adjuvant. It provides a visual representation of the differences in IL-10, IL-4, and IFN-γ score

The results provide a clear indication that the Altered peptide ETETYSLGSALRPSTSRSLY, in combination with the adjuvant alpha-melanocyte stimulating hormone, represented by SYSMEHFRWGKPVEAAAKETETYSLGSALRPSTSRSLY, holds strong promise as a therapeutic vaccine for addressing rheumatoid arthritis (RA).

Chapter 5

Discussion, Conclusion and Future Perspectives

5.1 Discussion:

In this study, the success of developing a targeted APL vaccine against Rheumatoid Arthritis (RA) through the use of the reverse vaccinology strategy has been demonstrated. Strong results have been observed against various autoimmune diseases, including RA, cancer, and diabetes, through the application of modern vaccinology techniques based on the utilization of cellular epitopes {Oyarzun, 2015 #65}.

Therefore, the primary focus of the present study was the design of an APL vaccine capable of conferring immunity against RA. In contrast to existing drugs for RA that suppress the immune system, an APL vaccine can elicit a regulatory response, making it a superior alternative {Zhang, 2018 #66}.

The design of an APL vaccine in our study was centered around Vimentin proteins, which serve as autoantigens for RA following citrullination. This focus was chosen due to the pivotal role of Vimentin proteins in RA. Similarly, in a study conducted by N. Lorenzo *et al*. in 2003, heat shock proteins were employed as autoantigens, and they enhanced the regulatory response while reducing the inflammatory response in RA. This approach involved creating altered peptides, and the effectiveness of this strategy was demonstrated, mirroring the positive outcomes observed in our own study {Lorenzo, 2017 #67}.

The epitopes predicted from the Vimentin protein exhibit strong binding capabilities with the immune system and can serve as promising targets in vaccine development. This aligns with findings from other research, such as the work conducted by J. Spieling's *et al*., where epitopes derived from autoantigens in heat shock proteins for RA were utilized. The results obtained in our study closely mirror the positive outcomes achieved in their research {Spieling's, 2017 #69}.

The evaluation of epitopes was conducted using a range of online tools, and the prioritized epitopes were found to be specifically capable of eliciting responses from B cells. These

selected epitopes were chosen due to their homology with the human body, as the therapeutic vaccine was designed with this compatibility in mind. The alteration of these epitopes was later carried out to prevent the occurrence of autoimmune responses and immune reactions against one's own bodily components. This careful selection and modification of epitopes were crucial to ensure a robust and safe immune response {Stoppelenburg, 2023 #70}.

The chosen B cell epitopes demonstrated exceptional characteristics, including high immunogenicity and antigenicity, as well as a non-allergenic nature. They exhibited the best binding affinity with HLADRB4 and HLADRB1 receptors, which play crucial roles in Rheumatoid Arthritis (RA). Consequently, these epitopes emerged as ideal candidates for vaccine development, holding the potential to stimulate a highly effective, focused immune response.

Moreover, these selected B cell epitopes possess the capability to induce immune responses involving key molecules such as IFN-γ, IL-10, and IL-4. This implies that these epitopes can simultaneously trigger both B cell and T cell responses. IFN-γ is known for its ability to contribute to cartilage disruption during RA, while IL-10 and IL-4 act as regulators of the immune response, maintaining a balanced and controlled reaction. This dual mechanism enhances the therapeutic potential of the selected epitopes in combating RA {Pedrosa, 2020 #71}.

In 2017, T. Hirota and their colleagues designed an APL vaccine against Rheumatoid Arthritis (RA) using criteria similar to those employed in our study for epitope selection {Hirota, 2017 #72}.

Similarly, D. Prada and their team developed an APL vaccine against Rheumatoid Arthritis (RA) using a comparable approach for epitope prediction, evaluation, and prioritization, as employed in this study {Prada, 2018 #73}.

In a study conducted by Ohnishi et al., they found that certain Japanese patients with rheumatoid arthritis (RA) had a strong immune reaction to a specific segment of type II collagen (CII) called the 256–271 peptide. To manage this reactivity, they explored the use of altered peptide ligands (APLs). These APLs are basically modified versions of the CII

256–271 peptide, with slight changes in the amino acid sequence. The researchers tested 21 different APLs and discovered that one in particular, known as the 262 (G→A) APL of CII 256–271, displayed antagonistic activity across all their T-cell lines. This suggested that using CII APLs could potentially be a novel strategy for regulating RA {Ohnishi, 2006}.

In our own research, we followed a similar approach by designing an APL vaccine against RA. However, our study has certain advantages. Firstly, we targeted not just one but two important receptors, HLADRB1 and HLADRB4, which could enhance the effectiveness of the vaccine. Additionally, our predicted scores for immune-regulating cytokines such as IL10 and IL4 with the altered peptide are more favorable than those reported in the previous study. To further boost the vaccine's potential, we incorporated an adjuvant, which is known to enhance immune responses, resulting in even better outcomes.

Our research builds upon the promising concept of APLs as a therapeutic strategy for RA, offering improvements in targeting and immune response modulation.

Michael Sela and his colleagues conducted research highlighting the growing significance of therapeutic vaccines, particularly in an era of increasing life expectancy. While efforts to develop vaccines against diseases like cancer, AIDS, hepatitis, and Alzheimer's disease are still ongoing, progress has been substantial in the field of autoimmune diseases.

They suggested that their success is an immunomodulatory vaccine for multiple sclerosis (MS), known as Copaxone. This vaccine, used by approximately 100,000 patients daily, is a copolymer made up of four amino acid residues related to myelin basic protein. It has proven effective against the relapsing-remitting type of MS. Copaxone operates by inducing T helper 2 (Th2) regulatory cells, which play a key role in controlling the immune response, in both mice and humans.

Another vaccine candidate discussed in the research is aimed at myasthenia gravis (MG), an autoimmune disease related to the nicotinic acetylcholine receptor. This vaccine utilizes altered peptide ligands, composed of amino acid analogs, to inhibit autoimmune responses associated with MG. The mechanism behind this inhibition involves CD4+ CD25+ immunoregulatory cells, which suppress the immune response and lead to a shift from pro-

inflammatory to anti-inflammatory cytokines like IL-10 and transforming growth factor β{Sela, 2006 }.

These findings are particularly relevant to our own research, as they underscore the potential of immune modulation as a successful approach to treating diseases like rheumatoid arthritis (RA).

In our study, the process of 3D structure prediction played a pivotal role in providing a deeper understanding of the potential folding and three-dimensional structure of the peptide we intended to utilize as a vaccine against Rheumatoid Arthritis (RA). To achieve this, we employed Rosetta, a powerful computational tool capable of generating accurate structural models. Trrosetta allowed us to explore the intricate spatial arrangement of atoms within the peptide, unveiling crucial insights into its conformation {Du, 2021 #74}. This information is invaluable when designing a vaccine, as the precise 3D structure of an antigen is often a key determinant of its immunogenicity and effectiveness in eliciting a robust immune response. However, predicting the static structure was only one facet of our investigation. To gain a comprehensive understanding of the peptide's behavior and stability, we took our analysis a step further. We subjected the predicted 3D structure to Molecular Dynamics (MD) simulations, a sophisticated computational technique carried out using the GROMACS software.

MD simulations involve a detailed, dynamic examination of the peptide's behavior over time, essentially providing a molecular movie of its movements. This approach allows us to assess the stability of the peptide's structure and explore its dynamic interactions with its surroundings, such as solvent molecules and ions. Through MD simulations, we not only gauged the structural integrity of the peptide but also gained valuable insights into its flexibility, fluctuation, and potential binding interactions with relevant biomolecules. These simulations serve as a critical tool in vaccine development, as they provide essential information about the peptide's behavior in a biological environment {Hospital, 2015 #75}.

Docking analysis was a crucial component of our research, enabling us to thoroughly examine the binding affinity of the altered peptide, which served as the foundation for our vaccine construct. We specifically focused on its interaction with the receptors HLADRB4 and HLADRB1, both of which are intimately involved in Rheumatoid Arthritis (RA) and

play pivotal roles in immune responses. Through docking analysis, we scrutinized the molecular interactions and binding energies between the altered peptide and these receptors. This assessment provided vital insights into the strength and specificity of the peptide's attachment to HLADRB4 and HLADRB1.Understanding the binding affinity is of utmost importance in vaccine development, as it helps us determine whether the altered peptide can effectively engage with these receptors to initiate a targeted immune response against RA. These docking studies contribute valuable information that informs the design and optimization of the vaccine construct, ultimately paving the way for potential therapeutic advancements in RA treatment {Nagafuchi, 2016 #76} {Koning, 2015 #77}.

Indeed, the evaluation of vaccine constructs through docking analysis with specific receptors has been a widely employed strategy in previous research endeavors, especially in the design of synthetic epitope-peptide based vaccines. This approach has been repeatedly chosen because it offers a comprehensive understanding of the molecular interactions and binding affinities between the vaccine components and their target receptors {Naqvi, 2018 #78} {Vidal-Limon, 2022 #79} {Shu, 2020 #80} {Xia, 2020 #81}.

5.2 Conclusion:

In silico-designed APL vaccine for Rheumatoid Arthritis (RA) shows promise as a potential solution. We've identified a strong candidate epitope fragment derived from the Vimentin protein, with the sequence ETETYSLGSALRPSTSRSLY, which is 20 amino acids long. When combined with Alpha-Melanocyte Stimulating Hormone, the sequence SYSMEHFRWGKPVEAAAKETETYSLGSALRPSTSRSLY, totaling 34 amino acids, presents a compelling option. It has strong potential to elevate strong regulatory immune response by elevating the production of IL10 and IL4 cytokines and also has potential to suppress the inflammatory cytokines such as IFNgamma. While further testing is needed, our research underscores the value of computer-based methods in crafting improved vaccines for diseases like RA, offering hope for enhanced treatments.

5.3 Future perspectives:

In future Conduction of *in vitro* and *in vivo* experiments to validate the efficacy and safety of the vaccine candidate, including animal studies and clinical trials will be done Adaptation the vaccine to address different strains or variants of RA-causing agents, ensuring broader coverage and adaptability to changing disease patterns. This vaccine will also be Investigated the vaccine's compatibility with other RA treatments, such as disease-modifying antirheumatic drugs (DMARDs) or biologics, to develop combination therapies that offer comprehensive disease management.

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Abstract

Rheumatoid Arthritis (RA) is a prevalent autoimmune disease, affecting millions worldwide. Its treatment is often costly, placing a significant economic burden on patients and healthcare systems. Current therapies primarily rely on immunosuppression, which can have drawbacks, such as increased susceptibility to infections and long-term medication dependency. The development of targeted APL vaccines represents a promising avenue for more effective and safer RA management, offering hope for improved patient outcomes and reduced treatment costs in the future.

In this research, an Altered Peptide Ligand (APL) therapeutic vaccine against Rheumatoid Arthritis (RA) was designed through *in silico* methods. The Vimentin protein sequence, sourced from NCBI, served as the starting point. A specific B cell epitope, "STRTYSLGSALRPSTSRSLY," which exhibited strong binding with both HLADRB1 and HLADRB4 receptors, was the focus. However, it demonstrated high immunogenicity and IFN-γ production, coupled with reduced IL10 and IL4 levels. To enhance its regulatory response while reducing inflammation, a double substitution was performed. At positions 1 and 3, S was replaced by E, and R was substituted with E, respectively. Remarkably, these alterations did not compromise binding to HLADRB1. Furthermore, the peptide was linked to Alpha-Melanocyte Stimulating Hormone through an EAAAK linker. The resultant sequence, "SYSMEHFRWGKPVEAAAKETETYSLGSALRPSTSRSLY," exhibited reduced IFN-γ production and increased IL10 and IL4 levels. This innovative peptide is proposed as a potential APL vaccine candidate against RA, underscoring the efficacy of *in silico* methodologies in therapeutic vaccine design.

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