Fall 2021

HLA Blockers for potentially treating Rheumatoid arthritis

Samaneh Mehri
University of Windsor

HLA Blockers for potentially treating Rheumatoid arthritis

By

Samaneh Mehri

A Thesis

Submitted to the Faculty of Graduate Studies

Through the Department of Chemistry and Biochemistry

In Partial Fulfillment of the Requirements for

The Degree of Master of Science

At the University of Windsor

Windsor, Ontario, Canada

HLA Blockers for potentially treating Rheumatoid arthritis

by

Samaneh Mehri

APPROVED BY:

_____

M. Rahim
Department of Biomedical Sciences

_____

Y. Tong
Department of Chemistry & Biochemistry

_____

L. Porter, Co-Advisor
Department of Biomedical Sciences

_____

J. Trant, Co-Advisor
Department of Chemistry & Biochemistry

September 2, 2021

Declaration of Co-Authorship

I hereby declare that this thesis incorporates material that is result of joint research as follows: Project design was developed by Dr. John Trant. Biology experiments were designed by the author with additional input given by Dr. John Trant and Dr. Elizabeth Fidalgo da Silva. All experiments and characterization were conducted by the author.

I am aware of the University of Windsor Senate Policy on Authorship, and I certify that I have properly acknowledged the contribution of other researchers to my thesis and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances in my thesis.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other university or institution.

Abstract

Autoimmune diseases occurs when the immune system recognize self antigens in the body as foreign invaders leads to dysfunction of tissue. RA is an autoimmune disease, caused by improper recognition of self-peptides, particularly human cartilage glycoprotein and type II collagen, by specific human leukocyte antigen (HLA) receptors. Normally T-cell specific for these peptides are destroyed in the thymus before they are released, preventing autoimmunity. However, certain post-translational modifications, especially citrullination, can lead to "self-peptide" recognition by non-self T cells: in the case of RA, one HLA protein (DRB*0401), out of about 1700 possible ones, is responsible for 65% of RA cases. If this protein could be blocked, drugs could be developed that interrupt the disease at its root cause without affecting the rest of the immune system; this is the focus of research in the Trant Lab. This thesis will briefly overview the approach, including the drug design, and will focus on the molecular biology work accomplished to date. The main finding of our thesis are as follows. Human leukocyte antigen is a protein needed to advance the research and drug development in autoimmune diseases and cancers. In these early stages of this project, we showed that choice of HEK293 cell as a host to express HLA protein and IMAC chromatography as a purification system gives us a reasonable amount of α/β heterodimer glycosylated membrane HLA protein.

Acknowledgements

The path toward this dissertation has been circuitous. Its completion is largely thanks to the special people who challenged, supported, and stuck with me along the way. I am tremendously fortunate to have supervisors, Dr. John Trant and Dr. Lisa Porter. I would thank Dr. Elizabeth Fidalgo da Silva for her guidance throughout my graduate studies. I thank them for supporting this project and giving thoughtful feedback and helping me to get this degree to move forward in my career.

Beside my advisors, I would like to thank the rest of my thesis committee members Dr. Munir Rahim and Dr. Yufeng Tong for their time and advice.

My sincere thanks also go to my lab mates, Jillian, Adam, Jackie, Philip, Nick, Isabelle, Sanaz, Sarah and Mana, who are not only my friends but also, they are my family members. I would never forget their warm supports, especially during the pandemic. I thank Trantteam and Porter lab members for all the help during this hard time. Thank you to the research associates of the Porter lab, Dr. Dorota Lubanska, Dr. Bre-Anne Fifield and post doctoral in Trantteam, Dr. Nazanin Taimoory, Dr. John Heyward who have fostered my development as a scientist and as a professional. I would like to thank my lovely family especially my parents for supported me through all hard pathways in my life.

Last but not least, I would like to thank my husband, Mehdi Anfasi for supporting me spiritually throughout my life with whom I would never be here.

Table of Contents

List of Figures

List of Tables

List of Abbreviations/Symbols

AD	Autoimmune Diseases
ACPAs	Anti-Citrullinated Protein Antibodies
APL	Altered Peptide Ligands
BHK	Baby Hamster Kidney
BiP	Binding Protein
CCP	Cyclic citrullinated peptides
CHO	Chinese Hamster Ovary
COX	cyclooxygenase
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
EBNNA-1	Epstein-barr Nuclear Antigen
ELS	Ectopic Lymphoid Structures
ER	Endoplasmic Reticulum
FLSs	Fibroblast like Synoviocytes
HCgp-39	Human Cartilage Glycoprotein-39
HLA	Human Leukocyte Antigen
HLAB	Human Leukocyte Antigen Blockers
MHC	Major Histocompatibility Complex
mTECs	Medullary Thymic Epithelial Cells
MMPs	Matrix Metalloproteinases
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
PBC	Primary Biliary Cirrhosis
PBMCs	Peripheral Blood Mononuclear Cells
RF	Rheumatoid Factors
SDS-PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel
SE	Shared Epitope
SF	Synovial Fluid
SPR	Surface Plasmon Resonance

TAP	Transporters associated with antigen presentation
T1D	Type 1 Diabetes
YAC	Yeast Artificial Chromosomes

Introduction:

I. Autoimmunity

i. Autoimmune diseases

In recent decades, the prevalence and severity of autoimmune diseases (AD) such as type I diabetes, multiple sclerosis, rheumatoid arthritis, autoimmune encephalomyelitis, and celiac disease has increased dramatically in the western world: 7% of the population of the United States, and 1.2% in the world as a whole, and the prevalence is still rising, possibly due to increases in accurate diagnosis [1,2]. Autoimmune diseases occurs when the immune system recognize self antigens in the body as foreign invaders leads to dysfunction of tissue. Some autoimmune diseases may be initiated by an infectious agent or a specific environmental exposure[3]. Autoimmune diseases can impact a specific organ, or they can be systemic which leads to a wide range of symptoms and organ injuries depending on the site of autoimmune attack. The main factor causing the autoimmune disease is still unknown, however genetic factors certainly play a key role at least in susceptibility [4].

Most autoimmune diseases do not have cure, and the available treatments have detrimental effects on the quality of life for these patients. Many of these patients around the world losing their job because of less productivity at work, loss of organ function and enduring debilitating symptoms. Generally, females are affected more frequently than men and autoimmune diseases remain among the leading causes of death for young and middle-aged women which imposes a heavy burden on the patient's family and on society [3,5]. Today, autoimmune diseases are treated with immunosuppressive agents that non-specifically reduce the severity of symptoms by weakening the whole immune system which make the patients more susceptible to infection and cancer. As a result, a promising therapeutic could be the one which can cure the autoimmune disease specifically by targeting the origin of the disease without affecting the rest of the immune system.

ii. Tolerance and autoimmunity

The key role of the immune system is to recognize self from non-self antigens [3]. The immune system is tolerant of self-antigens; as a result the immune system should not attack the body's own cells, tissues, and organs[6]. The presence of antigens in immune cell-inaccessible anatomic sites like the central nervous system and eye tissues prevents lymphocytes from interacting with them. The main consequence of antigen ignorance by lymphocytes is the absence of any type of immune response [7]. Immune tolerance refers to unresponsiveness of the immune system toward certain substances or tissues that are normally capable of stimulating an immune response. Self-tolerance is essential for normal immune balance, and failure or breakdown of that tolerance results in autoimmunity and autoimmune diseases[8].

Immune tolerance comprises both central and peripheral tolerance[9]. The major mechanism of the central immune tolerance is the negative selection in the thymus. The complementary mechanism which abandons the expansion and reactivity of mature self-reactive cells is peripheral tolerance.

Central tolerance, known as negative selection occurring in the thymus for T cells and bone marrow for B cells, is the primary way that the immune system discriminates self from non-self. Central tolerance refers to the elimination of autoreactive lymphocyte clones before they become fully immunocompetent, of which the main mechanism is negative selection [10]. This procedure occurs during lymphocyte development in the thymus and bone marrow for T and B lymphocytes, respectively. After T and B lymphocytes enter the peripheral tissues and lymph nodes, peripheral tolerance will occur to inhibit immune responses against the body's own tissues, which occurs primarily in the secondary lymphoid organs, such as the spleen and lymph nodes. Peripheral tolerance, which occurs in tissues and lymph nodes after lymphocyte maturation, controls self-reactive immune cells and prevents overreactive immune responses to various environmental factors. Mechanisms of peripheral tolerance include anergy (functional unresponsiveness), deletion (apoptotic cell death) and suppression by regulatory T cells [11,10]. Loss of tolerance results in autoimmune disorders, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1 diabetes (T1D) and primary biliary cirrhosis (PBC)[12]. Autoimmune diseases may develop when self-reactive lymphocytes escape from tolerance control mechanisms; thereby the loss of tolerance leads to autoimmune diseases[13].

iii. The mechanisms of T cell selection in the thymus in autoimmunity

T lymphocytes are one of the important components of adaptive immune system response, containing T-cell receptor on their cell surface. These T cells can recognize antigens and are activated when they bind to preformed complexes between peptide antigens and the major histocompatibility complex (MHC) protein on the surface of antigen presenting cells. As a result of activation, T-cells initiate a series of downstream signalling pathways that lead to cytokine secretion and T cell proliferation[14]. T cells arise in the bone marrow; however, they migrate to the thymus gland to mature. In the cortex of the thymus, T cell precursors known as thymocytes proliferate and differentiate into CD4+ and CD8+ cell types. When CD4+ and CD8+ T cells recognize the peptide-MHC on cortical thymic epithelial cells, based on the affinity with different classes of MHC, they differentiate into CD4- and CD8- single positive T cells. This process is called positive selection. So, positive selection ensures that the developing thymocytes have assembled proper T cell receptors and are capable of recognizing peptide-MHC complexes.

Negative selection happens when developing thymocyte recognize self-antigen-MHC complexes with very high affinity. High affinity of binding means that this particular T cell will most likely react against this self-antigen-MHC complex and should be eliminated. Low affinity interactions are not eliminated as they would be insufficient to active Tcells(Figure 1). In the medulla, CCR7 ligands CCL19 and CCL21 are expressed by medullary thymic epithelial cells (mTECs), and they interact with the chemokine receptor CCR7 expressed by CD4 or CD8 T cells These interactions lead to the deletion of autoreactive T cells. This activity is essential to protect the body from circulating T-cells that would initiate an immune response against the self. This is extremely dangerous and leads to autoimmune disease. However, occasionally self-reactive T-cells escape these control mechanisms and can lead to diseases like rheumatoid arthritis should they react to a series of synovium or collagen-related peptide sequences.

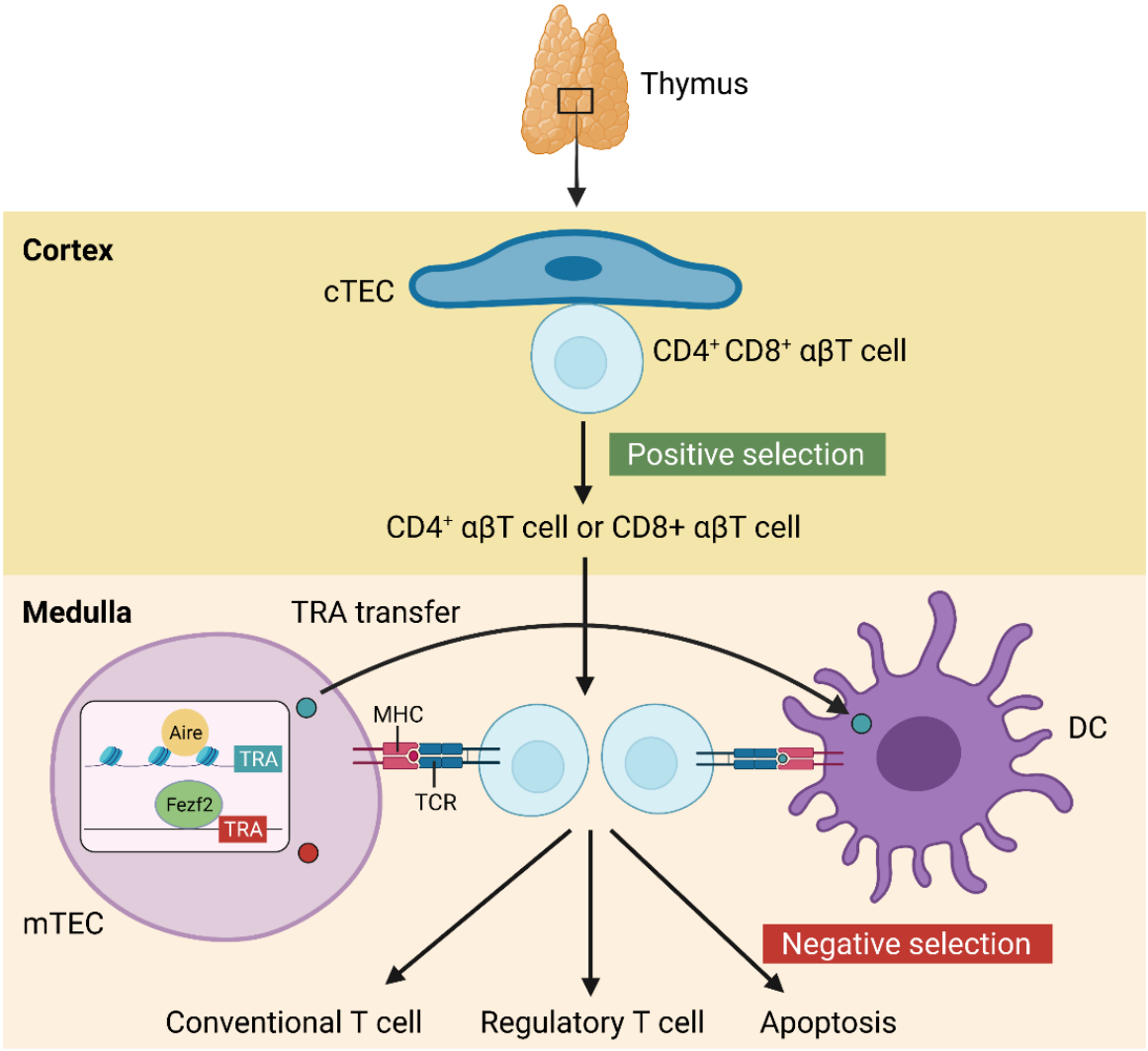


Figure 1 The mechanism of positive and negative selection in Thymus

II. Rheumatoid Arthritis

A healthy joint consists of two adjacent bony ends completely covered in cartilage to prevent friction. The articular cavity is the space between these two ends containing synovial fluid and a thin layer of cells forming by two types of synoviocytes: type A or macrophage-like synovial cells and type B or fibroblast like synoviocytes (FLSs). Together these cells make the synovial membrane. The synovial membrane is responsible for producing synovial fluid, the lubricant between the cartilage (Figure 2).

Rheumatoid arthritis, or RA, is an inflammatory autoimmune disease. that causes chronic synovial inflammation which leads to joint destruction and disability [15]. The confluence of genetic susceptibility and environmental factors recruit multiple components and steps in both the innate immunity, a nonspecific defense mechanism of the body and adaptive immune system, a specific defence process which are involved in the initiation of autoimmune diseases. In RA, synovial fluid is invaded by macrophages, dendric cells, plasma cells and T cells which are components of innate and adaptive immune systems involved in pathobiology of RA.[4]

Over the years, the prevalence of rheumatoid arthritis has increased globally. According to the Arthritis Society of Canada, 1.2% of Canadians has RA (with 20% have some form of arthritis). RA leads to swelling in the small joints of the hands and feet in which it can damage the joint and affect other organs such as eyes, skin, lungs, and the heart if the inflammation continues untreated. The symptoms of RA vary in different patients. Many patients, experience non-specific symptoms such as fatigue, soreness in joints and muscles, weight loss. On the other hand, some RA patients experience severe symptoms dramatic morbidity and premature mortality[16]. Unfortunately, there is no cure for RA and current therapies can only avoid pain and joint damage to help the patients live longer. As a result, it is important to find a cure for RA as most of the patients suffer physical and social consequences. Many patients with RA are at the higher risk of chronic diseases such as heart disease, diabetes, blood pressure and high cholesterol.

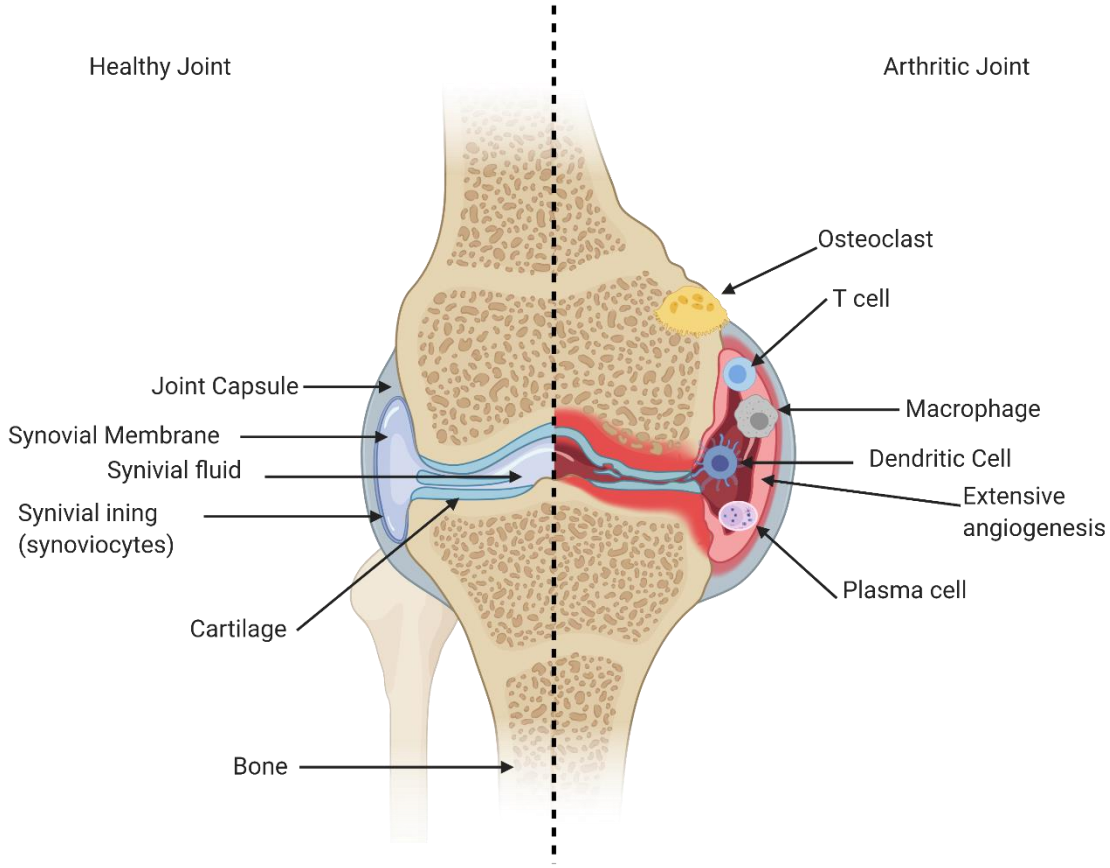


Figure 2. A cartoon comparing healthy and Arthritic joints. [17]

ii. Major Histocompatibility Complex

The major histocompatibility complex (MHC) is located on chromosome 6 at locus p21.3 in humans (Figure 3A), and on chromosome 17 in mice[18]. MHCs are a class of molecules that bind peptides derived from cellular proteins and display them for surveillance by the immune system[19]. MHC molecules are the key elements in the development of humoral and cell-mediated immune responses[20]. Each person has a limited repertoire of MHC proteins, also call the human leukocyte antigen(HLA) inherited in a Mendelian fashion for each class. Fortunately, each HLA protein can host various peptides, and since everyone has one or two alleles at each of the classical loci (HLA-A, B, and C for class I, and HLA-DP, DQ, and DR for class II), a large number of antigens can be effectively eliminated [21]. However, compared to the number of possible pathogen antigens, and, as we shall see, the number of possible T-cell receptors that can recognize antigen-HLA complexes, the number of HLAs remains very low. Important in terms of terminology, in humans, the MHC proteins are called the Human Leukocyte Antigen receptor proteins (HLA), and HLA and MHC will be used throughout the rest of this thesis. In general HLA will be used when discussing specific proteins or clinical ramifications, while the more species-non-specific term MHC will be used for discussions of the protein class in isolation.

The HLA gene products are divided into two main classes: class I and class II. Both share a similar three-dimensional structure in which the antigen binding groove is composed of two domains originating from a single heavy α-chain in HLA class I and from two chains (α-chain and β-chain) in HLA class II.

HLA class I genes consist of three classic loci and three non-classic ones that are less polymorphic. The classic loci are HLA-A, -B, and -C, whereas the non-classics are HLA-E, -F and -G[19,22]. HLA class I molecules consist of one membrane-spanning α chain (heavy chain) and one β2-microglobulin (light chain) (Figure 3B). They are expressed on almost all nucleated cells, and they present 8-10 amino acid peptides to cytotoxic T cells [23]. HLA class II genes, on the other hand, are expressed only on antigen-presenting cells, such as macrophages, dendritic cells, and B cells (Figure 3C). They present 14-18 amino acid peptides to helper T cells [24].

The source of antigens is cytosolic proteins for HLA class I and endosomal and lysosomal proteins for HLA class II [25]. In HLA class I, the antigens are degraded by cytosolic and nuclear proteasomes. Transporters associated with antigen presentation (TAP) translocate the antigen into the endoplasmic reticulum (ER) to access HLA class I. TAP is a member of the ATP-binding cassette family of transport proteins, and each TAP has two subunits (TAP1 and TAP2)[26]. Once the antigens are in the ER lumen, ER aminopeptidases ERAAP/ERAP1 and ERAP2 trim them to HLA I's preferred length of 8-10 residues. TAP also associates with the HLA-I heavy chain, β2-microglobulin, tapasin, calreticulin, and ERp57. This collection of proteins is called the peptide-loading complex, and it facilitates the binding of an antigen to an HLA I molecule. Once the two components join, the loaded HLA I molecule travels to the cell surface. Following the assembly of α and β heterodimers, HLA class I needs the peptide as a third part for stability-without the

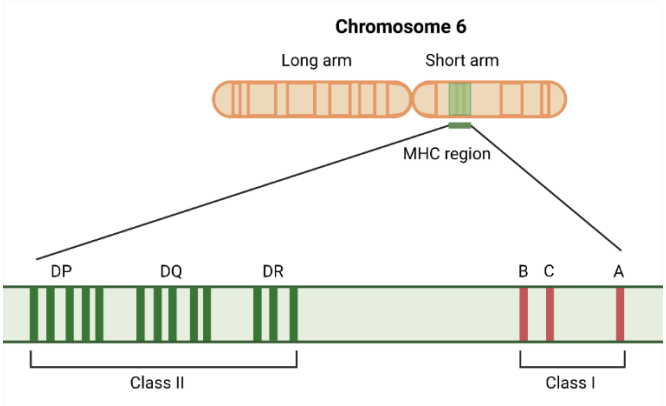
peptide it dissociates, misfolds, and is marked for degradation. After accommodating the peptide in the peptide binding groove, the assembled peptide-HLA complexes leave the ER to present the peptide to CD8+ T cells [27],[28](Figure 4).

HLA class II consists of three subclasses of genes: DR, DQ, and DP (Figure 3A). While the DNA sequences for α-chain are almost conserved in each class, those for β-chain present polymorphism, resulting in the diversity and specificity of peptide binding [29]. In the DR subclass of HLA (HLA-DR), the α-chain is exclusively coded by the DRA*01:01 allele whereas the number of allelic variants of the β-chain (DRB) exceeds 1700 [30]. Individuals can also have multiple copies of each locus, although the number of copies varies between individuals.

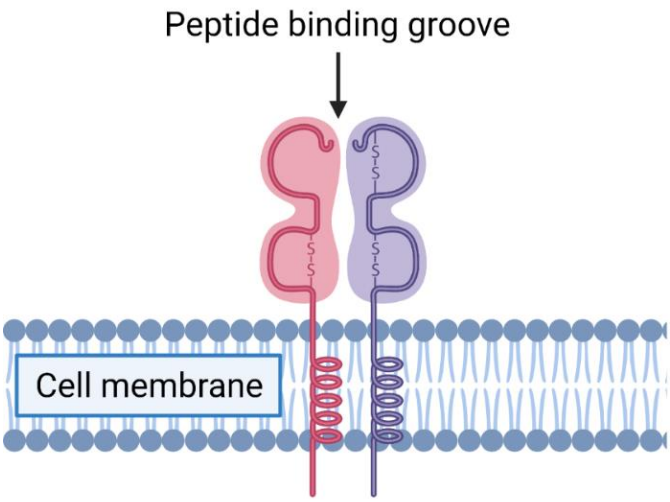
HLA class II molecules are assembled in the ER with a peptide called the invariant chain (Ii), which acts as a pseudo antigen by filling the HLA class II peptide-binding groove during this assembly and keeping them intact before they associate with antigens. These antigens for the HLA class II are endocytosed from the external environment, degraded in the endosomal pathway, and then transported in the form of vesicles or tubules to the plasma membrane [31] (Figure 5).

This series of molecular interactions is essential for a properly working immune system. It is when the antigen, instead of being derived from a foreign pathogen, is self, and when a T-cell with a receptor that recognizes self peptide-MHC complexes manages to escape the thymus, that autoimmune disease is initiated.

A



B



C

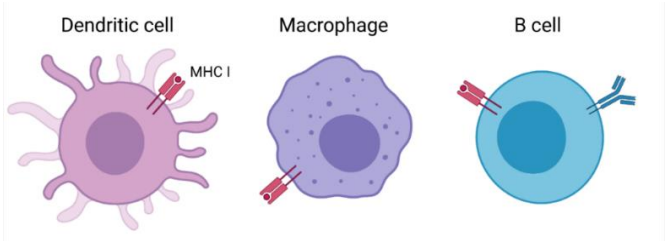


Figure 3 (A) MHC genes in humans, (B), MHC class II molecule, (C) Antigen Presenting Cells

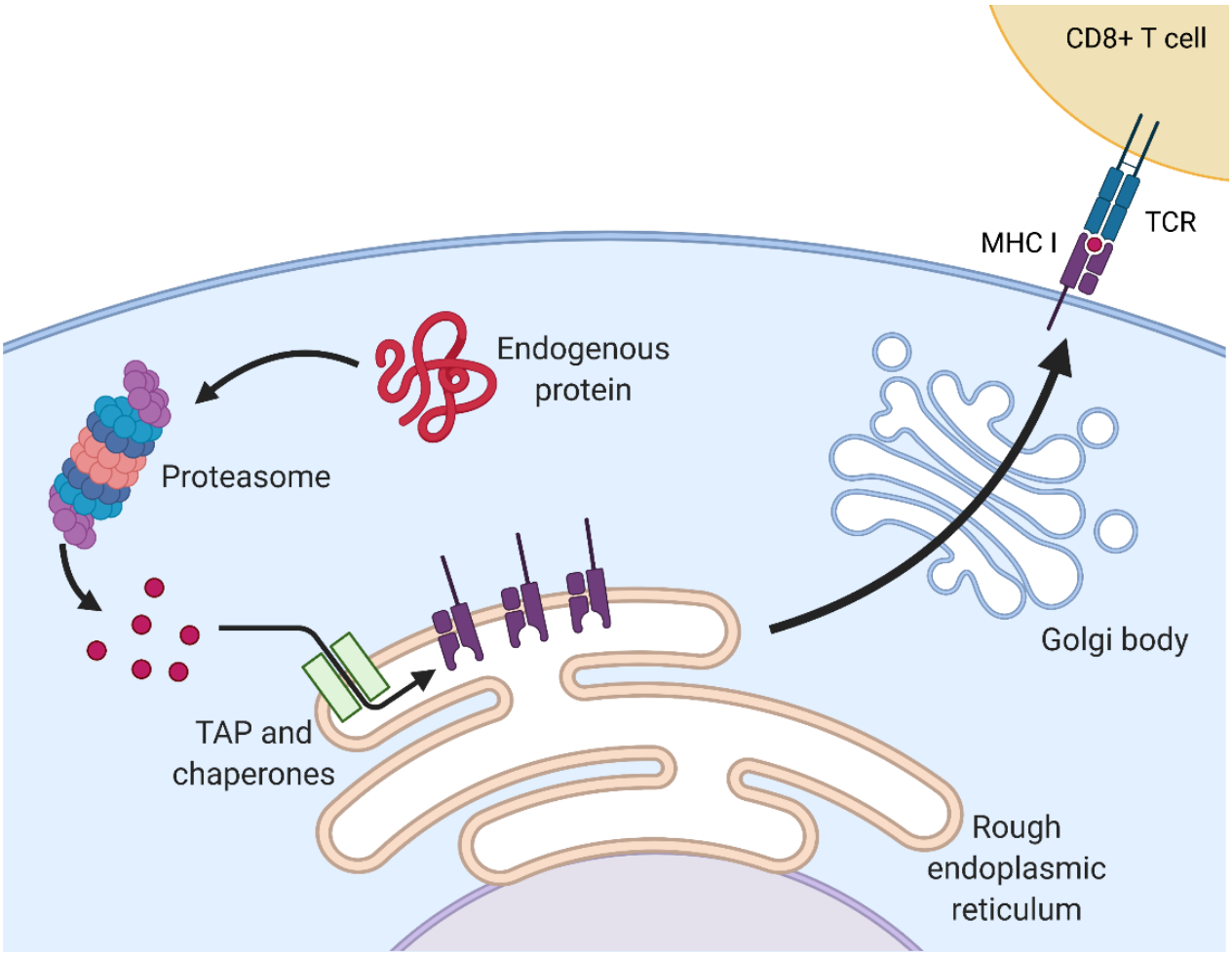


Figure 4 MHC class I assembly[32]

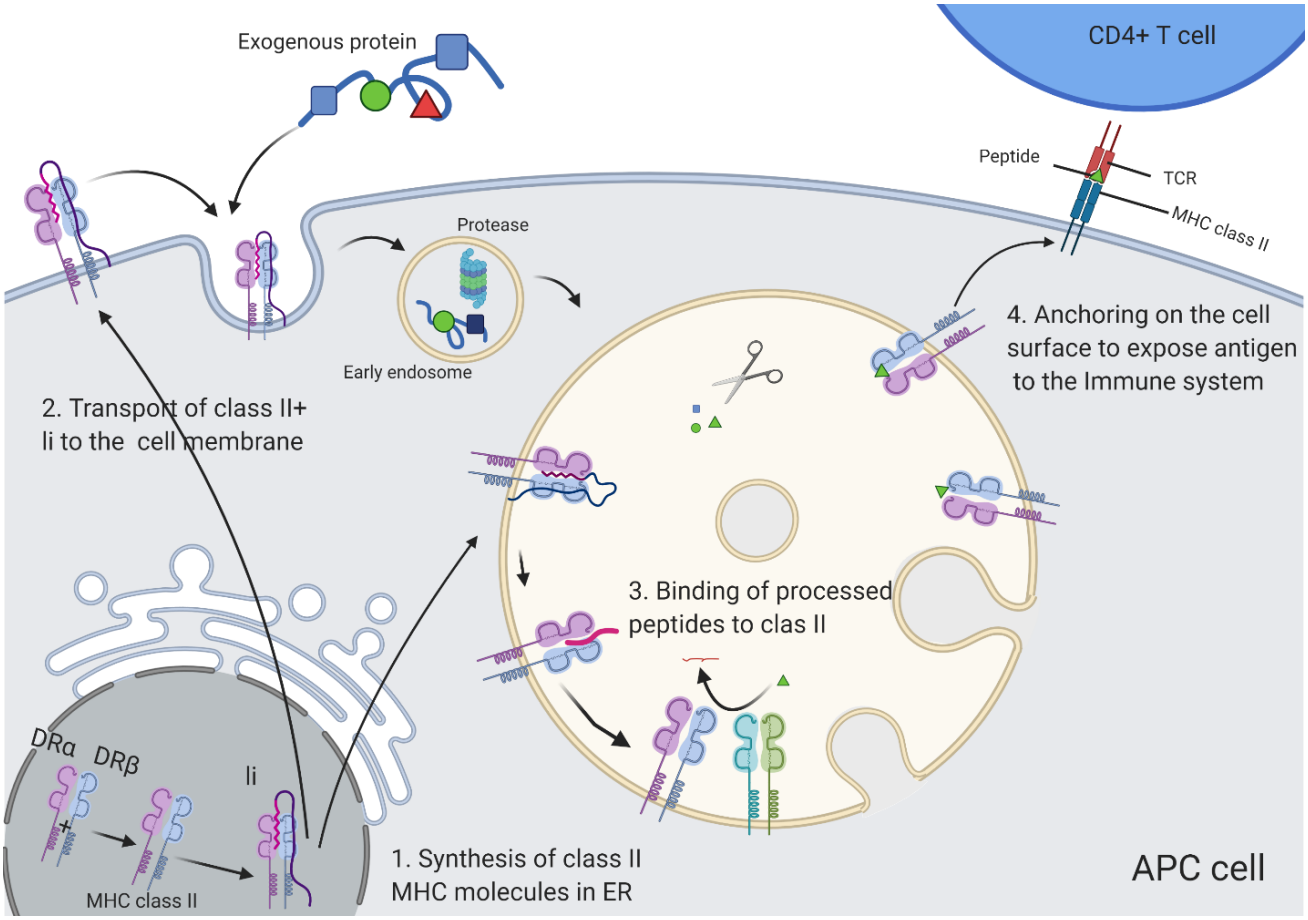


Figure 5 MHC class II pathway (picture from Nature Reviews Immunology)

iv. Pathogenesis of Rheumatoid Arthritis

An interaction between genetic and environmental elements contributes to the pathogenesis of RA. Environmental risk factors such as smoking, infectious agents, and hormones have a role in causing RA; however, genetic risk factors are responsible for about 60% of the risk in developing RA.[33] This autoimmune disease is caused by improper recognition of self-peptides, particularly human cartilage glycoprotein and type II collagen, by specific human leukocyte antigen (HLA) receptors[34]. Major histocompatibility antigens/human leukocyte antigens (specifically those at the HLA-DR loci) and some non-HLA genes have been linked with the pathogenesis of RA. It is well established that HLA-DRB1*01, HLA-DRB1*04, HLA-DRB1*1001, HLA-DRB1*13 and HLA-DRB1*15 containing shared epitope (SE), a five amino acid motif (QKRAA) in the third allelic hypervariable region of HLA-DRβ chain, are associated with the pathogenesis of RA.

Autoimmunity and autoimmune diseases are considered to be driven mainly by adaptive immune responses, namely by auto-reactive B and T cell over-activity[35]. T lymphocytes contribute in multiple steps to the immunopathogenesis of RA [36]. The diversity of the disease process and lymphoid microstructure illustrates that multiple T cell activation pathways are involved. CD4 T cells are the main key lymphocyte in the synovial infiltrate and play a crucial role in pathologic immune response. Antigen-presenting cells including macrophages, dendritic, and active B cells present autoantigens to CD4+ T cells in the context of MHC class II. These cells are known to be the most potent activators and modulators of the adaptive immune system. They reside in peripheral tissue, and they capture and transfer antigens from the outside world to the cells of the adaptive immune system [37,38]. Therefore, CD4+ T cells secrete IL-2 and IFN-γ and infiltrate the synovial membrane, and results in increasing of the lining layer of the joints. T cells in rheumatoid arthritis infiltrate the synovial membrane to transform the macrophages and synovial fibroblasts into tissue destructive effector cells[39]. IL-5 is the main cytokine growth factor for synovial T cells; however, rheumatoid arthritis is considered a T helper 1 cell-mediated disorder, and some studies showed the presence of T helper 17 in synovial membrane. There are different cytokines contributing to expansion and differentiation of TH1 and TH17 cells such as IL-1β, IL-6, IL-17, IL-12, IL-15, IL-18, IL-23P19 and TGFβ.[40] This list of exacerbating cytokines provides many possible targets for therapy; but simultaneously, these are high risk targets as they are all also involved in the normal immune response to pathogenic infection: any modulation of their activity must involve very careful balance as dysregulating them can lead to significant immunocompromisation, infection, or increased tumorigenesis.

Moreover, B cells play a key role in pathogenesis of RA by producing antibodies, autoantibodies, and cytokines. The most common autoantibodies in RA patients are RF and anti-CCP (cyclic citrullinated peptides). Rheumatoid factors (RF) are a family of autoantibodies against Fc portion of IgG. B cells in the lymphoid follicles and germinal center produce RF in RA patients. RF have been detected in 60-80% of RA patients. Anti-citrullinated protein antibodies (ACPAs) are the

other main diagnosis factors with high sensitivity (97%) in RA patients. ACPA is a result of an antibody response to a variety of citrullinated proteins such as fibrin, vimentin, Epstein-barr Nuclear Antigen 1 (EBNNA-1), type II collagen in the body. HLA-DR1 and HLA-DR4 are found as the strongest genetic risk factor associated with ACPA- positive RA. As a result, RF and ACPA are the most significant autoantibodies which results in different pathophysiological information[41], [17]. The continuous stimulation of dendritic cells by autoantigens (fibrin, vimentin, Epstein-barr Nuclear Antigen 1 (EBNNA-1), type II collagen increases B cell activity drives auto-reactive B cells to increase the production of autoantibodies and pro-inflammatory cytokines. Macrophages, besides presenting autoantigens to the immune system, are involved in osteoclast genesis which are multinucleated cells in charge of resorption of bone. [42]

On the other hand, pro-inflammatory cytokines such as IL-6 and TNF-α, IFN-γ, and IL-1, have a significant effect on the pathogenesis of RA by osteoclast activation, increasing monocyte activation and production of synovial fibroblast cytokines, respectively. Cartilage degradation in RA patients happens when these pro-inflammatory cytokines activate synoviocytes, , which leads to MMPs (Matrix metalloproteinases) secretion into SF (Synovial fluids).[43] All in all, we can conclude that CD4 T cells are the main mediators in pathobiology of Rheumatoid Arthritis and targeting this mechanism will be essential for the development of promising therapeutic for RA. The molecular mechanisms and feedback loops are complicated and disorienting: however, it is important to note that once the initial response is initiated, triggered by the presence of the antigen, the downstream cytokine responses discussed here are common to almost all inflammatory responses to pathogen or antigen presence. Again, although this apparently provides a myriad of drug targets, all of these molecular targets are common to the rest of the immune system and are required for proper function. Targeting these systems needs to be done very cautiously; it can be seen as akin to a police force, trying to catch a thief to keep a city running well, arresting the entire population. It will certainly capture the thief, but it will also prevent the city from running well. A more targeted approach is required (Figure 6).

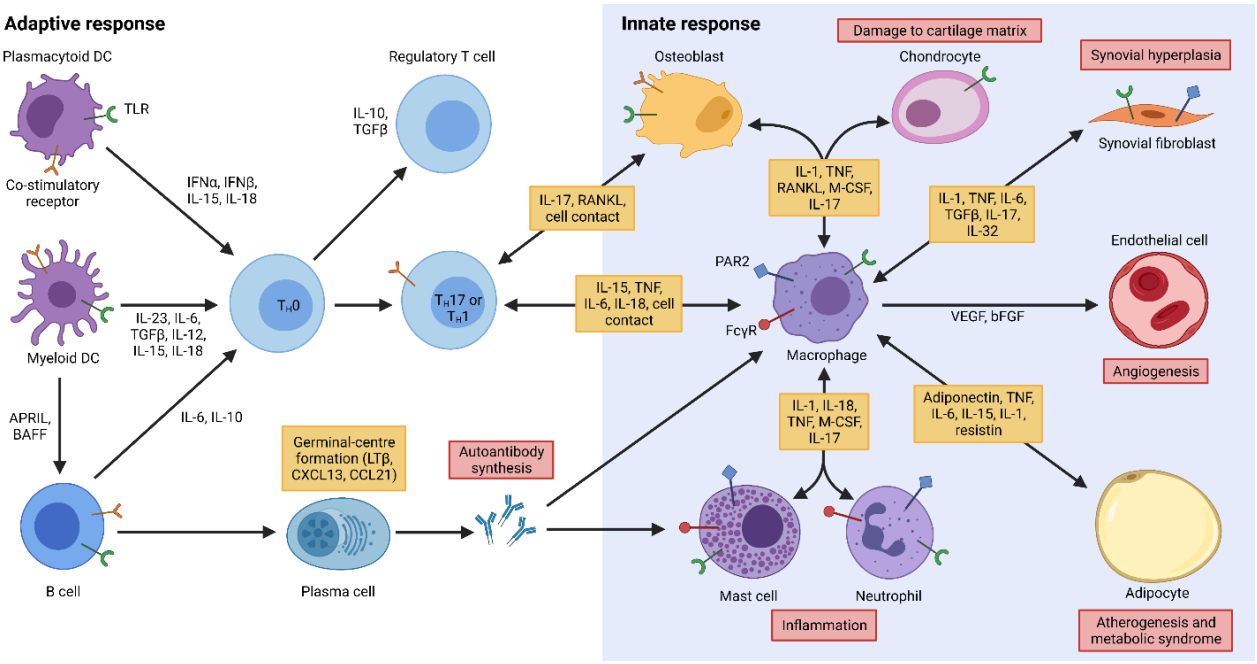


Figure 6 Components of adaptive and innate immune system involved in the pathogenesis of RA.

iii. Ectopic Lymphoid Structures in Rheumatoid arthritis

Ectopic (or tertiary) lymphoid structures (ELS) are organized aggregates of lymphocytes resembling secondary lymphoid organs and developing in the target organs of patients affected by autoimmune diseases, such as salivary glands in SS [44], synovial tissue in RA [45], kidneys in SLE [46], meninges in MS [47], and thyroids in Hashimoto's thyroiditis. In autoimmune diseases, ELS play a role in chronic inflammation and maintaining the disease process leading to a severe disease course[48].

Innate immunity cells and FLS contribute to the formation of ELS by infiltrating lymphoid cells into synovia and making secondary B-cell follicles with germinal cells and T cell compartments containing dendritic cells. It is believed that these immunological components facilitate the antigen presentation at the place of inflammation to trigger the immune system. ELS can destroy the tissue specifically, cartilage and joints by exposing continuously with autoantigens at the site of inflammation. In RA, the synovium, the soft tissue that lines the inner surface of joints, is the place of heterogeneous infiltration of leukocyte, synovial lining hyperplasia, and neovascularization resulting in the destruction of cartilage, bone erosion and joint disability.

The mediators involved in the process of ELS formation are lymphotoxin-β (LTβ), CXCL13, CCL19, and CCL21[49]. These lymphoid chemokines play a key role in ELS development. The lymphoid chemokines, CXCL13, CCL19 and CCL21 can be upregulated in rheumatoid synovium. The high level of these chemokines is also associated with the upstream regulation of ELS organization. Studies have shown that in rheumatoid synovium haematopoietic cells including CD14+/CD68+ monocyte/macrophages and memory T cells are producing CXCL13. So, all the cells in the innate and adaptive immune system help to recruit and organize the B cells. Particularly, B-cell aggregates with the feature of ELS is associated with the expression of CXCL13. As a result, CXCL13 and CCL21 are the main chemokines contributed in the maintain and function of ELS.

vi. Treatment strategies in rheumatoid arthritis

As referenced above, current, and emerging therapeutics either treat the symptoms of the disease or non-specifically weaken the immune response to reduce symptom severity by targeting some of the cytokines involved in the immune response. Some nonsteroidal anti-inflammatory drugs (NSAIDs) like aspirin, ibuprofen, or naproxen are used by some patients on a regular basis to relieve the pain. This family of drugs blocks the cyclooxygenase (COX) enzymes. COX trigger the production of prostaglandins, which are the mediators causing inflammation and pain. The main potential side effect of NSAID overuse is inhibiting platelet aggregation leading to dramatic gastrointestinal disorders such as bleeding, ulcers, and perforation.[50] The other group of drugs which is common between patients with severe RA is glucocorticoids. These drugs bind to their specific receptors and inhibit cellular signaling pathways[51]. Although this family of drugs is significantly effective in the treatment of RA, the adverse side effects are one of the main concerns such as infection, gastrointestinal problems, ophthalmologic effects.[52]. To reduce inflammation, disease-modifying anti-rheumatic drugs (DMARDs) were developed. DMARDs are common in the treatment of autoimmune diseases like RA. Methotrexate, an analog and antagonist of folic acid, inhibits folate-dependent enzymes, it also inhibits purine and pyrimidine synthesis which are necessary for DNA and RNA synthesis. Leflunomide is another drug from DMARDs family drug abandon the synthesis of de novo pyrimidine ribonucleotides As a result they supress the production of pro inflammatory cytokines [53]. As mentioned in the pathogenesis of RA, pro-inflammatory cytokines such as IL-6 and TNF-α, IFN-γ, and IL-1 are abundant in these patients in which TNF is considered a key proinflammatory cytokine in autoimmune diseases, as a result discovery of anti-TNF biologics such as infliximab, etanercept, adalimumab, golimumab, and certolizumab pegol has been a gold standard therapeutic drugs in the past decades for autoimmune diseases [54 55].

In addition to anti-TNF biologics, other biological drugs suppressing proinflammatory cytokines have been developed. Abatacept, a humanized fusion protein of extracellular domain of TLA-4 and Fc fraction if IgG1, selectively inhibits CD80-CD86 and activation of T cells. The other biologic drug is Tocilizumab: a humanized anti-IL-6 receptor monoclonal antibody that blocks the transmembrane signaling of IL-6. Other biological drugs targeting other pro-inflammatory cytokines such as IL-1, IL-12, IL-23, IL-17, and CD20 have been approved as a complementary treatment of anti-TNF therapy.

Although anti-TNF biologic drugs have been used in the treatment of AD progressively, the adverse side effects of these drugs should be taken into consideration. Studies have shown that anti-TNF biologics may, unsurprisingly, have major impacts on TNF expression in patients. Moreover, patients with TNF-alpha 308G > A polymorphism do not respond to ani-TNF therapy[56].

TNF is one of the major arms of immune system to defend the body against pathogens by inducing nitric oxide in macrophages, triggering IFN-γ to induce inducible nitric oxide synthase (iNOS). As a result, using the anti-TNF biologics to block TNF expression, is certainly a great concern due to reactivation of tuberculosis and pneumonia leading to new bacterial infection. Anti inflammatory treatment promotes the risk of cancer, so patients with malignancy should be carefully studied after anti-TNF biologics [57]. Consequently, effort has been made to target the HLA-antigen interaction, the one molecular event that is unique to RA and is not involved in any other immune response. Preventing self-peptides from initiating an immune response would mean that we would be able to prevent RA without affecting any other normal component of the immune system.

Family of Drugs	Drugs	Target	Side effects
Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)	Traditional non-selective NSAIDs	Inhibit cyclooxygenase (COX) enzymes	Gastrointestinal disorders, renal toxicity, cardiovascular system
	Selective cyclooxygenase-2 (COX-2) inhibitors	Inhibit cyclooxygenase (COX) enzymes	
Glucocorticoids, (corticosteroids)	Dexamethasone and betamethasone	Inhibit cellular signaling pathway such as AP-1 and NF-κB	Gastrointestinal ulcers and bleeding, infection, immunosuppression, and bone damage
Conventional Disease-Modifying Anti-Rheumatic Drugs (cDMARDs)	Methotrexate	Inhibit the activity of folate-dependent enzymes, and synthesis of purine and pyrimidine which are required for DNA and RNA synthesis	Adverse effect on skin and mucosa, reversible and irreversible renal disease, hypertension, and heurism
	Leflunomide	Inhibit the synthesis of de	

		novo pyrimidine ribonucleotides	
Biologics	Abatacept	Suppress T cells	
Biologics	Rituximab	Suppress B cells	
Anti-TNF Biologics	Infliximab	Inactive binding of TNF to its soluble and membrane receptors	Not all patients respond equally well to the treatment, infections, such as tuberculosis and pneumonia, are common adverse event of patients treated with anti-TNF biologics, malignancy, side effects on the neurological system
	Etanercept	Inactivates soluble form and membrane form of TNF and lymphotoxin	
	Adalimumab	Block human TNF binding to its receptors	
	Golimumab	Neutralization of soluble and trans-membrane forms of TNF	
	Certolizumab Pegol	TNF inhibitor	

III. Definition of Altered Peptide Ligands

Peptides derived from antigens are the major element in causing the autoimmune diseases which have been a potential target of treating autoimmune diseases. Two general approaches are possible. The first, altered peptide ligands (APLs), are analogues derived from the original antigenic peptide that aim at triggering different classes of T-cells to balance the pro-inflammatory response with an anti-inflammatory response. The second are HLA-blockers (HLABs), which seek to stop the immune response by quantitatively displacing antigenic peptides and also preventing any T-cell receptor interaction with the MHC-HLAB complex. (Figure 7).

Specific recognition of an immunogenic peptide epitope by T cell receptor triggers the immune system. A strategy for immune evasion would be helpful to prevent the T cell response. Altered peptide ligands, analogues derived from the native peptides with single or few amino acid changes can regulate the immune responses. APLs can antagonize and inhibit T cell activation and discovery of APLs have been an immune therapeutic peptide to stop the immune response where the T cell activation can play a key role in the immunopathology of autoimmune diseases. Many APLs have been designed in peptide-based research by replacing the single amino acids in an immunogenic peptide to check the outcome of T cell proliferation and cytokine production.[58] The modification of Alanine to Leucine in the melanoma-associated Mart-1/Melan-A(26-35) epitope EAAGIGILTV that cause enhances MHC-binding is a famous example of APLs[59]. Three different APLs with a couple of substitutions of key residues have been designed for TCR and HLA-DR recognition in rheumatoid arthritis patients. These APLs are produced based on native peptides such as Collagen II (CII), human cartilage glycoprotein-39 (HCgp-39) and binding protein (BiP). Results on peripheral blood mononuclear cells (PBMCs) of RA patients have been illustrated that mutation in the main contact residues are able to abandon the immune responses; However, APLs may result in unanticipated and unwanted side effects and have adverse events in the patients[60]. TCR polymorphism means that a peptide that initiates a strong anti-inflammatory Th2 response in one individual can initiate a strong pro-inflammatory Th1 response in another (Figure 7). Some studies have been showed the APLs generated from myelin sheath are able to change the responses from Th1 the stimulator of cellular immune response by macrophage inhibition to Th2, the subgroup of T cell stimulating humoral immune response and promoting cell proliferation and antibody production and inhibit EAE. Moreover, several patients developed immediate hypersensitivity and immune responses due to the cross reactivity with the native antigens and high production of IFNγ and low IL-4 were reported in some cases[60]. The primary risk for these systems is that they are highly personal: similar TCRs able to recognize the same MHC-APL complex might be anti inflammatory in many patients, but proinflammatory in others, or highly immunogenic in a third group. Although careful ex vivo testing of an individual's response to APLs might be able to mitigate the risk and identity those likely to benefit from those likely to exhibit extreme adverse, and potentially fatal, responses, the approach still retains risk as reactive

TCRs could be present in very low concentrations and not immediately respond to the testing of the therapy. This inherent risk has been one of the reasons that no APL has ever been approved for clinical use.

APLs have one major benefit though: as they are designed to balance the immune response, they do not need to prevent antigen binding, they only need to balance it. This means that they do not need to exhibit exceptional affinity for the binding groove in comparison to the natural antigens. This does make their design a lot more flexible and easier.

Glatiramer acetate is an approved drug for the treatment of relapsing and remitting of Multiple sclerosis. It consists of salts of synthetic polypeptides containing random chains of four naturally amino acids. This drug competes with myelin basic protein for biding to MHC molecules (HLA-DRB1) and downregulate inflammation by secretion of anti-inflammatory cytokines. However, it is generally received that this is only one of the mechanisms of action of this drug, and not likely the one primarily responsible for the observed anti-autoimmune effects. But it does indicate that peptide drugs which could be accommodated in the MHC binding groove with strong and high affinity could be a promising peptide drug which cure the autoimmune diseases [61].

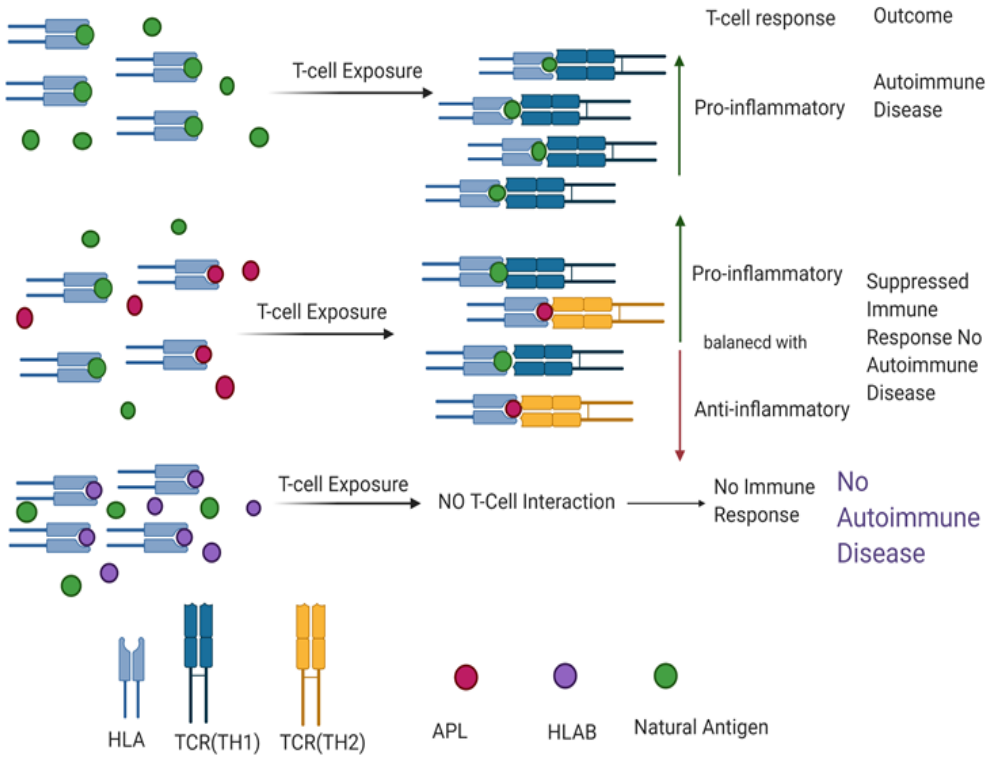


Figure 7 Schematic picture of the different outcomes of APLs and HLABs[61]

IV. Human Leukocyte Antigen Blockers

Some new strategies such as Human Leukocyte Antigen Blockers (HLABs) are needed to block the HLA with high and strong affinity aim to displace the antigenic peptides from the HLA quantitively could cure autoimmune diseases.

HLABs are peptide drugs that possess several features: exceptional binding affinity (we estimate >10,000-fold that of immunogenic antigens), no immunogenicity, high protease resistance, and facile up-take by antigen-presenting cells. The HLABs can displace the disease-causing peptides with higher affinity. As a result, they prevent T cells from recognizing the HLAB-HLA complex to ensure that that they do not initiate the immune responses and halt the immune response in autoimmune diseases without any impact on the rest of the immune system.

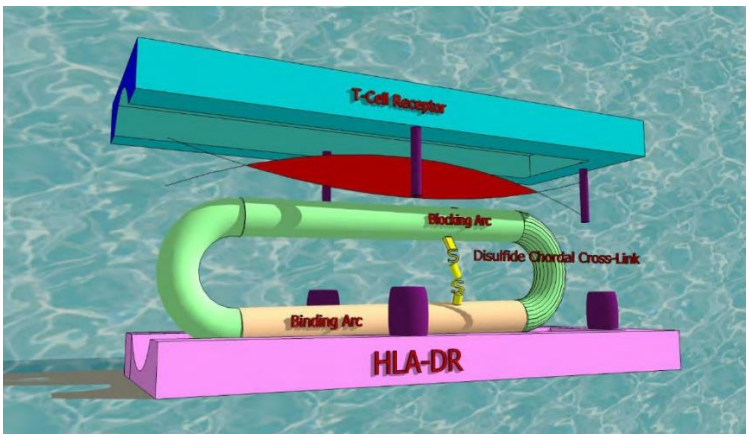
The natural antigen must be quantitatively displaced for an HLAB strategy to work. This requires the drug to have exceptional affinity for the HLA; however, after a decade of effort, it is now clear that the required increase in affinity is not attainable using natural residues. HLA binding pockets are not specific for any given residue, the HLA must be able to bind a wide variety of peptide ligands and so no pocket can be specific for any amino acid. The required relative improvements in binding affinity are simply not available using natural amino acids. Exhaustive screens have been conducted for celiac disease by the groups of Khosla, Sollid, and Konigs over the past couple of decades demonstrating this is unfeasible. But this does not mean that the HLA binding groove's pockets do not have a defined 3-dimensional structure. It just means that no natural residue fits very well. Consequently, we need to design new residues to fit these pockets. This is the core innovation of the Trant lab's research approach[62]. Our computational chemists have designed peptides with non-conical amino acids which can bind with high affinity and accumulate in the empty binding groove space of HLA-DR4, and that will be able to quantitatively displace any antigen present in the groove due to their exceptional affinity.

However strong binding is insufficient: this would just lead to a new antigen-HLA complex that would likely be recognizable by circulating T-cells and we are left with the same issue faced by APLs. However, TCRs must recognize key points on both the antigen and the HLA to bind. Should the TCR be physically prevented from binding, then it can't recognize the receptor and we can render it invisible to the immune system. This can be accomplished by cyclizing the peptide. Figure 8b shows a cyclic peptide resting in an HLA (DR1). The peptide surface is presented as a blue wire mesh, the HLA as a ribbon structure and the TCR as a string structure. In 8c, the TCR surface of the same view is now provided. It needs to occupy the precise space occupied by the "blocking arc" of the peptide. Figure 8a provides a cartoon representation of this strategy. The group is focusing on building new, and using established methods, to cyclize peptides and build them from

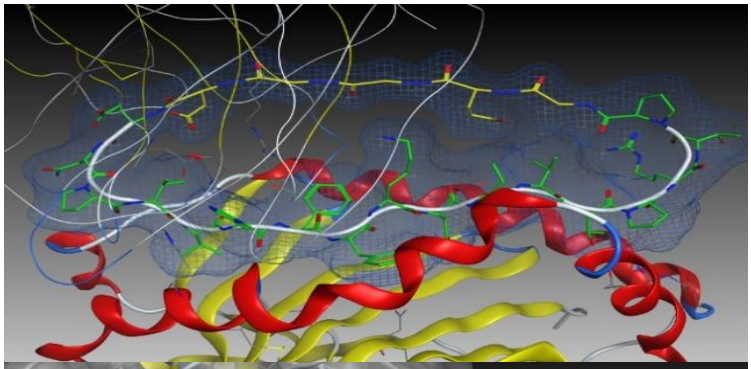
unnatural amino acids to make these types of drugs. However, the drugs are useless without a means to evaluate them.

The main goal of this thesis is to develop a reliable method to express and purify the HLA-DR4 protein, so that we can evaluate the binding affinity of the purified protein with the peptide drugs designed in our lab to aid in the design of these novel therapeutics.

A



B



C

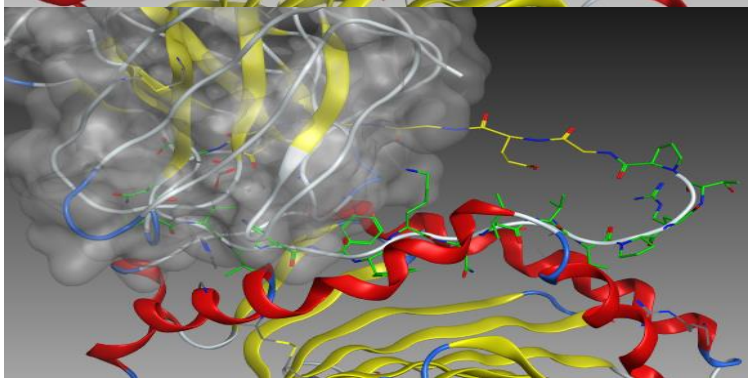


Figure 8 A cartoon illustrating HLABs. (Made by John Trant, unpublished figure, 2015 Figure) B. a cyclic peptide resting in an HLA (DR1). C. the TCR surface (Made by Taimoory and Trant, unpublished result, 2018).

Hypothesis

We hypothesize that because Human Leukocyte Antigen is a human α/β glycosylated protein, mammalian cell culture will be the best expression system and also the Human Leukocyte Antigen Blockers (HLAB) will be able to competitively displace and prevent the binding of disease-causing peptides in the HLA. We hypothesize that our HLABs will prove to be non-immunogenic in a DR4 mouse model, and that, when co-administered with the immunogenic peptide, will prevent the emergence of RA compared to positive control models (where the peptide is administered). We employed computational design to identify non-natural amino acids that fit the HLA binding pockets unsatisfied by natural amino acids. After synthesis we will measure our HLABs' experimental affinity for the HLA, and their efficacy in vitro, in a novel human ex vivo model, and in vivo in mice.

Objectives

Long-Term Objectives:

To generate new HLAB peptide therapies for RA, and to develop chemical tools that will allow immunologists, for the first time, inhibit an HLA-TCR interaction.

Medium-Term Objectives for the Trantteam:

- Design, synthesize and determine the in vitro binding affinity of HLABs targeting RA associated HLA-DR4.
- 3-D print synovium (the tissue inflamed in rheumatoid arthritis) and use it as an ex vivo model of RA
- Determine the efficacy of HLABs in vitro and in vivo.

Short term:

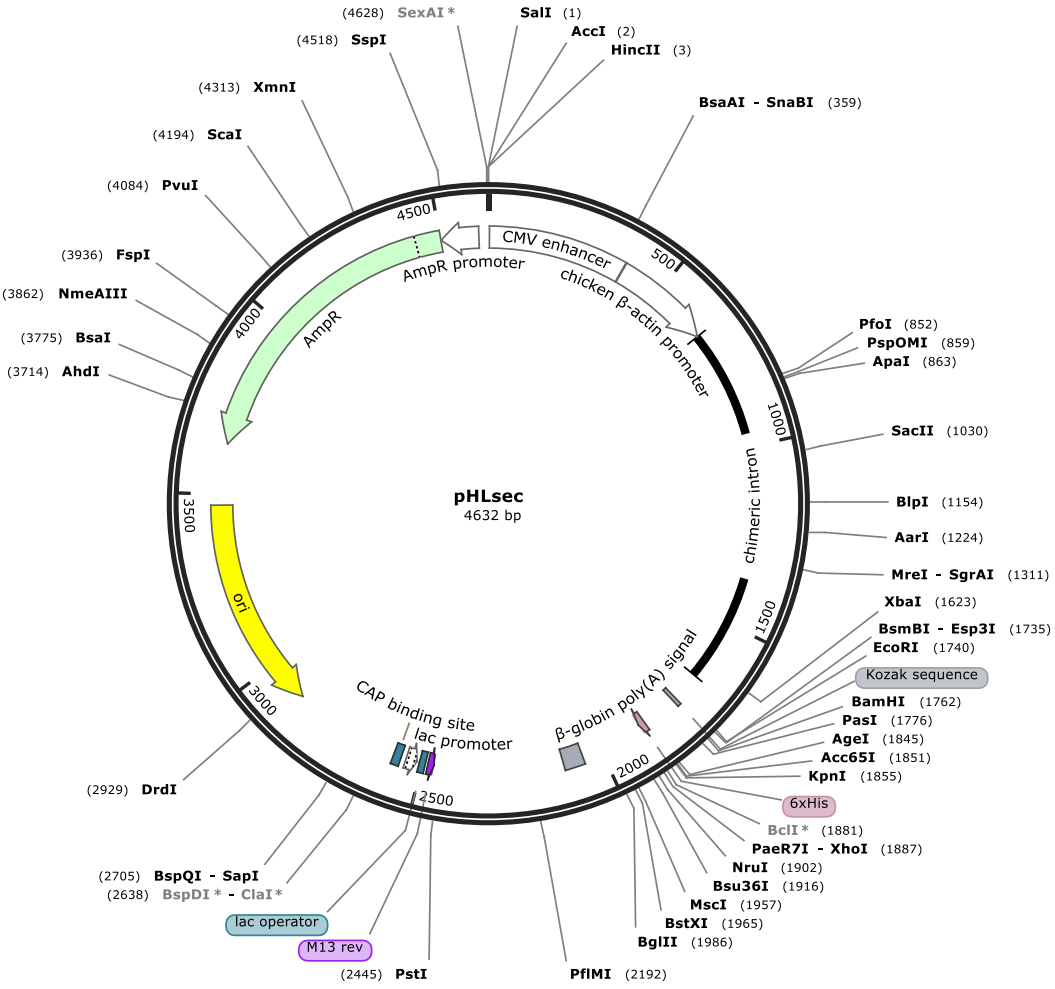
To generate a proper method to express and purify Human leukocyte Antigens for structural and biophysical studies, functional assays, drug development and for therapeutic applications assays.

Materials & Methods:

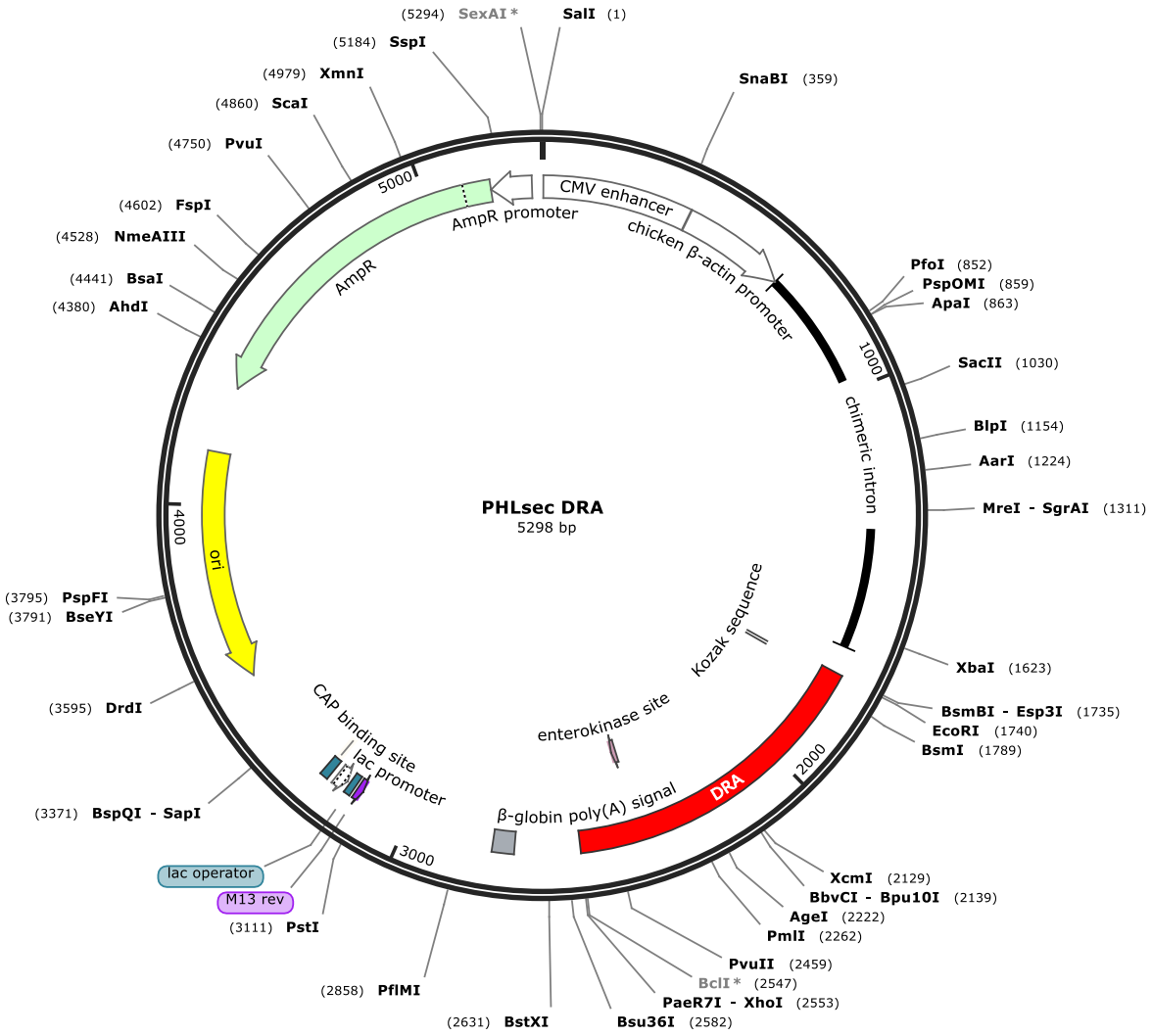
Plasmids

The pHLsec DRA DNA & pHLsec DRB1*0401 DNA plasmids were a gift from Stephen W. Scally, Monash University, Australia. The pHLsec vector contains the extracellular domains of HLA -DR4 (DRA*01:01/DRB1*0401) α and β chains and C-terminal enterokinase cleavable fos/jun zippers to promote dimerization. The β chain contains a BirA site for biotinylation and tetramer generation and a Histidine tag for Immobilized Metal Affinity Chromatography (IMAC) purification. An ampicillin resistance gene is included in both DRA DNA & pHLsec DRB1*04:01 DNA plasmids. The maps below were designed using SnapGene Viewer, version 5.1.5, from GSL Biotech; available at www.snapgene.com.

A. pHLsec plasmid map



B. pHLsec DRA map



C. pHLsec DRB1 04 map

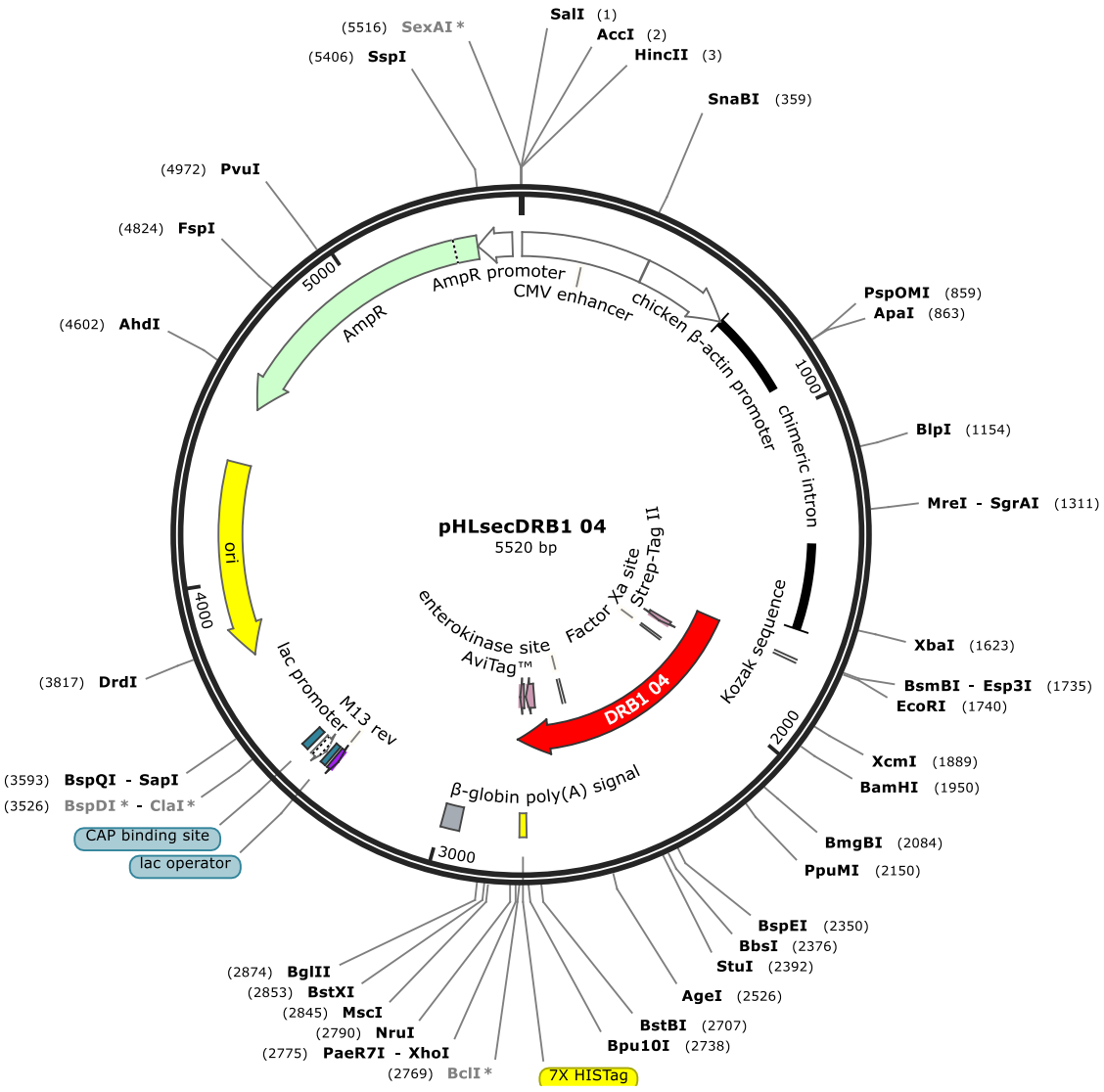


Table 1 DNA Sequence of DRA and DRB1 04

Vector/DNA Sequence	Sequence
pHLsec DRA	GAATTCAAGCTTGCCGCCACCATGGGAATCCTGCCTTCACCCG GAATGCCTGCGCTTCTGTCACTGGTGTCGTTGCTTTCGGTACTC CTGATGGGATGCGTGGCCATTAAGGAGGAACACGTCATCATTC AGGCGGAGTTCTATCTGAACCCGGATCAATCGGGCGAGTTTAT GTTCGACTTCGACGGTGATGAGATCTTCCATGTGGATATGGCA AAGAAAGAGACAGTCTGGCGCCTTGAAGAATTTGGGAGGTTCG CCAGCTTTGAGGCCCAAGGTGCCCTCGCGAATATCGCTGTAGA CAAAGCGAACCTTGAAATCATGACGAAGAGATCTAACTATACT CCGATTACGAACGTGCCACCGGAGGTCACGGTGCTCACCAACT CCCCGGTGGAGCTGAGGGAGCCAAATGTGTTGATCTGTTTCAT CGATAAGTTTACCCCTCCCGTCGTCAACGTAACATGGTTGCGA AATGGTAAACCGGTGACAACAGGCGTTAGCGAAACTGTATTCC TTCCACGTGAGGATCACCTCTTTCGGAAATTCCATTACCTCCCT TTTCTGCCCTCCACGGAGGACGTGTACGACTGTCGAGTTGAAC ACTGGGGACTTGACGAACCCTTGTTGAAGCATTGGGAGTTTGA TACTAGTGGGGACGATGATGATAAGGGAAGTGGGAGCGGGCT CACAGACACCCTCCAAGCCGAAACGGACCAGCTGGAAGATGA AAAGTCAGCACTTCAGACGGAGATTGCGAATCTGTTGAAAGAA AAAGAAAAACTTGAGTTTATCCTTGCAGCGTAATGATCACTCG AG
PHLsecDRB1 04	GAATTCAAGCTTGCCGCCACCATGGGGATTCTGCCTTCACCTGG GATGCCTGCTCTGCTGTCTCTGGTCTCTCTGCTGTCAGTCCTGCT GATGGGATGCGTCGCTTGGAGCCACCCACAGTTCGAGAAGGGA GCACCTGTGTCCAAAATGAGAATGGCTACACCACTGCTGATGC AGGCAAGCGGGGGAAGCGGAAGTATTGAGGGGCGGGGATCCG GGGACACCCGACCACGTTTCTTGGAGCAGGTTAAACATGAGTG TCATTTCTTCAACGGGACGGAGCGGGTGCGGTTCCTGGACAGA TACTTCTATCACCAAGAGGAGTACGTGCGCTTCGACAGCGACG TGGGGGAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTGATG CCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAAGC GGGCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGG TGAGAGCTTCACAGTGCAGCGGCGAGTCTATCCTGAGGTGACT GTGTATCCTGCAAAGACCCAGCCCCTGCAGCACCACAACCTCC TGGTCTGCTCTGTGAATGGTTTCTATCCAGGCAGCATTGAAGTC AGGTGGTTCCGGAACGGCCAGGAAGAGAAGACTGGGGTGGTG TCCACAGGCCTGATCCAGAATGGAGACTGGACCTTCCAGACCC

	TGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACAC CTGCCAAGTGGAGCACCCAAGCCTGACGAGCCCTCTCACAGTG GAATGGAGAGCAACCGGTGGCGACGATGATGACAAGGGTTCA GGGTCTGGGAGAATTGCCCGACTCGAAGAGAAAGTCAAAACG TTGAAAGCGCAAAACTCGGAGCTGGCTTCAACCGCGAATATGC TCCGTGAACAGGTCGCGCAGTTGAAACAGAAGGTGGGAAGCG GTTCGGGAGGGTTGAATGACATTTTCGAAGCGCAGAAGATCGA GTGGCACGAGGGCTCAGGCCATCATCATCACCATCACCACTAA TGATCACTCGAG

Table 2 Protein sequence of DRA and DRB1.04

Vector/Protein sequence	Sequence
pHLsec DRA	MGILPSPGMPALLSLVSLLSVLLMGCVAIKEEHVIIQAEFYLNPDQ SGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFASFEAQGALANI AVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFID KFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPS TEDVYDCRVEHWGLDEPLLKHWEFDTSGDDDDKGSGSGLTDTL QAETDQLEDEKSALQTEIANLLKEKEKLEFILAA--
PHLsecDRB1.04	MGILPSPGMPALLSLVSLLSVLLMGCVAWSHPQFEKGAPVSKMR MATPLLMQASGGSGSIEGRGSGDTRPRFLEQVKHECHFFNGTERV RFLDRYFYHQEEYVRFDSDVGEYRAVTELGRPDAEYWNSQKDLL EQKRAAVDTYCRHNYGVGESFTVQRRVYPEVTVYPAKTQPLQHH NLLVCSVNGFYPGSIEVRWFRNGQEEKTGVVSTGLIQNGDWTFQT LVMLETVPRSGEVYTCQVEHPSLTSPLTVEWRATGGDDDDKGSG SGRIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVGSGSG GLNDIFEAQKIEWHEGSGHHHHHHH

Transformation into competent *E. coli*

The plasmids containing DRB1*04 and DRA*0101 genes were separately transformed into NEB-STBL (NEB; Cat#C3040H) competent cells. 2ul of each plasmid (see supplementary) was added to 25 µL of the competent cells and incubated at 4 °C for 30 minutes, then they were heat shocked at 42°C for 42 seconds and returned to ice 4°C for 2 minutes. The transformation mix was diluted into 1 ml of SOC and incubated at 37°C with shaking at 250 rpm for 1 hour. After incubation, the 10uL or 100 µL of transformation was each plated onto 10 cm LB agar plates containing ampicillin antibiotic (see supplementary) and incubated at 37°C overnight.

After 24 hours, colonies of E. coli containing the plasmid of interest was grown in 3mL of LB media plus 50 ug/ml of ampicillin antibiotic overnight. The plasmid DNA was extracted using DNA miniprep kit (Biobasic; Cat#BS88503. The plasmid DNA were digested with EcoRI restriction enzyme to check the purity of the preparation and the correct size of each plasmid. One of the plasmids preps, presenting the corrected size and the cleanest band was transformed again to do a maxiprep using the maxiprep kit (Biobasic; Cat#9K0060023). After maxi prepping, the products underwent restriction digestion using EcoR Ⅰ restriction enzyme to again check for the size and purity of the preparation.

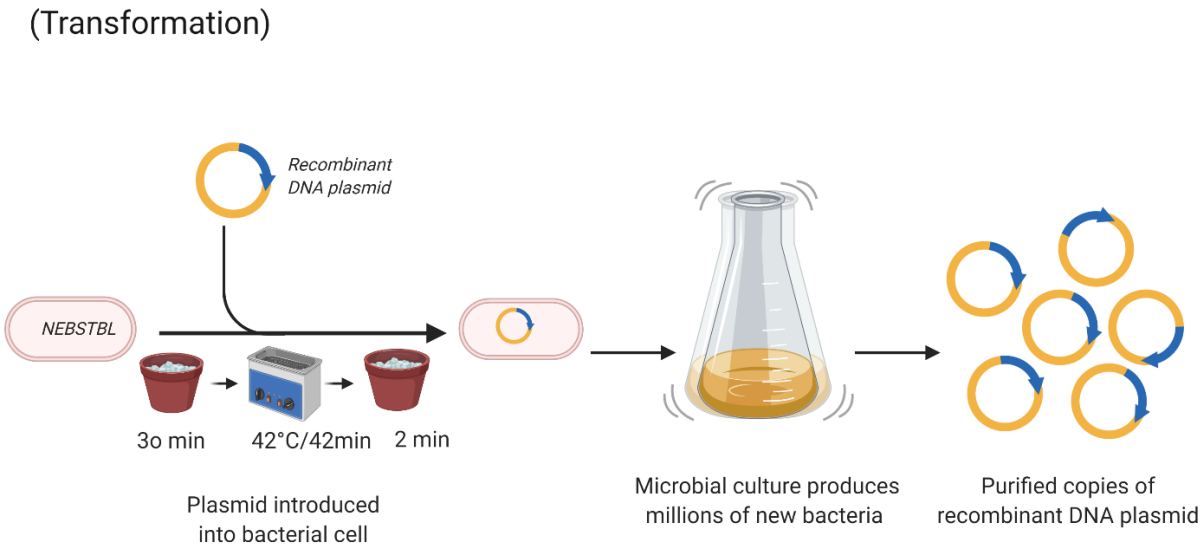


Figure 9 Transformation of *E. coli*

Digestion of Plasmids

Restriction digestion of the plasmids was performed in 10 µl of solution containing: 5 µl of plasmid DNA, 1 µl of EcoRI, 1 µl of fast digest green buffer and 3µl H_2O for miniprep extraction and 10 µl of solution containing: 2 µl of plasmid DNA, 1 µl of EcoRI, 1 µl of Fast Digest green buffer and 6µl H_2O for maxiprep extraction. Reactions were incubated at 37°C for 30 minutes and analysed by agarose gel.

Agarose DNA gels:

Agarose gels were made up to 1% (w/v) Agarose in 1X TAE containing 1 µl ethidium bromide. After solidifying the gels, prepared samples were loaded into the wells. Gels were run in a tank containing 1X TAE for 30 min at 100 V. Bands were visualised by UV using Alpha Innotech gel imaging system.

Antibodies

The following antibodies were used in western blotting and immunofluorescence analysis:

Anti His rabbit (Santa Cruz Biotechnology – Cat # sc-803)

Anti HLA-DR human Clone L243 (Thermofisher, Cat #5011313)

Alexa 488 rabbit (Thermo - Life Technologies Cat # A11008)

α-rabbit IgG peroxidase conjugated (Sigma Cat # A0545)

Mammalian Cell Culture

HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; Thermo-Fisher; Lot#906532) and 1% Penicillin-Streptomycin (Gibco; Cat#1514122) at 37°C in 5% CO2 overnight (see the supplementary).

Protein expression & concentration determination

Cells at the confluency of 70% were transfected with 10 µg of DNA and 30 µg branched polyethyleneimine reagent (PEI, Sigma; Cat# 408719). After 24 hours the media was changed with the fresh growth media and the transfected cells were incubated for 48 hours at 37 °C in 5%. CO_2. The transfected cells were collected by pipetting and spinning at 1000 rpm for 5 min at 4°C, washed once with 1X PBS, and were used for cell lysis. Cells were lysed with TNE (50mM Tris pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1% Triton). Bradford assay were used to determine the protein concentrations. Briefly, 995 µL of Bradford reagent was quickly added to 5µL of lysate. The standard curve was made just before the measurement of the sample using Bovine Serum Albumin and needed regression of 0.960 or higher before proceeding.

Western Blotting

SDS-PAGE or non denature PAGE (see supplementary section for recipe) was used to evaluate the presence of recombinant proteins (DRα, DRβ) separately and both chains together (DRαβ), respectively. In SDS-PAGE, western blotting the samples were denatured and run on a 10% sodium dodecyl sulfate poly acrylamide gel at 120 V for 2 hours. Then the proteins were transferred onto a 0.45 um polyvinylidene fluoride (PVDF) membrane (Millipore Cat# IPVH00010) at 30 V for 2 hours. After the transfer, the membrane was blocked with 1% milk in Tris-buffered saline with 10% Tween-20 (TBST) for 1 hour at room temperature while it is on the rotator. Following of the blocking the membrane, membrane was incubated with primary antibody (1:1000) anti his tag at 4°C overnight on the rotator. The next day, the membranes were washed 3 times with TBST for 10 minutes and then was incubated 1 hour with the second antibody anti rabbit (Santa Cruz Biotechnology Cat # sc-803) (1:10000) at 4°C on the rotator. Afterwards the membrane was washed with TBST for 5 minutes. To make the membrane ready for the chemiluminescence imaging, a solution of (1:1) ECL (enhanced chemiluminescent substrate) and 0.01% H_2O_2 reagents (Thermo-Fisher; Cat#32106) were used right before the imaging by Alpha Innotech equipment.

Immobilized Metal affinity chromatography

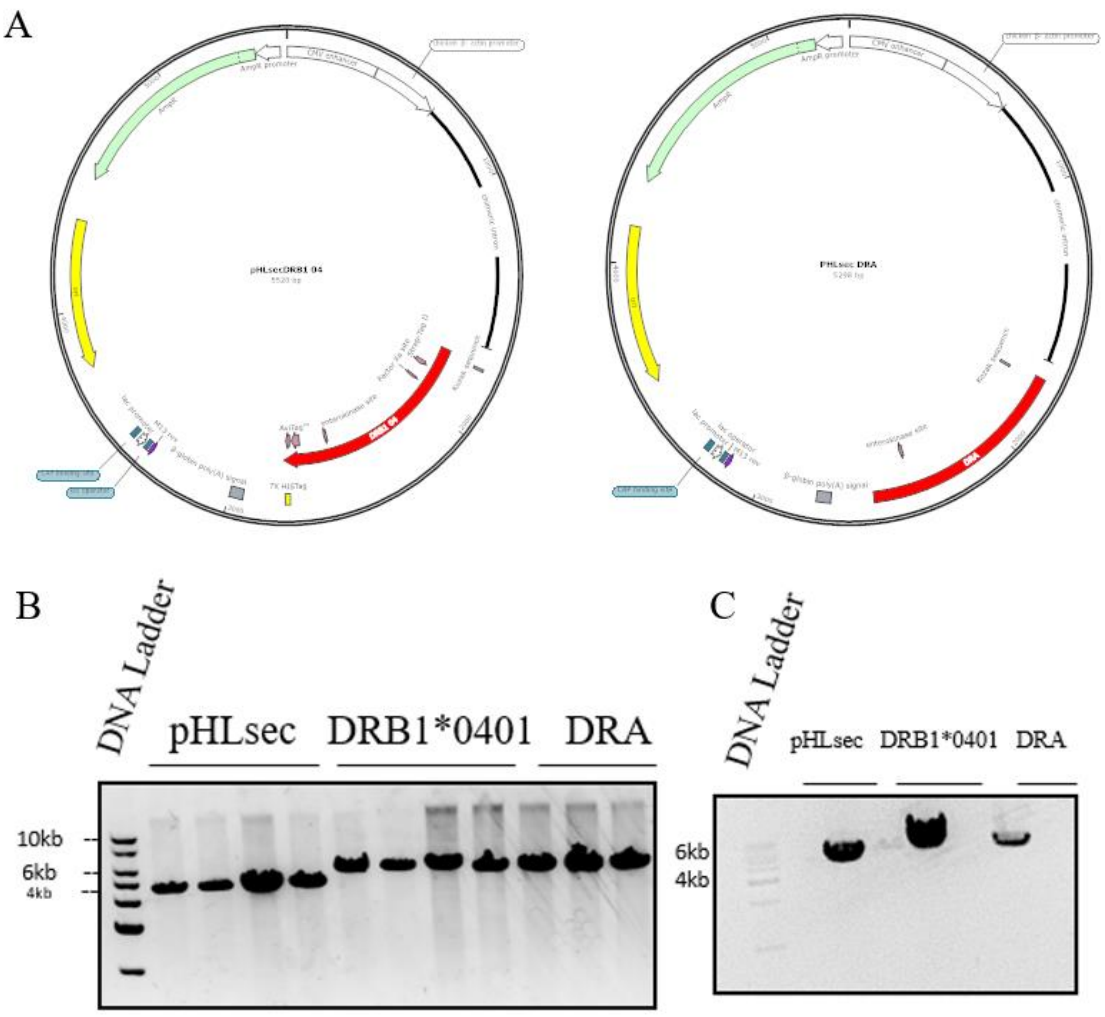
Poly-histidine tagged (HLA-DRαβ) protein was purified using Immobilized metal affinity chromatography. 2 ml Thermo Pierce Centrifuge columns were packed with IMAC Sepharose 6 Fast Flow (GE Healthcare) and were washed with 5 column volumes of distilled water to remove the 20% ethanol. Afterwards, the resin was charged with 0.1 M solution of Ni^{2+} ion. Then the columns were washed with at least 2 column volumes of distilled water to remove the excess metal ions. The columns were equilibrating with 5 column volumes of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4 which was followed by applying the protein lysate. Then the columns were washed with binding buffer followed by using 5 column volumes of the elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4.). After elution, the columns were regenerate the columns by washing them 5 to 10 column volume of binding buffer and the samples were kept in -80 ºC freezer for the next experiments.

Flow Cytometry

HEK-293 cells were cultured and transfected as described previously. 1 x 10^6 cells were resuspended in staining buffer (BD 554656) and incubated with Anti Hu HLA-DR (L243 PE) or anti HIS plus Alexa 488 antibodies for 1 hour in 4°C. After staining the cells were washed three times with 1X PBS (see supplementary for the recipe) and analyzed using using BD Fortessa X20 cytometer using the 488 nm filter for His Tag-Alexa 488 and 561 nm filter for L243-PE dyes

Results

The plasmids containing DRB1*0401 and DRA*0101 genes were extracted using a mini prepping kit and maxi prepping kit. (A). Vector maps of the DRB1*0401 and DRA*0101. (B) Agarose DNA gels of successful mini prep extraction of DRB1*0401 and DRA*0101 DNA. (C) Agarose DNA gels of successful maxi prep extraction of DRB1*0401 and DRA*0101 DNA (D) The concentration and purity of DRB1*0401 and DRA*0101 DNA., control vector (pHLsec) was used as a control.in all the experiments.



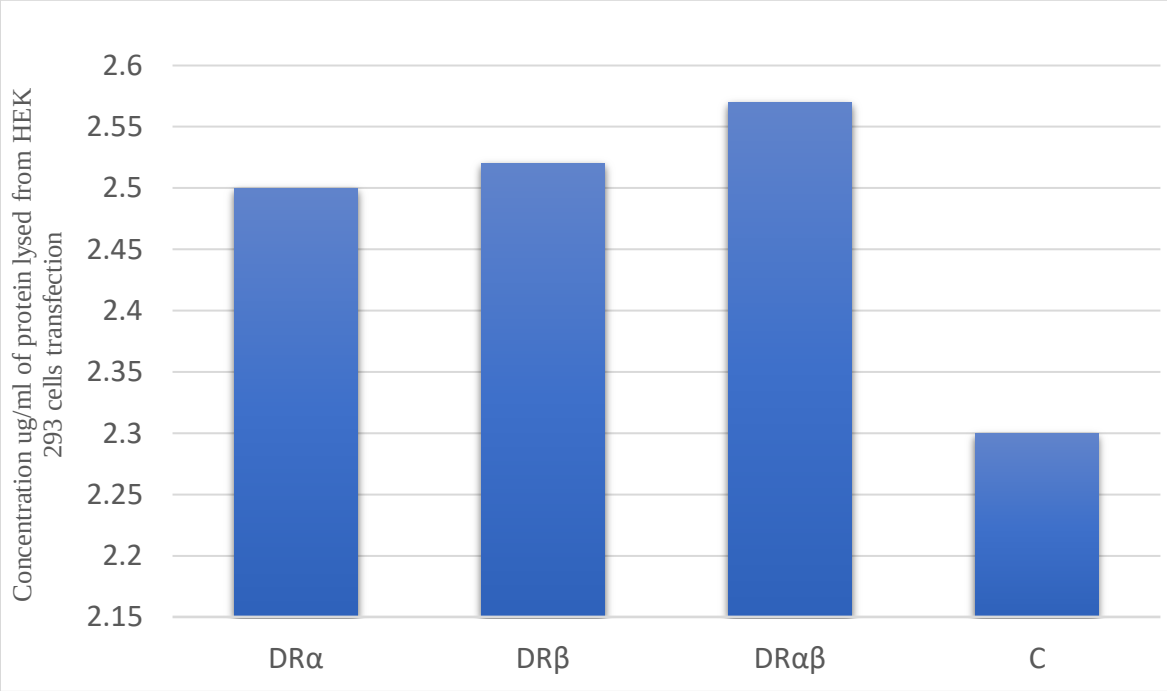
D

	C: ng/ul	purity
pHLsec	424.2	1.88
DRB*0401	655.4	1.88
DRA	654	1.88

Figure 10 The vectors consist of extracellular domain of the DRB1*0401 and DRA*0101 cloned into PHLsec were used for high yield DNA extraction.

The DRB1*0401 and DRA*0101 Extra Cellular Domain were used to transfect HEK293 cells when the cells reached the concentration of 6 $\times 10^6$ cells/mL. (A) The concentration of the protein in the lysis and, (B) successful western blot using anti his antibody results shows that the chains were expressed.

A



B

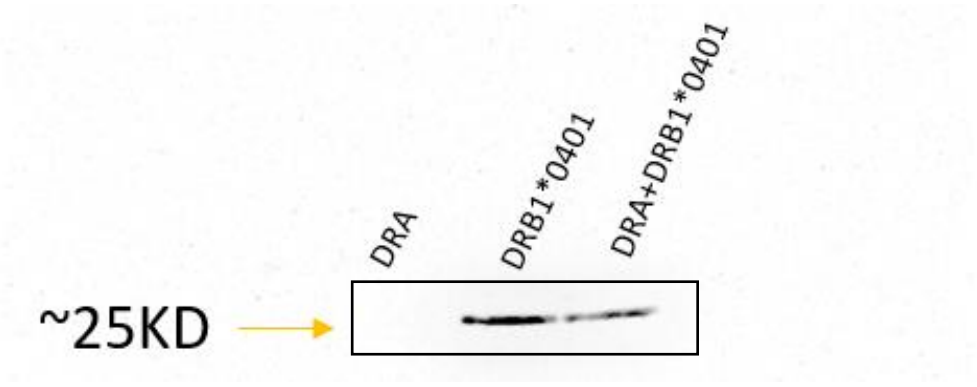


Figure 11 Expression of DRB*0401, DRA 0101, a combination of both chains in HEK293 mammalian cells and a control without transfection

FACS analysis of cell-surface expression of DRA molecules after transfection with vector for DRA, DRB, or the combination of both. L243 stain (top panel) and His tag stain (bottom panel) illustrate that both chains are assembled and located in the cell surface

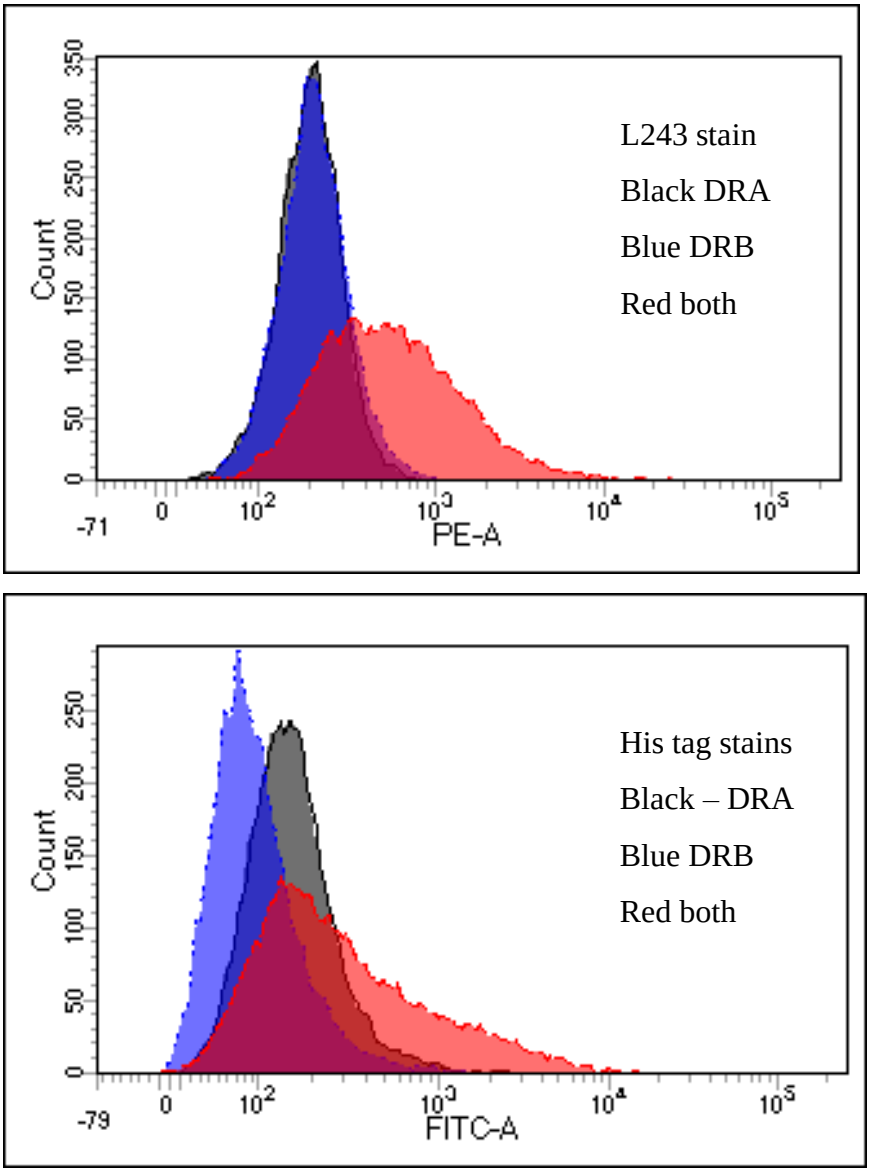
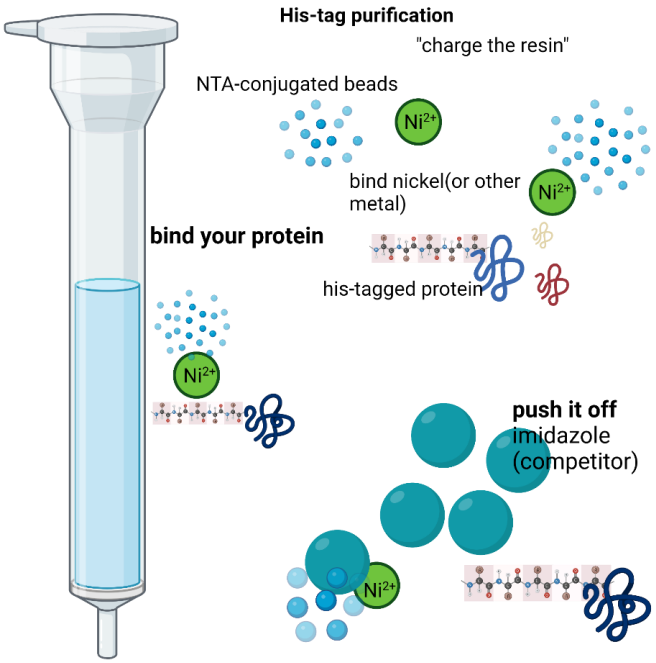


Figure 12 Detection of the HLA complex by Flow Cytometry using (A)L243 stain and (B) His tag stain illustrates that both chains are assembled.

A



B

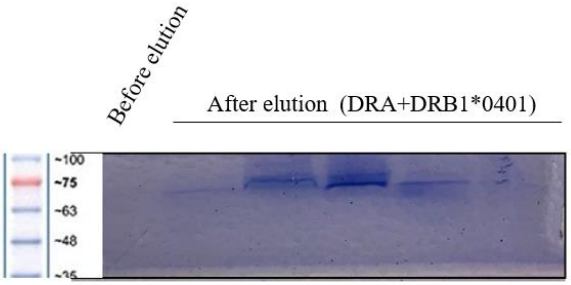


Figure 13 Purification of the HLA complex by IMAC.

Discussion

Biopharmaceutical companies produce many drugs to target proteins from specific pathways in autoimmune diseases and cancers. Proteins are expressed, or produced, by transcribing DNA to messenger RNA (mRNA); the translation of this mRNA into polypeptides is followed by their folding into the functional protein. Advent of recombinant protein become important for structural and biophysical studies, functional assays, drug development and for therapeutic applications in biopharmaceutivals[63]. Protein folding does certainly occur spontaneously as the protein is prepared; however, it often requires the intervention of chaperone proteins, complex aggregates of proteins (such as HLA class I noted in the introduction, or the need for MHC class II to have peptides present to maintain their structure), and often post-translational modifications such as glycosylation or phosphorylation that require other enzymes. The primary structure does not provide the post-translation information, and often the crystal data we have on these proteins involve stripping away glycosides or other post-translational modifications that are necessary for function. This is mainly a feature of the difficulty of interpreting these types of proteins, but it often needs to be reiterated: X-ray crystal structures are often simply modelling of the protein, stripped of some of its essential components, and frozen in a single conformation that may or may not be related to the biologically active conformation or species. It's important to take into account the post-translation modifications in each protein to better understand its structure for drug development.

Consequently, the choice of host species for the expression and purification of the desired protein plays an important role for the characterization of proteins and ligands interactions. MHC is studied in a several autoimmune disease s and cancers[64]. The first step to produce a MHC recombinant protein is getting its gene cloned and the protein amplified in the chosen expression system. As discussed in the introduction HLA DR4 is implicated in 65% of rheumatoid arthritis cases and is the genetic locus most highly associated with disease development. This protein complex consists of both an α and a β chain. The DNA sequence of the α chain is almost completely conserved in each class and between individuals. It is exclusively coded by DRA*0101 allele. However, the β chain is extremely polymorphic, coded by over 1700 different alleles. The allele DR4 is the most common one in rheumatoid arthritis patients and has excellent well-known affinity for the dominant immunogenic peptide sequence from type II collagen. For the studies in this thesis, we expressed and purified HLA-DR4 is our first peptide model to evaluate the peptide-drugs interaction

Success in expressing foreign genes in heterologous living organisms to produce the target proteins is sensitive to many different parameters such as choice of host, choice of plasmid and choice of purification tag[65]For our project, an appropriate protein expression system is the maximum importance to express this heterodimeric glycosylated protein HLA -DR4

(DRA*01:01/DRB1*0401). Determining which of these should be used was the first step of this thesis. There are broadly three ways to approach this problem. The first is to try the different systems. This is lengthy and perhaps unnecessary. The second is to identify a consensus in the literature of which approach should be used. This was impossible to find as there is no consensus. The third, and the one we used, was to conduct an exhaustive comparative review of the approaches outlined in the literature to compare and contrast the success of previous approaches. Upon starting my MSc in September 2019, determining the best route forward was the focus of my masters thesis for the first three months.

Various host systems have been previously used to express the HLA including mammalian cell culture, baculovirus system, bacteria, and yeast. Each host system has positive and negative points, with the balance towards being preferred depending on the purpose of the study. Choice of host is a crucial step in expression of this protein which has a great impact on the yield, functionality, and stability of the protein. The location of HLA complex, expressed as it is on the surface of antigen presenting cells including macrophages, dendric cells and B cells, will affect the choice of method of isolation and purification of the product. This choice was the critical first decision of the thesis. When the project began, our lab was leaning towards a baculovirus-insect cell system; however, we were aware that this was a partially arbitrary decision, and consequently the first task was to determine the preferred system for HLA expression. There is no consensus in the literature, and there is no body of literature comparing the relative merits of the different systems. One of the expected deliverables of this thesis is a systematic critical review of HLA expression tools; the article is currently being finalized and is expected to be submitted to *HLA*, the journal of record for MHC-related immunology, following correspondence between Dr. Trant and the editorial board. This publication is the result of my efforts to outline and contrast the various strategies.

Expression Conditions

Mammalian cell culture is one the most common methods to express recombinant human proteins. This method has become the preferred system to express recombinant proteins due to the presence of all the proper machinery required for proper protein folding and posttranslational glycosylation which are carried out in the endoplasmic reticulum and Golgi apparatus of mammalian cells. HLA is an α/β heterodimer glycosylated membrane protein; functional HLA molecules only can be produced in mammalian cells[66]. So, it is crucial to use a proper host to express this protein to include post translational modifications.

There are a couple of different mammalian cell lines which have been used to express proteins including Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, myeloma (NS0, SP2/0), HeLa, Vero, murine 3t3 fibroblast cells and HEK293 cells[67]. CHO cells are used for

approximately 60% of all expressions of the therapeutic proteins because they are able to produce a productive and high titre yield and also lower biosafety risk however, they can not be a great host choice to express proteins because of generating non-human glycan structures including in N-glycolylneuraminic acid (Neu5Gc) and galactose-alpha-1,3-galactose group (α-Gal) onto glycoprotein products which confers increased risk of immunogenicity.[68] [67] To produce more human-like protein therapeutics , efforts have been made to produce human cell lines such as HEK293 , HT-1080[67]. In particular, the HEK293 cell line has become one of the most common human cell lines to express the proteins due to easy manipulation and rapid production of recombinant proteins, however, the total yield is one of the main challenges in using this specific cell line[69]. As you see in table number 10 the cell lines used in different studies is listed. Nobuo Watanabe and his colleagues have shown that using mammalian cell culture is the best method to produce α/β heterodimeric glycosylated membrane proteins; this was confirmed by us in our results. (Table 10). Moreover, the selection of HEK293 expression was so favorable to IMAC purification as the harvest lysate can be loaded directly to Ni charged columns before buffer exchange. Therefore. IMAC was chosen to purify the protein based on the acceptable yield and purity (Figure 13)

Other methods such as Baculovirus mediated insect cells or yeast has been used to produce α/β heterodimeric glycosylated membrane However, the baculovirus system has several limitations. The lytic infection process leads to the release of host & viral proteases which affects the protein quality. Some viral proteins such as v-cathepsin and v-chitinase stop the secretary pathway of the protein resulting in repeated rounds of virus amplification and they also make defective particles and reduce the yield. Some studies have shown that deleting these viral proteins can improve the production of secreted proteins. Although, this method has been widely used to express human leukocyte antigens, purification can be challenging. IMAC chromatography, exploiting the His tag on our construct, is the preferred method for purification. However, IMAC chromatography is rarely successful from baculovirus expression systems as the high molecular weight o-Pluronic acid, standard in the baculovirus media, can strip to the column.

Past studies have used yeast for the expression of human genes, supporting use of HLA expression in yeast. The most common yeast vectors for HLA expression have been Pichia pastoris and yeast artificial chromosomes (YACs). Though no studies have explicitly stated their reasoning for using YACs for HLA expression, it has been shown to be a successful and favoured vector for HLA expression (table 12). However, YACs do have limitations. Protein expression has been shown to vary and have inconsistencies between various YAC clones. The inconsistency may be due to alternative splicing of exons of the HLA gene or the lack of tissue specificity in YACs expressing a human gene. YACs are also prone to chimerism and lack of stability, which may contribute to overall difficulty with reproducibility and high-volume HLA expression.

There are not many reports providing head-to-head comparisons of these different vectors, so it is not trivial to identify the best method to express and purify the HLA protein. As the research steps forward for clinical trials more factors will be taken into consideration. Different studies in the production of recombinant proteins have been shown that mammalian cells are the best method among different expression systems to produce the protein because they can perform complex post-translational modifications that are necessary for efficient secretion, drug efficacy, and stability. These modifications consist of misfolding and aggregation, oxidation of methionine, deamidation of asparagine and glutamine, variable glycosylation, and proteolysis. These modifications are not only input so many challenges to produce an accurate protein, but also may lead to incorrect results in the study of human leukocyte antigen. Unfortunately, the yields, titres, and costs of this method are all unfavourable. Our results showed that the concentration of purified protein 0.14 ug/ul from 1 ml of lysate is sufficient for use with our biophysical evaluation tools we wish to employ in our drug-screening such as surface plasmon resonance and fluorescence displacement assays. Thus, we chose to use this route, as proper folding is essential for our purposes, using HLA was already a significant compromise, we could not accept further deviations from the in vivo conformation of the protein.

Protein purification

IMAC has the advantage of purifying HLA with high affinity, specific binding and selectivity by using imidazole in chromatography buffers. Native PAGE bands in our results are showing specificity and purity of using IMAC to purify HLA. Moreover, Histidine tags between four to ten residues provides the strongest affinity for the chromatography. Ni2+ is one the most common metals used for his-tag purification which gives a high yield.

SDS-PAGE and Native PAGE analysis

After protein expression and purification, a sample was evaluated by SDS-PAGE analysis. The blotting with anti Histag antibody confirmed that DRα, DRβ has been expressed using HEK293 cell line. However, SDS-PAGE was not able to confirm that the complex DRαβ has been formed. SDS-PAGE gel is a denaturant system. In order to confirm that both chains are assembled and expressed on the surface of membrane, Flow Cytometry using L243 stain (anti-HLA antibody recognize DRαβ when they are conformationally assembled) and His tag stain illustrates that both chains are assembled (fig 13B.) Our Native-PAGE using Coomassie blue dye with the intensity of the bands showing DRαβ are assembled after purification

The main finding of our thesis are as follows. Human leukocyte antigen is a protein needed to advance the research and drug development in autoimmune diseases and cancers. In these early stages of this project, we showed that choice of HEK293 cell as a host to express HLA protein and

IMAC chromatography as a purification system gives us a reasonable amount of α/β heterodimer glycosylated membrane HLA protein.

Surface Plasmon Resonance

One of the commonly used technologies is Surface plasmon resonance (SPR) for detailed and quantitative studies of protein-peptide interactions and determination of their equilibrium and kinetic parameters. Tracking the change in the SPR signal over time generates a sensogram, a plot of the binding response (RU) versus time which allows different stages of a binding event to be visualized and evaluated. During the injection of an analyte, the binding response increase because of the formation of analyte(peptide)–ligand(protein) complexes at the surface and the sensorgram is dominated by the association phase. After a certain time of injection, a steady state is reached, in which binding and dissociating molecules are in equilibrium. The decrease in response after analyte injection is terminated because of dissociation of the complexes, defining the dissociation phase. Depending on the dissociation rate of the tested ligand, some assays may require a regeneration step in order to reach the baseline again. Fitting the sensorgram data to an appropriate kinetic binding model allows calculation of kinetic parameters such as the association (k_a) and dissociation (k_d) rate constants, and the binding affinity of the tested interactions. The SPR (surface plasmon resonance) analysis is currently underway in our lab to confirm the binding of HLA*DR4 to human cartilage glycoprotein 39 as a control to evaluate the binding affinity of this self peptide to the target protein. This whole research was conducted under COVID restriction at the University of Windsor for over 16 month of the last two years and for personnel reasons restrict my time in the lab since February 2021.

Supplementary methods

Transformation

1. Place NEB-STBL (NEB; Cat#C3040H) competent cells vials into ice immediately from -80°C.
2. They should stay in the ice to get thawed for approximately 5 minutes.
3. Add 2µl of 701.62 µg/µl pHLsec DRA DNA, 892.67 ng/µl pHLsec DRB1*0401 DNA and 165.88 ng/µl empty pHLsec vector plasmids to 25 µL of the competent cells and incubated at 4 °C for 30 minutes.
4. Place them into the water bath at 42°C for 42 seconds for heat shock.
5. Return them immediately to ice 4°C for 2 minutes.
6. Dilute the transformation mix into 1 ml of SOC media and incubate them at 37°C with shaking at 250 rpm for 1 hour.
7. Plate 10 µL or 100 µL of transformation each onto 10 cm LB agar plates containing ampicillin antibiotic and incubate them at 37°C overnight.
8. After 24 hours, add a couple of colonies of *E. coli* containing the plasmid of interest to 3mL of LB media plus 50 µg/ml of ampicillin antibiotic overnight.
9. Extract the plasmid DNA using DNA miniprep kit (Biobasic; Cat#BS88503) and the maxiprep kit (Biobasic; Cat#9K0060023).
10. Mini prepping and Maxi prepping should be follow using the company instruction kit.

LB agar plates containing ampicillin antibiotic recipe

LB Agar Powder, Miller, (Biobasic, CAT. #: SD7003) is made following manufacture's protocol. It should be autoclaved. After it gets cool mix with 100 ug/ml Ampicillin antibiotic.

Fast Digest Restriction enzyme

The 10X Fast Digest Green Buffer includes a density reagent and two tracking dyes for direct loading. The blue dye migrates with 3–5 kb DNA fragments in a 1% agarose gel and has an excitation peak of 424 nm. The yellow dye migrates faster than 10 bp DNA fragments in a 1% agarose gel and has an excitation peak of 615 nm.

Thaw and split HEK293 cell (Human embryonal kidney cells: (HEK293, ATCC [Cat. No. CRC-1573])

Growth media: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS; Thermo-Fisher; Lot#906532) and 1% Penicillin-Streptomycin (Gibco; Cat#1514122).

Thaw

Preparatory Work: Warm media to 37°C in water bath

1. Frozen cells completely should get thaw in hands.
2. Transfer the cells to 15 ml tube and spin at 1000 rpm for 5 min.
3. Remove the Supernatant and resuspend the pellet with 1 ml of media and transfer the cell suspension to 10 cm plate containing 7 ml of media.
4. Incubate the Cells at 37°C and 5% CO2.

Seeding the Cells

Preparatory Work: Warm media and PBS to 37°C in water bath

PBS1X (make and keep it as a stock)

1. Check the Cells on plate by microscope to see if they are healthy and see confluency of cells (usually good to have about 70-80% coverage of plate.
2. Remove the media and wash the cells with 1ml PBS and place them into a 15ml test tube and spin them in the centrifuge at 1000RPM for 5 minutes.
3. Discard the supernatant and add fresh room-temperature growth media to the pellet (amount of media depends on size of pellet, but usually 4-5mls) and resuspend pellet.
4. Add 50 µl Trypan Blue into Eppendorf tube to 50 µl of the media and cells and count cells using hemocytometer.

5. Place 10 µl in hemocytometer and counted the four quadrants. Average of cells from the four quadrants and times by $2x10^4$. (Since 1:1 ratio solution and want ml. This gave us cells/ml). To figure out how much resuspended media to add on new/seeding plates to get certain number of cells (ml).
6. The calculated number of resuspended cells placed into media around the plate, cells and spread around so they have room to grow.
7. The doubling time for HEK293 cells is 24 hours.

SDS PAGE analysis

Prepare Gel Electrophoresis:

1. Make 10 % running gel (Table 3) and stacking gel (Table 4).

Table 3 Running Gel Recipe

Running Gel %	10%
H_2O	15.5mL
Acrylamide	7mL
Running/Lower Gel Buffer	7.5mL
10% APS	112.5uL
TEMED (add last)	33.75uL

Table 4 Stacking Gel Recipe

Stacking Gel	
H_2O	6.4mL
Acrylamide	1.125mL
Upper Gel Buffer	2.5mL
10% APS	30uL
TEMED (add last)	20uL

Preparing Protein Samples:

1. Pipette 80 ug of each protein (DRα, DRβ and DRαβ) lysate into an empty Eppendorf tube
2. Add TNE and sample buffer to each tube so that the max volume is ¼ sample buffer

3. place the samples on a 95°C heat block for 5 minutes.
4. Spin down the samples and add the samples to the well.
5. Run the sample at 120 V for 2 hours in the gel electrophoresis buffer (Table 5).
6. Transfer the proteins onto a 0.45 um polyvinylidene fluoride (PVDF) membrane (Millipore Cat# IPVH00010) at 30 V for 2 hours in transfer buffer (Table 6).
7. Block the membrane with 1% milk in Tris-buffered saline with 10% Tween-20 (TBST) for 1 hour at room temperature while it is on the rotator.
8. Then incubate the membrane with primary antibody (1:1000) anti his tag at 4°C overnight on the rotator.
9. The next day, wash the membranes 3 times with TBST for 10 minutes and then incubate 1 hour with the second antibody anti rabbit (Santa Cruz Biotechnology Cat # sc-803) (1:10000) at 4°C on the rotator.
10. Afterwards wash the membrane with TBST for 5 minutes.
11. To make the membrane ready for the chemiluminescence imaging, use a solution of (1:1) ECL (enhanced chemiluminescent substrate) and 0.01% H_2O_2 reagents (Thermo-Fisher; Cat#32106) right before the imaging by Alpha Innotech equipment.

Table 5 Running Buffer Recipe

1x Running Buffer Recipe (1L)	
Tris Base	3.02g
Glycine:	18.8g
10% SDS	10mL
H2O	Fill container with water until solution reaches 1L

Table 6 Transfer Buffer Recipe

1x Transfer Buffer Recipe (1L)	
Tris Base:	5.8g
Glycine:	2.93g
10% SDS	10mL
Methanol (MeOH)	200mL
H2O	Add last, fill container with water until solution reaches 1L

Non denature PAGE

Table 7 Running Gel Recipe for Non-denatured PAGE

Running Gel %:	10%
H_2O	15.5mL
Acrylamide	7mL
Running/Lower Gel Buffer (1.5 M Tris-HCl, pH 8.8)	7.5mL
10% APS	112.5uL
TEMED (add last)	33.75uL

Table 8 Stacking Gel Recipe for Non-denatured PAGE

Stacking Gel Recipe	
H_2O	6.4mL
Acrylamide	1.125mL
Upper Gel Buffer (0.5 M Tris-HCl, pH 6.8)	2.5mL
10% APS	30uL
TEMED (add last)	20uL

Table 9 Gel Electrophoresis Running Buffer Recipe for Non-denatured PAGE

1x Running Buffer Recipe (1L)	
Tris Base:	3.g
Glycine:	14.4g
H2O	Fill container with water until solution reaches 1L pH 8.3

Samples should be diluted accordingly with 5X sample buffer: 0.1M Tris-HCl (pH 6.8), 30% glycerol, 80 µg/ml bromophenol blue to give a solution that is 1X sample buffer and run on a 10% native poly acrylamide gel at 120 V for 3 hours After 3 hours the gels were stained in protein stain consist of 0.25 g Coomassie brilliant blue, 125 mL methanol, 25 mL glacial acetic acid, and 100 mL water. After staining overnight, the gels were washed with distaining solution (100 mL methanol, 100 mL glacial acetic acid, and 800 mL water) for 1 hour.

Table 10 Studies used mammalian cell culture to express human leukocyte antigen

HLA Allele	Cell Line	Author(s)
A2	Human lymphoblastoid cell line JY	Bjorkman[70]
B27	Human B lymphoblastoid cell line LG-2	Gorga[71]
DRA	CHO	Serra[72]
	HeLa	Lotteau[73], Salamero[74]
	Dap.3 subclone of class II-negative murine L fibroblast	Panina-Bordignon[75]
	NIH 3T3	Korman[76]
	Rat2	Hitzel[77]
	Human B lymphoblastoid cell line LG-2	Gorga[78]
	L929	Höpner[79]
DRB	CHO	Serra[72]
	HeLa	Lotteau[73], Salamero[74]
	Dap.3 subclone of class II-negative murine L fibroblast	Panina-Bordignon[75]
	NIH 3T3	Korman[76]
	L929	Höpner[79]
	Human B lymphoblastoid cell line LG-2	Gorga[78], Valli[80]
	TR81.19 fibroblast	Sette[81]
	L257.6 fibroblast	Sette[81]
	L466.1 fibroblast	Valli[80], Wucherpfennig[82]
	L416.3 fibroblast	Valli[80], Wucherpfennig[82]
	L242.5 fibroblast	Valli[80]
	L255.1 fibroblast	Valli[80]

	MAT	Valli[80]
	Priess	Valli[80]
	BIN40	Valli[80]
	SWEIG	Valli[80]
	Pitout	Valli[80]
DR1	Human B lymphoblastoid cell line LG-2	Gorga[83]
	Dap.3 subclone of class II-negative murine L fibroblast	Klohe[84], Koehler[85]
	LG-2	O'Sullivan[86–88], Sette[81,89]
	HeLa	van Lith[90]
DR2	MST	Gorga[83]
	GMO 3107 lymphoblastoid cell line	Nag[91]
	3107 EBV cells	O'Sullivan[86–88]
	L416.3 fibroblast	Sette[81]
DR3	WT20	Gorga[83]
	Rat2	Hitzel[77]
	GMO 8067 lymphoblastoid cell line	Nag[91]
	MAT	O'Sullivan[86,88], Sette[81]
DR4	Murine fibroblast	Boen[92], Klohe[84]
	Priess	Gorga[83], Sette[81,89]
	GMO 6821A lymphoblastoid cell line	Nag[91]
	BIN40	Sette[81,89]
DR5	Dap.3 subclone of class II-negative murine L fibroblast	Klohe[84]
	SWEIG	O'Sullivan[86–88], Sette[81]
DR7	Mann	Gorga[83]
	Dap.3 subclone of class II-negative murine L fibroblast	Klohe[84]

	Pitout	Sette[81,89]
DR8	23.1	Gorga[83]
DRB	CHO	Day[93]
	Human lymphoblastoid cell lines BSM, 2046, and MT	Hammer[94]
	Priess	Liu[95]
DO	HeLa	Brunet[96]
DQ	Human B lymphoblastoid cell line LG-2	Gorga[78]
	Dap.3 subclone of class II-negative murine L fibroblast	Klohe[84]
	HeLa	van Lith[90]
DP	Human B lymphoblastoid cell line LG-2	Gorga[78]
	Dap.3 subclone of class II-negative murine L fibroblast	Klohe[84]
	HeLa	van Lith[90]

Table 11 Studies used baculovirus system to express Human leukocyte antigen

HLA Allele	Cell Line	Author(s)
A1	Sf9 insect	Smith[97]
A2	Sf9 insect	Smith[97]
	High Five insect	Mancino[98]
B27	Sf9 insect	Lévy[99]
B35	High Five insect	Thammavongsa[100]
DR1	Sf9 insect	Chou[101], Narayan[102], Natarajan[103], Sadegh-Nasseri[104], Stern[105]

DR2	High Five insect	Smith[97]
	Sf9 insect	Gauthier[106]
DR4	Sf9 insect	Fourneau[107], Scheirle[108]
DQ2	Sf9 insect	Jüse[109], Kim[110], Quarsten[111]
	High Five insect	Xia[112]

Table 12 YAC clones used for HLA expression and their respective HLA gene incorporation, insert size and reference.

HLA fragment	YAC Clone	Author
HLA-DRA	A95C5	Kozono et al.[113]
From HLA-DQB2 locus through DRA locus	B1D12	Chen[114].; Kozono[113]
Centromeric of HLA-DOB to interval between HLA-DQA1 and HLA-DRB1	A148A7	Demmer and Chaplin[115]; Kozono [113]
HLA-B and -C	B38D3	Bronson [116]
HLA-B and -1.7p	B92H5	Bronson
HLA-C	B209D7	Bronson
HLA-DMA, -DMB, -DOB, -DQA2, -DQB2, -DQB3, -DQB1 and -DRB1	11.2	Ragoussis, Trowsdale, and Markie[117]
HLA-DOB, -DQA2, -DQB2, -DQB3, -DQA1, -DQB1, -DRB1, -DRB2, -DRB3, and -DRA	4D1	Ragoussis, Trowsdale, and Markie [117]
HLA-E and -A	225B1	Wei et al. [118]
HLA-B and -C	152G3	Wei et al.
HLA-E	A231G12	Wei et al.
HLA-G and -F	A190C8	Wei et al.
HLA-A	B30H3	Wei et al.
HLA-DQB1 to -DRA	4D1D11	Fabb et al. [119]
LMP2-DRA	4D1D10	Fabb et al.
DQB3-DRB1	11.2C10	Fabb et al.
LMP2-DRB1	11.2A1	Fabb et al.
HLA-DQw2 (DQA*0501/DQB1*00201) and -DR3 (DRA*0101/DRB1*0301/DRB3*52)	4D1	Chen et al. [120]
HLA-DR4 to -DQ3	748D9	Chen et al.

References

1. Lohi, S. *et al.* Increasing prevalence of coeliac disease over time: INCREASING PREVALENCE OF COELIAC DISEASE. *Aliment. Pharmacol. Ther.* **26**, 1217–1225 (2007).
2. Lerner, A., Jeremias, P. & Matthias, T. The World Incidence and Prevalence of Autoimmune Diseases is Increasing. *Int. J. Celiac Dis.* **3**, 151–155 (2016).
3. Lleo, A., Invernizzi, P., Gao, B., Podda, M. & Gershwin, M. E. Definition of human autoimmunity — autoantibodies versus autoimmune disease. *Autoimmun. Rev.* **9**, A259–A266 (2010).
4. Tsonis, I. A., Avrameas, S. & Moutsopoulos, H. M. Autoimmunity and pathophysiology. *J. Autoimmun.* **29**, 203–205 (2007).
5. Ngo, S. T., Steyn, F. J. & McCombe, P. A. Gender differences in autoimmune disease. *Front. Neuroendocrinol.* **35**, 347–369 (2014).
6. Rosenblum, M. D., Remedios, K. A. & Abbas, A. K. Mechanisms of human autoimmunity. *J. Clin. Invest.* **125**, 2228–2233 (2015).
7. Romagnani, S. Immunological tolerance and autoimmunity. *Intern Emerg Med* **1**, 10 (2006).
8. Luo, X., Miller, S. D. & Shea, L. D. Immune Tolerance for Autoimmune Disease and Cell Transplantation. *Annu. Rev. Biomed. Eng.* **18**, 181–205 (2016).
9. Xing, Y. & Hogquist, K. A. T-Cell Tolerance: Central and Peripheral. *Cold Spring Harb. Perspect. Biol.* **4**, a006957–a006957 (2012).
10. Nemazee, D. Mechanisms of central tolerance for B cells. *Nat. Rev. Immunol.* **17**, 281–294 (2017).

11. Klinman, N. R. The “Clonal Selection Hypothesis” and Current Concepts of B Cell Tolerance. *Immunity* **5**, 189–195 (1996).
12. Zhang, P. & Lu, Q. Genetic and epigenetic influences on the loss of tolerance in autoimmunity. *Cell. Mol. Immunol.* **15**, 575–585 (2018).
13. Walker, L. S. K. & Abbas, A. K. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* **2**, 11–19 (2002).
14. Cochran, J. R., Cameron, T. O. & Stern, L. J. The Relationship of MHC-Peptide Binding and T Cell Activation Probed Using Chemically Defined MHC Class II Oligomers. *Immunity* **12**, 241–250 (2000).
15. Kurkó, J. *et al.* Genetics of Rheumatoid Arthritis — A Comprehensive Review. *Clin. Rev. Allergy Immunol.* **45**, 170–179 (2013).
16. Pincus, T. & Callahan, L. F. Reassessment of Twelve Traditional Paradigms Concerning the Diagnosis, Prevalence, Morbidity and Mortality of Rheumatoid Arthritis. *Scand. J. Rheumatol.* **18**, 67–96 (1989).
17. Hansson, G. K., Libby, P., Schönbeck, U. & Yan, Z.-Q. Innate and Adaptive Immunity in the Pathogenesis of Atherosclerosis. *Circ. Res.* **91**, 281–291 (2002).
18. Cunliffe, V. & Trowsdale, J. The molecular genetics of human chromosome 6. *J. Med. Genet.* **24**, 649–658 (1987).
19. Wieczorek, M. *et al.* Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. *Front. Immunol.* **8**, (2017).
20. Wraith, D. C. & Nicholson, L. B. The adaptive immune system in diseases of the central nervous system. *J. Clin. Invest.* **122**, 1172–1179 (2012).

21. James, L. & Georgopoulos, A. Persistent Antigens Hypothesis: The Human Leukocyte Antigen (HLA) Connection. *J. Neurol. Neuromedicine* **3**, 27–31 (2018).

22. Shiina, T., Hosomichi, K., Inoko, H. & Kulski, J. K. The HLA genomic loci map: expression, interaction, diversity and disease. *J. Hum. Genet.* **54**, 15–39 (2009).

23. Hewitt, E. W. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* **110**, 163–169 (2003).

24. Roche, P. A. & Furuta, K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat. Rev. Immunol.* **15**, 203–216 (2015).

25. Mantegazza, A. R., Magalhaes, J. G., Amigorena, S. & Marks, M. S. Presentation of Phagocytosed Antigens by MHC Class I and II: Presentation of Phagocytosed Antigens. *Traffic* **14**, 135–152 (2013).

26. Powis, S. J. Major histocompatibility complex class I molecules interact with both subunits of the transporter associated with antigen processing, TAP1 and TAP2. *Eur. J. Immunol.* **27**, 2744–2747 (1997).

27. Neefjes, J., Jongsma, M. L. M., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat. Rev. Immunol.* **11**, 823–836 (2011).

28. York, I. A. & Rock, K. L. ANTIGEN PROCESSING AND PRESENTATION BY THE CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX. *Annu. Rev. Immunol.* **14**, 369–396 (1996).

29. Watanabe, N. *et al.* A cell-based high-throughput screening assay system for inhibitor compounds of antigen presentation by HLA class II molecule. *Sci. Rep.* **7**, 6798 (2017).

30. Pociot, F. & Lernmark, Å. Genetic risk factors for type 1 diabetes. *The Lancet* **387**, 2331–2339 (2016).

31. Vollers, S. S. & Stern, L. J. Class II major histocompatibility complex tetramer staining: progress, problems, and prospects. *Immunology* **123**, 305–313 (2008).

32. Kobayashi, K. S. & van den Elsen, P. J. NLRC5: a key regulator of MHC class I-dependent immune responses. *Nat. Rev. Immunol.* **12**, 813–820 (2012).

33. van Delft, M. A. M. & Huizinga, T. W. J. An overview of autoantibodies in rheumatoid arthritis. *J. Autoimmun.* **110**, 102392 (2020).

34. Weyand, C. M. & Goronzy, J. J. PATHOGENESIS OF RHEUMATOID ARTHRITIS. *Med. Clin. North Am.* **81**, 29–55 (1997).

35. Toubi, E. & Vadasz, Z. Innate immune-responses and their role in driving autoimmunity. *Autoimmun. Rev.* **18**, 306–311 (2019).

36. Weyand, C. M. & Goronzy, J. J. HLA Polymorphisms and T Cells in Rheumatoid Arthritis. *Int. Rev. Immunol.* **18**, 37–59 (1999).

37. Waldner, H. The role of innate immune responses in autoimmune disease development. *Autoimmun. Rev.* **8**, 400–404 (2009).

38. Banchereau, J. *et al.* Immunobiology of Dendritic Cells. *Annu. Rev. Immunol.* **18**, 767–811 (2000).

39. Weyand, C. M., Bryl, E. & Goronzy, J. J. The role of T cells in rheumatoid arthritis. *Arch. Immunol. Ther. Exp. (Warsz.)* **48**, 429–435 (2000).

40. McInnes, I. B. & Schett, G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat. Rev. Immunol.* **7**, 429–442 (2007).

41. Smolen, J. S. & Steiner, G. Therapeutic strategies for rheumatoid arthritis. *Nat. Rev. Drug Discov.* **2**, 473–488 (2003).

42. Choy, E. Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis. *Rheumatology* **51**, v3–v11 (2012).

43. Smolen, J. S. & Steiner, G. Therapeutic strategies for rheumatoid arthritis. *Nat. Rev. Drug Discov.* **2**, 473–488 (2003).

44. Link, A. *et al.* Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat. Immunol.* **8**, 1255–1265 (2007).

45. Manzo, A., Bombardieri, M., Humby, F. & Pitzalis, C. Secondary and ectopic lymphoid tissue responses in rheumatoid arthritis: from inflammation to autoimmunity and tissue damage/remodeling. *Immunol. Rev.* **233**, 267–285 (2010).

46. Bird, A. K., Meednu, N. & Anolik, J. H. New insights into B cell biology in systemic lupus erythematosus and Sjögren's syndrome. *Curr. Opin. Rheumatol.* **27**, 461–467 (2015).

47. Magliozzi, R. *et al.* Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain* **130**, 1089–1104 (2006).

48. Corsiero, E. *et al.* Role of lymphoid chemokines in the development of functional ectopic lymphoid structures in rheumatic autoimmune diseases. *Immunol. Lett.* **145**, 62–67 (2012).

49. Rivellese, F., Pontarini, E. & Pitzalis, C. Tertiary Lymphoid Organs in Rheumatoid Arthritis. in *Inducible Lymphoid Organs* (eds. Kabashima, K. & Egawa, G.) vol. 426 119–141 (Springer International Publishing, 2020).

50. Fujita, T., Kutsumi, H., Sanuki, T., Hayakumo, T. & Azuma, T. Adherence to the preventive strategies for nonsteroidal anti-inflammatory drug- or low-dose aspirin-induced gastrointestinal injuries. *J. Gastroenterol.* **48**, 559–573 (2013).

51. Barnes, P. J. Anti-inflammatory Actions of Glucocorticoids: Molecular Mechanisms. *Clin. Sci.* **94**, 557–572 (1998).

52. Curtis, J. R. *et al.* Population-based assessment of adverse events associated with long-term glucocorticoid use. *Arthritis Rheum.* **55**, 420–426 (2006).

53. Kremer, J. *et al.* Combination leflunomide and methotrexate (MTX) therapy for patients with active rheumatoid arthritis failing MTX monotherapy: open-label extension of a randomized, double-blind, placebo controlled trial. *J. Rheumatol.* **31**, 1521 (2004).

54. Monaco, C., Nanchahal, J., Taylor, P. & Feldmann, M. Anti-TNF therapy: past, present and future. *Int. Immunol.* **27**, 55–62 (2015).

55. Feldmann, M., Brennan, F. M. & Maini, R. N. ROLE OF CYTOKINES IN RHEUMATOID ARTHRITIS. *Annu. Rev. Immunol.* **14**, 397–440 (1996).

56. Wijnen, P. A. *et al.* Association of the TNF- G-308A polymorphism with TNF-inhibitor response in sarcoidosis. *Eur. Respir. J.* **43**, 1730–1739 (2014).

57. Li, P., Zheng, Y. & Chen, X. Drugs for Autoimmune Inflammatory Diseases: From Small Molecule Compounds to Anti-TNF Biologics. *Front. Pharmacol.* **8**, 460 (2017).

58. Candia, M., Kratzer, B. & Pickl, W. F. On Peptides and Altered Peptide Ligands: From Origin, Mode of Action and Design to Clinical Application (Immunotherapy). *Int. Arch. Allergy Immunol.* **170**, 211–233 (2016).

59. Valmori, D. *et al.* Enhanced Generation of Specific Tumor-Reactive CTL In Vitro by Selected Melan-A/MART-1 Immunodominant Peptide Analogues. *J. Immunol.* **160**, 1750 (1998).

60. Katsara, M., Minigo, G., Plebanski, M. & Apostolopoulos, V. The good, the bad and the ugly: how altered peptide ligands modulate immunity. *Expert Opin. Biol. Ther.* **8**, 1873–1884 (2008).

61. Meister, D., Taimoory, S. M. & Trant, J. F. Unnatural amino acids improve affinity and modulate immunogenicity: Developing peptides to treat MHC type II autoimmune disorders. *Pept. Sci.* **111**, e24058 (2019).

62. Andersson, I. E. *et al.* Design of Glycopeptides Used to Investigate Class II MHC Binding and T-Cell Responses Associated with Autoimmune Arthritis. *PLoS ONE* **6**, e17881 (2011).

63. Assenberg, R., Wan, P. T., Geisse, S. & Mayr, L. M. Advances in recombinant protein expression for use in pharmaceutical research. *Curr. Opin. Struct. Biol.* **23**, 393–402 (2013).

64. Valencia, J. C., Egbukichi, N. & Erwin-Cohen, R. A. Autoimmunity and Cancer, the Paradox Comorbidities Challenging Therapy in the Context of Preexisting Autoimmunity. *J. Interferon Cytokine Res. Off. J. Int. Soc. Interferon Cytokine Res.* **39**, 72–84 (2019).

65. Structural Genomics Consortium *et al.* Protein production and purification. *Nat. Methods* **5**, 135–146 (2008).

66. Watanabe, N. *et al.* A cell-based high-throughput screening assay system for inhibitor compounds of antigen presentation by HLA class II molecule. *Sci. Rep.* **7**, 6798 (2017).

67. Dumont, J., Euwart, D., Mei, B., Estes, S. & Kshirsagar, R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit. Rev. Biotechnol.* **36**, 1110–1122 (2016).

68. Goh, J. B. & Ng, S. K. Impact of host cell line choice on glycan profile. *Crit. Rev. Biotechnol.* **38**, 851–867 (2018).

69. Chin, C. L. *et al.* A human expression system based on HEK293 for the stable production of recombinant erythropoietin. *Sci. Rep.* **9**, 16768 (2019).

70. Bjorkman, P. J. *et al.* Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506–512 (1987).

71. Gorga, J. C., Madden, D. R., Prendergast, J. K., Wiley, D. C. & Strominger, J. L. Crystallization and preliminary X-ray diffraction studies of the human major histocompatibility antigen HLA-B27. *Proteins Struct. Funct. Bioinforma.* **12**, 87–90 (1992).

72. Serra, P. *et al.* Increased yields and biological potency of knob-into-hole-based soluble MHC class II molecules. *Nat. Commun.* **10**, 1–14 (2019).

73. Lotteau, V. *et al.* Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348**, 600–605 (1990).

74. Salamero, J., Borgne, R. Le, Saudrais, C., Goud, B. & Hoflack, B. Expression of major histocompatibility complex class II molecules in HeLa cells promotes the recruitment of AP-1 Golgi-specific assembly proteins on Golgi membranes. *J. Biol. Chem.* **271**, 30318–30321 (1996).

75. Panina-Bordignon, P., Fu, X. T., Lanzavecchia, A. & Karr, R. W. Identification of HLA-DRα chain residues critical for binding of the toxic shock syndrome toxin superantigen. *J. Exp. Med.* **176**, 1779–1784 (1992).

76. Korman, A. J., Frantz, J. D., Strominger, J. L. & Mulligan, R. C. Expression of human class II major histocompatability complex antigens using retrovirus vectors. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2150–2154 (1987).

77. Hitzel, C., Van Endert, P. & Koch, N. Acquisition of peptides by MHC class II polypeptides in the absence of the invariant chain. *J. Immunol.* **154**, 1048–1056 (1995).

78. Gorga, J. C., Horejsi, V., Johnson, D. R., Raghupathy, R. & Strominger, J. L. Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. *J. Biol. Chem.* **262**, 16087–16094 (1987).

79. Höpner, S. *et al.* Small organic compounds enhance antigen loading of class II major histocompatibility complex proteins by targeting the polymorphic P1 pocket. *J. Biol. Chem.* **281**, 38535–38542 (2006).

80. Valli, A. *et al.* Binding of myelin basic protein peptides to human histocompatibility leukocyte antigen class II molecules and their recognition by T cells from multiple sclerosis patients. *J. Clin. Invest.* **91**, 616–628 (1993).

81. Sette, A., Southwood, S., Miller, J. & Appella, E. Binding of Major Histocompatibility Complex Class II to the Invariant Chain-derived Peptide, CLIP, Is Regulated by Allelic Polymorphism in Class II By Allelic Polymorphism in Class II. *J. Exp. Med.* **181**, 677–683 (1995).

82. Wucherpfennig, K. W. *et al.* Structural Requirements for Binding of an Immunodominant Myelin Basic Protein Peptide to DR2 Isotypes and for Its Recognition by Human T Cell Clones. *J. Exp. Med.* **179**, 279–290 (1994).

83. C. Gorga, J., H. Brown, J., Jardetzky, T., C. Wiley, D. & L. Strominger, J. Crystallization of HLA-DR antigens. *J. Immunol. Res.* **142**, 401–407 (1991).

84. Klohe, E. P. *et al.* Analysis of the molecular specificities of anti-class II monoclonal antibodies by using L cell transfectants expressing HLA class II molecules. *J. Immunol.* **141**, 2158–2164 (1988).

85. Koehler, N. K. U. *et al.* Structure-based discovery of nonpeptidic small organic compounds to block the T cell response to myelin basic protein. *J. Med. Chem.* **47**, 4989–4997 (2004).

86. O'Sullivan, D. *et al.* Characterization of the specificity of peptide binding to four DR haplotypes. *J. Immunol.* **145**, 1799–1808 (1990).

87. O'Sullivan, D. *et al.* On the interaction of promiscuous antigenic peptides with different DR alleles: Identification of common structural motifs. *J. Immunol.* **147**, 2663–2669 (1991).

88. O'Sullivan, D., Sidney, J., Del Guercio, M.-F., Colón, S. M. & Sette, A. Truncation analysis of several DR binding epitopes. *J. Immunol.* **146**, 1240–1246 (1991).

89. Sette, A. *et al.* HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. *J. Immunol.* **151**, 3163–3170 (1993).

90. Van Lith, M., McEwen-Smith, R. M. & Benham, A. M. HLA-DP, HLA-DQ, and HLA-DR have different requirements for invariant chain and HLA-DM. *J. Biol. Chem.* **285**, 40800–40808 (2010).

91. Nag, B. *et al.* Intramolecular charge heterogeneity in purified major histocompatibility class II α and β polypeptide chains. *J. Biol. Chem.* **269**, 10061–10070 (1994).

92. Boen, E., Crownover, A. R., McIlhaney, M., Korman, A. J. & Bill, J. Identification of T Cell Ligands in a Library of Peptides Covalently Attached to HLA-DR4. *J. Immunol.* **165**, 2040–2047 (2000).

93. Day, C. L. *et al.* Ex vivo analysis of human memory CD4 T cells specific for hepatitis C virus using MHC class II tetramers. *J. Clin. Invest.* **112**, 831–842 (2003).

94. Hammer, J. *et al.* Peptide Binding Specificity of HLA-DR4 Molecules: Correlation with Rheumatoid Arthritis Association. *J Exp Med* **181**, 1847–1855 (1995).

95. Liu, Z., Li, B., Li, X., Zhang, L. & Lai, L. Identification of small-molecule inhibitors against human leukocyte antigen-death receptor 4 (HLA-DR4) through a comprehensive strategy. *J. Chem. Inf. Model.* **51**, 326–334 (2011).

96. Brunet, A., Samaan, A., Deshaies, F., Kindt, T. J. & Thibodeau, J. Functional characterization of a lysosomal sorting motif in the cytoplasmic tail of HLA-DOβ. *J. Biol. Chem.* **275**, 37062–37071 (2000).

97. Smith, M. Baculoviral expressed hla class i heavy chains used to screen a synthetic peptide library for Allele–Specific peptide binding motifs. *Mol. Immunol.* **35**, 1033–1043 (1998).

98. Mancino, L., Rizvi, S. M., Lapinski, P. E. & Raghavan, M. Calreticulin recognizes misfolded HLA-A2 heavy chains. *Proc. Natl. Acad. Sci.* **99**, 5931–5936 (2002).

99. Lévy, F. & Kvist, sune. Co-expression of the human HLA-B27 class I antigen and the E3/19K protien of adenovirus-2 in insect cells using a baculovirus vector. *Int. Immunol.* **2**, 995–1002 (1990).

100. Thammavongsa, V. *et al.* Assembly and intracellular trafficking of HLA-B*3501 and HLA-B*3503. *Immunogenetics* **61**, 703–716 (2009).

101. Chou, C.-L. *et al.* Short peptide sequences mimic HLA-DM functions. *Mol. Immunol.* **45**, 1935–1943 (2008).

102. Narayan, K. *et al.* HLA-DM targets the hydrogen bond between the histidine at position β81 and peptide to dissociate HLA-DR–peptide complexes. *Nat. Immunol.* **8**, 92–100 (2007).

103. Spiekerman, A. M., Hetherly, M. E. & Hall, F. F. Improved high-resolution high-voltage paper electrophoresis system for use in screening for aminoacidopathies. *Clin. Chem.* **21**, 1981–1982 (1975).

104. Sadegh-Nasseri, S., Stern, L. J., Wiley, D. C. & Germain, R. N. MHC class II function preserved by low-affinity peptide interactions preceding stable binding. *Nature* **370**, 647–650 (1994).

105. Stern, L. J. & Wiley, D. C. The human class II MHC protein HLA-DR1 assembles as empty αβ heterodimers in the absence of antigenic peptide. *Cell* **68**, 465–477 (1992).

106. Gauthier, L. *et al.* Expression and crystallization of the complex of HLA-DR2 (DRA, DRB1*1501) and an immunodominant peptide of human myelin basic protein. *Proc. Natl. Acad. Sci.* **95**, 11828–11833 (1998).

107. Fourneau, J.-M., Cohen, H. & van Endert, P. M. A chaperone-assisted high yield system for the production of HLA-DR4 tetramers in insect cells. *J. Immunol. Methods* **285**, 253–264 (2004).

108. Autrup, H. & Warwick, G. P. Some characteristics of two azoreductase systems in rat liver. Relevance to the activity of 2-[4’-di(2"-bromopropyl)-aminophenylazo]benzoic acid (CB10-252), a compound possessing latent cytotoxic activity. *Chem. Biol. Interact.* **11**, 329–342 (1975).

109. Jüse, U., Fleckenstein, B., Bergseng, E. & Sollid, L. M. Soluble HLA-DQ2 expressed in S2 cells copurifies with a high affinity insect cell derived protein. *Immunogenetics* **61**, 81–89 (2009).

110. Kim, C.-Y., Quarsten, H., Bergseng, E., Khosla, C. & Sollid, L. M. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proc. Natl. Acad. Sci.* **101**, 4175–4179 (2004).

111. Quarsten, H. *et al.* Staining of Celiac Disease-Relevant T Cells by Peptide-DQ2 Multimers. *J. Immunol.* **167**, 4861–4868 (2001).

112. Xia, J., Sollid, L. M. & Khosla, C. Equilibrium and Kinetic Analysis of the Unusual Binding Behavior of a Highly Immunogenic Gluten Peptide to HLA-DQ2 †. *Biochemistry* **44**, 4442–4449 (2005).

113. Kozono, H. *et al.* Molecular linkage of the HLA-DR, HLA-DQ, and HLA-DO genes in yeast artificial chromosomes. *Genomics* **11**, 577–586 (1991).

114. Chen, Z. *et al.* Humanized transgenic mice expressing HLA DR4-DQ3 haplotype: reconstitution of phenotype and HLA-restricted T-cell responses. *Tissue Antigens* **68**, 210–219 (2006).

115. Demmer, L. A. & Chaplin, D. D. Simultaneous transfer of four functional genes from the HLA class II region into mammalian cells by fusion with yeast spheroplasts carrying an artificial chromosome. *J. Immunol. Baltim. Md 1950* **150**, 5371–5378 (1993).

116. Bronson, S. K. *et al.* Isolation and characterization of yeast artificial chromosome clones linking the HLA-B and HLA-C loci. *Proc. Natl. Acad. Sci.* **88**, 1676–1680 (1991).

117. Ragoussis, J. *et al.* Cloning of the HLA class II region in yeast artificial chromosomes. *Proc. Natl. Acad. Sci.* **88**, 3753–3757 (1991).

118. Wei, H. *et al.* Genes in one megabase of the HLA class I region. *Proc. Natl. Acad. Sci.* **90**, 11870–11874 (1993).

119. Fabb, S. Generation of novel human MHC class II mutant B-cell lines by integrating YAC DNA into a cell line homozygously deleted for the MHC class II region. *Hum. Mol. Genet.* **6**, 1295–1304 (1997).

120. Chen, D. *et al.* Characterization of HLA DR3/DQ2 transgenic mice: a potential humanized animal model for autoimmune disease studies. *Eur. J. Immunol.* **33**, 172–182 (2003).

Vita Auctoris

NAME:	Samaneh Mehri
PLACE OF BIRTH:	TEHRAN, IRAN
YEAR OF BIRTH:	1988
EDUCATION:	Masoomin High School, Tehran, Iran 2001
	Tehran Azad University, Tehran, Iran, B.Sc 2006
	Tabriz University of Medical Sciences, Tabriz, Iran, M.Sc 2011