**ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# INVESTIGATION OF CLASS I and II ANTI-HUMAN LEUKOCYTE ANTIGEN ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS AND SCLERODERMA PATIENTS

**M.Sc.THESIS**

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**The Department of Molecular Biology-Genetics and Biotechnology**

**Molecular Biology-Genetics and Biotechnology Program**

**JUNE 2015**

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**JUNE 2015**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# SİSTEMİK LUPUS ERİTEMATOZUS ve SKLERODERMA HASTALARINDA ANTİ-İNSAN LÖKOSİT ANTİJEN ANTİKORLARININ İNCELENMESİ

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## ABBREVIATIONS

**SLE** : Systemic lupus erythematosus
**SSc** : Systemic sclerosis
**HLA** : Human Leukocyte antigen
**MHC** : Major histocompatibility complex
**DNA** : Deoxyribonucleic acid
**$T_c$** : Cytotoxic T cell
**$T_h$** : Helper T cell
**TNF** : Tumor necrosis factor
**LTA** : Lymphotoxin alpha
**LTB** : Lymphotoxin beta
**CD** : Cluster of differentiation
**PCR** : Polymerase chain reaction
**BCR** : B cell receptor
**Ig** : Immunoglobulin
**UPS** : Uninterruptible power supply
**SPSS** : Statistical Package for the Social Sciences
**ACR** : American college of Rheumatology
**ANA** : Antinuclear antibody
**Anti-dsDNA** : Anti-double-stranded DNA
**ENA** : Extractable nuclear antigen
**SLEDAI** : SLE Disease Activity Index
**Ml** : mililiter
**NTC** : No template control
**CI** : Coefficient Interval
**CNS** : Central nervous system
**APS** : Antiphospholipid syndrome
**anti-Sm** : Anti-Smith antibodies
**anti-RNP** : Anti- Ribonucleoprotein
**anti-RO** : Anti-Sjögren's-syndrome-related antigen A
**PRA** : Panel reactive antibody
**CREG** : Cross reactive group
**CDC** : Complement dependent cytotoxicity
**LCLs** : Lymphoblastic cell lines
**DTT** : Dithiothreitol
**ELISA** : Enzyme-linked immunosorbent assay
**RA** : Rheumatoid arthritis
**CIC** : Circulating immune complexes

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## SUMMARY

Human leukocyte antigen (HLA) is the human form of Major Histocompatibility Complex (MHC). Generally, HLA is a system of gene locus that encode for proteins on the surface of cells that are responsible for the regulation of the immune system. It is located on chromosome 6, encoded cell surface antigen presenting proteins. Anti-HLA antibodies are normally not occurring produced against self or non-self HLA antigens to form a immunologic response in blood transfusion, pregnancy and organ or tissue transplantation.

Systemic lupus erythematosus (SLE) is an autoimmunity disorder which inflammation along with tissue damage affecting multi organs such as skin, kidney, joint are the main cause of it. SLE is a multisystem autoimmune disorder that has variable clinic manifestation. Prior physiologic mechanism of SLE is polyclonal B cell activation and production of autoantibodies against nucleus, cytoplasmic and cell surface antigens. Although the main cause of SLE pathogenesis remains unclear, there have been several clinical and serological factors that define predisposition and risk for SLE. Systemic Sclerosis (SSc) that is characterized with vascular damage caused by immune system dysfunctions and collagen accumulation in several tissues and organs can cause smooth muscle atrophy result of neurological function loss and then motor function loss.

Generally anti-HLA antibodies can cause acute and chronic rejection in transplantation. Lately, there have some studies that associated between autoimmunity and anti-HLA antibodies. According to new theory, it has been indicated that there may be a similarity between alloimmune responses occur during transplantation and autoimmunity for self-antigen. Microchimerism is a well-known phenomenon effecting after solid transplantation, pregnancy and blood transfusion. The analogy between autoimmune diseases and alloimmune response in transplantation may explain role of microchimerism related with fetal or maternal origin.

Therefore, in our project, SLE and SSc were selected as a model autoimmune disease to investigate the relationship between autoimmunity and anti-HLA antibodies. First of all, three different group including SLE, SSc patients and healthy controls were formed. The clinical and demographic data were collected and analyzed. Then, anti-HLA antibodies screening were done to all groups with flourometric methods. After analysis of anti-HLA antibodies screening, statistical analysis were done. Also, anti-

HLA antibody identification analysis has been applied to anti-HLA antibody positive SLE, SSc patients and healthy controls. PRA ratios and CREG groups were determined statistically.

According to our findings, the frequencies of both class I and II anti-HLA antibodies were increased in both SLE and SSc patients when compared to controls. Also, anti-HLA antibodies frequencies were similar in both SLE and SSc patients. We also found that typical parameters for SLE patients such as photosensitivity, thrombocytopenia, anti-RNP positivity, anti-Ro positivity, anti-nuclear antibody (ANA) had slightly higher frequency than SSc patients. On the other hand, SSc parameters frequency such as digital ulcers, anti-centromere antibody, pulmonary hypertension, and pulmonary fibrosis were higher in SSc patients. Also, in comparison of degree of positivity of anti-HLA antibodies we found that patients with high positivity for anti-HLA antibodies in both SLE and SSc group was found higher than low positivity individuals in same groups.

In conclusion, anti-HLA class I and II antibodies were found as a significantly increased in patients of SLE and SSc that were chosen as model autoimmune diseases than healthy controls in which microchimerism may play a role. In order to focus the role of microchimerism in this relationship, more targeted studies should be done.

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## ÖZET

İnsan Lökosit Antijenleri (HLA), majör histokompabilite komplekslerinin insan formlarıdır. Genel olarak, HLA bir gen lokus sistem bütünü olup, immün sistem regülasyonunda görevli hücre yüzey proteinlerinin kodlanmasında görev alır. 6. Kromozomda bulunan HLA, hücre yüzey antijen sunucu proteinleri kodlamaktadır. Anti-HLA antikorları ise, normal olarak oluşmayıp, kan nakli, hamilelik, doku ya da organ nakillerinde kendi ya da kendi olmayan HLA antijenlerine karşı üretilen antikorlardır.

Sistemik lupus eritematozus (SLE), Sistemik Lupus Eritromatozus (SLE) otoimmün bir rahatsızlık olup başta cilt, eklem ve böbrek olmak üzere birçok organ ve dokuyu etkileyen iltihaplanmalara sebep olur. Multisistem bozukluğu olan SLE, değişken bir klinik tablo göstermektedir. SLE'nin öncelikli fizyolojik mekanizması poliklonal B-hücre aktivasyonu ve çekirdek, sitoplazmik ile hücre yüzey antijenlerine karşı üretilen otoantikor üzerinden gerçekleşmektedir. Patogenezinin anlaşılması için birçok araştırma yapılmasına rağmen, hala tam olarak aydınlatılmamıştır. Buna karşılık, SLE yatkınlığına etki eden ve hastalığın kliniksel ve serolojik karekteristiğini belirleyen birçok risk faktörü bulunmaktadır. İmmün sistem bozukluklarına bağlı olarak vasküler hasarlanma ile çeşitli doku ve organlarda kollajen birikimi ile karakterize edilen Sistemik Skleroz (SSc), nörolojik fonksiyon kaybı sonucu düz kas atrofisine, ilerleyen zamanlarda da motor fonksiyon kaybına neden olur.

Anti-HLA antikorları genel olarak organ nakillerinde akut ve kronik ret olgusuna neden olmaktadır. Son zamanlarda otoimmünite ile anti-HLA antikorları arasındaki ilişki üzerine yeni yeni çalışmalar yapılmaktadır. Yeni bir teoriye göre, transplantasyon sonrası süreçte gerçekleşen alloimmün cevap ile öz antijene yönelik gerçekleşen otoimmünite arasında benzerlik bulunmaktadır. Mikrokimerizm iyi bilinen bir olgu olup, organ nakillerinde, kan nakillerinde ve hamilelikte etkili olmaktadır. Otoimmün hastalıklarla, transplantasyondaki alloimmün cevabın arasındaki ilişkinin açıklanmasında, mikrokimerizmin rolü olduğu düşünülmektedir.

Bu nedenle, çalışmamızda otoimmün rahatsızlıklarla, anti-HLA antikoru arasındaki ilişkinin açıklanması için, SLE ve SSc hastalıkları model otoimmün hastalık olarak seçilmiştir. İlk olarak, SLE ve SSc hastaları ile sağlıklı kontrol grupları oluşturulmuştur. Bireylerin klinik ve demografik verileri toplanmış ve analiz

edilmiştir. Daha sonra, anti-HLA antikoru taraması flourometrik yöntemle yapıldı. Anti-HLA antikoru tarama sonuçlarının analizinin ardından istatistiksel analiz yapıldı. Bu aşamadan sonra, anti-HLA antikor tarama sonuçları pozitif olan SLE, SSc hasta ile sağlıklı kontrollere, anti-HLA antikor tanımlama yöntemi uygulandı. Bu yöntem için, tarama yönteminde olduğu gibi flurometrik yöntem olan Luminex/xMAP yöntemi kullanıldı. Olguların PRA oranları elde edildikten sonra, istatistiksel analiz yapıldı. Ayrıca, olguların belirlenen CREG epitop gruplarına göre, reaktif anti-HLA antikorları sınıflandırılarak, bütün olgular arasında en sık rastlanan CREG grupları belirlendi.

Çalışmamızdaki bulgularımıza göre, anti-HLA sınıf I ve II antikorları frekansı hem SLE hem de SSc hastaları gruplarında sağlıklı kontrollere göre yüksek olarak bulundu. SLE ve SSc hastaları grupları arasında anti-HLA antikoru karşılaştırılması sonucunda, önemli bir farka rastlanmadı. Ayrıca, SLE hastalığı için tipik parametrelerden olan, fotoduyarlılık, trombositopeni, anti-RNP antikoru pozitifliği, anti-RO ve anti-çekirdek antikoru pozitifliği frekansları SSc hastalarından daha fazla olarak bulunmuştur. Diğer yandan, SSc parametrelerinden olan parmak ülseri, anti-sentromer antikoru, pulmoner tansiyon ve pulmoner fibroz frekansları da SSc hastalarında daha yüksek olarak belirlenmiştir. Ayrıca, anti-HLA antikoru pozitiflik derecelerine göre karşılaştırılma yapıldığında, hem SLE hem de SSc hasta gruplarında yüksek pozitiflik frekansı sağlıklı kontrollere göre daha yüksek olarak bulunmuştur. Tanımlama sonuçlarında ise, sınıf-I anti-HLA antikor PRA oranlarının ortalaması SLE hastalarında, SSc hastalarında daha yüksek bulunurken, sınıf-II'de herhangi bir farklılık görülmemiştir. CREG epitop gruplarının sınıflandırmasında ise, anti-HLA antikor pozitif olarak bulunan SLE, SSc hastaları ile sağlıklı kontroller arasında en sık rastlanan CREG grupları B4 ve B6 olarak bulunmuştur.

Sonuç olarak, anti-HLA sınıf I ve II antikorları frekansı, otoimmün hastalık modelleri olarak seçilen SLE ve SSc hastalarında sağlıklı kontrollere göre daha yüksek bulunmuştur. Bu nedenle, mikrokimerizm rolü hakkında, bu rolün daha iyi belirlenmesi için daha hedefli çalışmaların yapılması düşünülmektedir.

# 1. INTRODUCTION

## 1.1 Major Histocompatibility Complex

Major histocompatibility complex is placed on chromosome 6 in humans, spanning over 4 centimorgans of DNA, approximately 4 x $10^{6}$ base pairs. Over 200 genes are incorporated in humans. Genes of α chain of MHC class I molecules and β chains of MHC class II are encoded in the same gene region complex, whereas the genes for β2-microglobulin and the invariant chain are on different chromosomes (Figure 1.1). In humans, these genes are named as Human Leukocyte Antigen or HLA genes, and in mouse they are known as H-2 genes (Janeway et al., 2001).

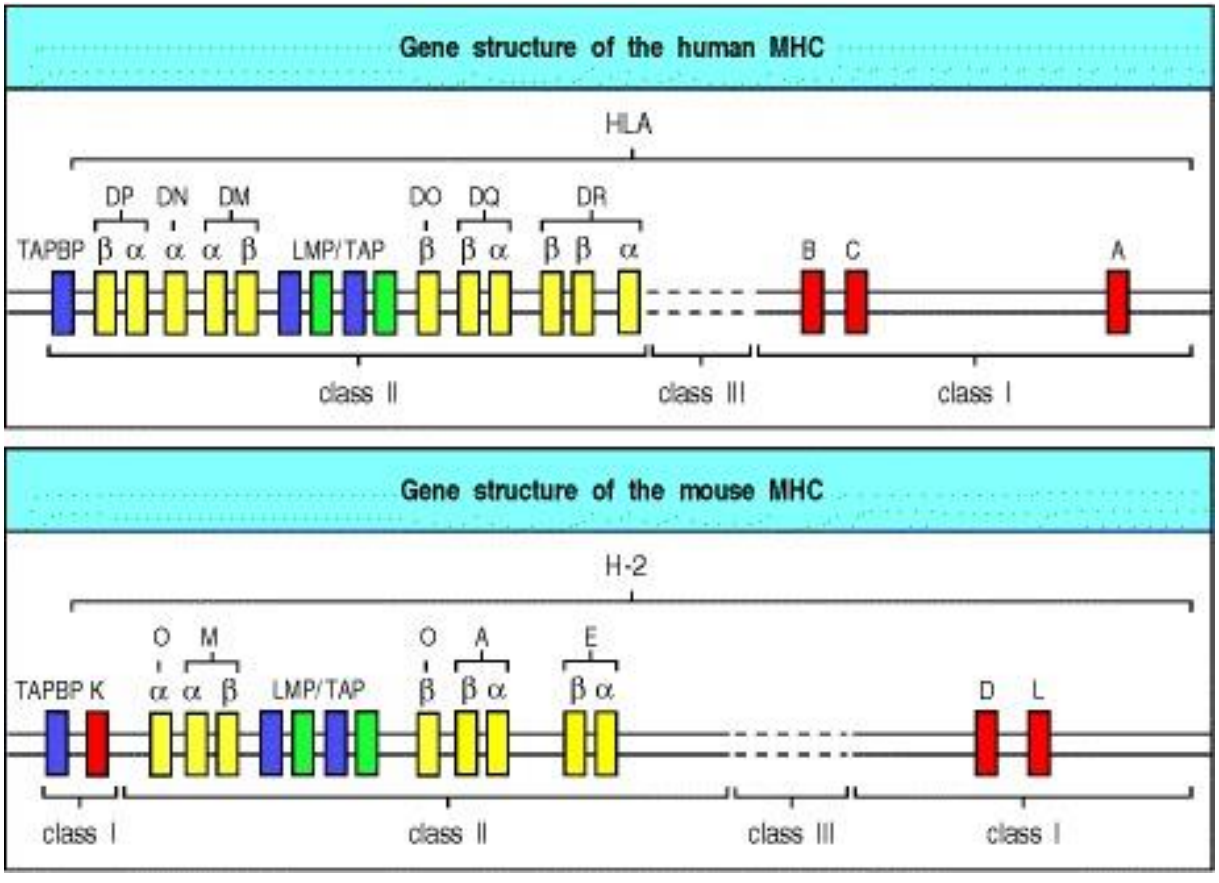



**Figure 1.1** Genetic organization of the major histocompatibility complex (MHC) in human and mouse (Adapted from Janeway et al., 2001)

Although the organization of genes can be varied differently, MHC genes are divided into regions encoded three types of molecules. Glycoproteins that are expressed on the surface of most of nucleated cells are encoded by class I MHC genes. The major role of product of these genes is antigen presentation to $T_c$ cells. Class II MHC genes encode glycoproteins expressed mainly on antigen presenting cells (macrophages, B cells and dendritic cells) and those antigen presenting cells present processed antigenic peptides to $T_h$ cells. Class III MHC genes are involved encoding some secreted proteins which have major immune functions such as components of complement system and inflammation molecules (Kindt et al., 2007).

As a general view, peptide (or antigen) presentation to T-cell receptors is the main function of MHC class I and II (Danchin et al.,2004) More specifically, general function of MHC molecules is to interact with peptide fragments from pathogens and present them on the cell surface in order to recognize by the appropriate T cells. The results of this process are always destructive to the pathogen and are concluded with macrophage activation to kill bacteria living in their intracellular vesicles and B cell activation to produce antibodies for elimination and neutralization of extracellular pathogens (Janeway et al., 2001).

### 1.1.1 Human Leukocyte Antigen

Human leukocyte antigen (HLA), is the human form of MHC with same function, are divided into 3 same groups as in MHC, Class I, II and III. HLA Class I genes contain 2 group of genes; one group is classical which consists of HLA-A, HLA-B and HLA-C and the non-classical group contains HLA E, HLA-F and HLA-G. Class II subregion also contain 3 separate group such as HLA-DR, HLA-DP and HLA-DQ. Class III region exclusively encodes some important immune response products like TNF genes, LTA and LTB genes (Mehraet al.2010).

Basically, HLA molecules were defined with serological methods by utilizing antibodies specific for variable HLA class I and II molecules. With that reaching, a terminology for nomenclature and definition of HLA antigens was established. For instance, HLA-A2 is different from HLA-A25, HLA-B18 from HLA B-27 etc. The development of PCR technologies leads to more informative about differences between HLA molecules. For example, present terminology indicate HLA-DRB1*1301, in which the letters to the left of asterisk define the HLA gene, and the

four digit to the right of asterisk point out the particular HLA allele. That major and informative nomenclature of HLA alleles has been quite useful for scientists and clinicians to try matching organ donors and recipients and also specify individuals who have been risk for autoimmunity (Coico& Sunshine, 2009).

## 1.2 Humoral immune system

### 1.2.1 B cells

Most of the pathogenic bacteria divide in human in the extracellular part of the body and many intracellular pathogens can be transported into all cells with the help of extracellular fluids. Those extracellular parts of human body are protected by humoral immune response by secreting antibodies in B cells for destruction of extracellular pathogens and block the spread of the intracellular infections. Differentiation of B cells into antigen secreting plasma cells after activation of them occur via antigen trigger and generally need helper T cells. Helper T cell is $Th_2$ class of CD4 cells, and a subgroup of $Th_1$ cells can be also effective in B cell activation (Janeway et al., 2001).

B lymphocytes have antibody molecules on their surface and antibodies can only interact with one specific antigen, especially antigenic determinant. T lymphocytes that are component of cell mediated immunity, express receptors specific to short peptide sequences related with MHC molecules present on the cell surface. Therefore, humoral immunity is specific to many different proteins, polysaccharides, lipids, toxins and lipopolysaccharides that secreted by spreading pathogens in the blood stream (Khan, 2009)

B lymphocytes are fundamental component of humoral immune system and basically, produce antibodies during immune response (Honjo et al., 2014).Generally, B lymphocytes are induced by antigens, and after activation they mainly differentiate into plasma cells. The plasma cell form of B cell is known as antibody-producing cells, with the help of T cell interaction. B cell receptor (BCR), which is comprised of surface immunoglobulin (Ig), can recognize antigen (Chaturvedi et al., 2012).

### 1.2.2 Antibody

Antibody, also known as immunoglobulin (Ig), is large Y-shaped protein structure produced by plasma B cells (Litman et.al, 1993) General functions of antibody are to bind foreign and non-self-molecules, basically antigens, neutralizing them or induction of their removal. Antibodies can be produced at vast variability, they can be structurally similar (all Y-shaped molecules) but they are unique. The chemical features of antibodies defines three functions of antibodies; 1) binding versatility, 2) binding specificity and 3) biological activities. An antibody molecule consist of four polypeptide chains with a molecular weight about 150 kD (Figure 1.2). These four chains are split into two identical light chains and two heavy chains (Elgert, 2009). The terms of heavy and light means the relative size of the proteins. Each light chain is covalently bound to a heavy chain and the heavy chains are also bound each other covalently. Both light and heavy chains of the antibody structure are similar. Each component has two regions, a variable (V) and a constant (C) region. The variable region of the light chain comprise two definite fragment named as 'V' and 'J'. However, the heavy chain contains three segments, named as 'V', 'D' and 'J'. Two variable regions from heavy and light chain define specificity of an antibody molecule (Jacqueline, 2012).

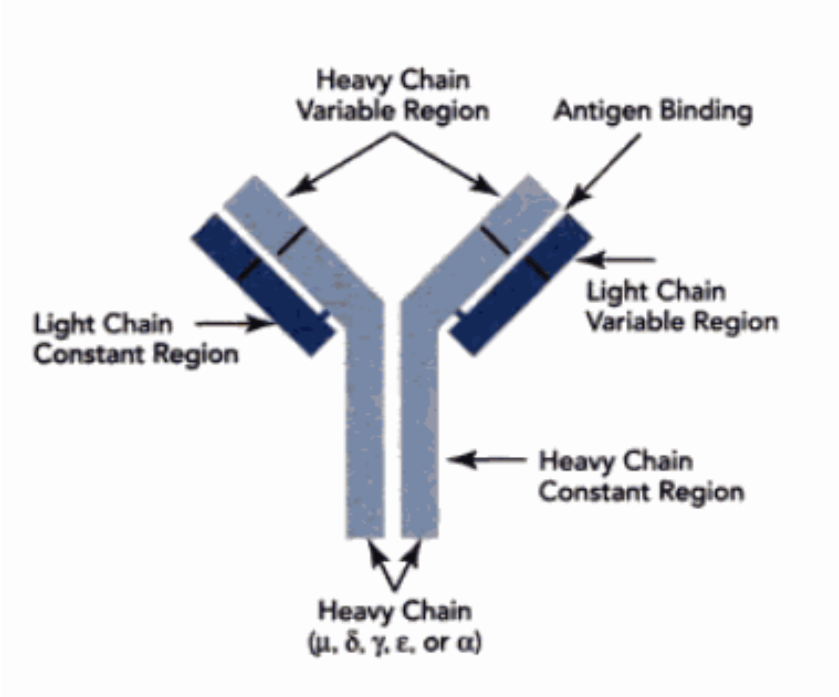


**Figure 1.2** Antibody (Imunoglobulin) structure (adapted from Jacqueline, 2012)

Antigenic determinants that characterize antibody classes are called isotypic determinants and the classes are indicated as isotypes (Figure 1.3). The five different classes of immunoglobulins are defined as IgG, IgM, IgD, IgE and IgA (Elgert, 2009).

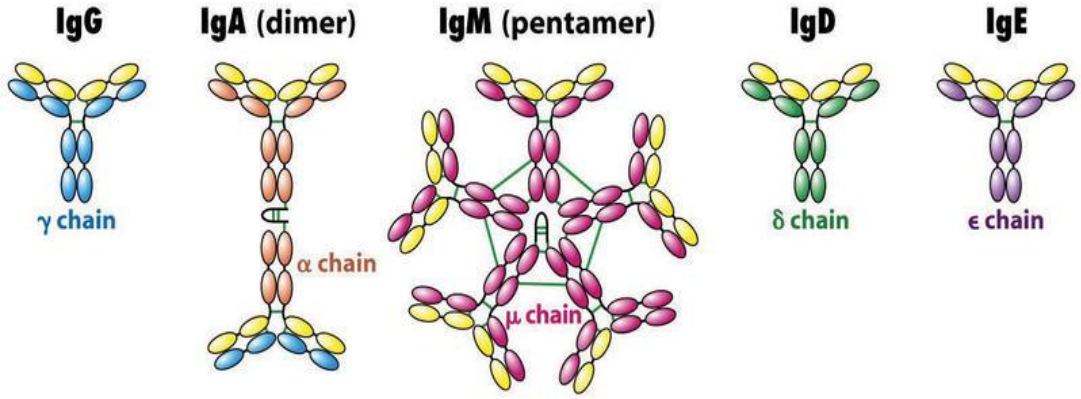



**Figure 1.3** Types of immunoglobulins (adapted from Berg et al., 2007)

### 1.2.2.1 Immunoglobulin E

Immunoglobulin E (IgE) antibodies are notably crucial in effective immune response to some parasites, especially in helminth infections in gastrointestinal tract. The Ig-E antibodies can interact to cells with FcεR receptors such as eosinophils, mast cells and basophils. The eosinophils secrete cytotoxic protein, after parasite/Ig-E complex binding to FcεR on eosinophils. Moreover, histamine released by mast cell and basophils since IgE make a crosslink with helminthes (Jacqueline, 2012).

### 1.2.2.2. Immunoglobulin D

Membrane form of immunoglobulin D (IgD) is primarily crucial since the soluble form is found in bloodstream and other body fluids moderately low. IgDtend to be capable of transducing activating and tolerizing signals, when it is found along with Ig-M on all mature, naïve B cells (Chaturvedi et al., 2012).

### 1.2.2.3. Immunoglobulin A

Immunoglobulin A (IgA) is produced in most amount among all antibody isotypes. The main function of IgA is to manage homeostasis at mucosal surface and immune response. Also, Ig-G plays an important role in avoiding tissue damage in several multiple autoimmune and inflammatory diseases (Mkaddem et al.,2014)

#### 1.2.2.4 Immunoglobulin M

Immunoglobulin M (IgM) is an antibody isotypes that has the highest molecular weight among all other isotypes. There are two types of IgM structurally; monomeric and pentameric. Monomeric IgM is used as an antigenic receptor by the B cells and is produced as membrane bound antibody on B cells. Pentamer IgM is produced by permablast cells. In spite of abundance of IgA in secretory fluids, IgM has an important function as a secretory immunoglobulin. Also, IgM is the first immunoglobulin that is secreted by the neonates (Rao, 2002). Moreover, IgM has more valency than other antibody isotypes since it has pentameric structure with the help of 10 antigen binding sites. However, IgM cannot diffuse easily due to its large molecular size, so the amount of IgM is very low at intercellular tissue fluids (Kindt et al., 2007)

#### 1.2.2.5 Immunoglobulin G

Immunoglobulin G (IgG) is the important class of immunoglobulins in serum. IgG can be found in extravascular spaces, not only the bloodstream. Also, IgG can be transported into the placenta so passive immunity in the fetus and neonates can be established. IgG is responsible for important function such as complement fixation, precipitation, neutralization of toxins and viruses (Mohanty&Leela, 2013). IgG can be investigated under four different subclasses according to their decreasing amount respectively, IgG1, IgG2, IgG3, and IgG4. Also, IgG antibody can give a response to an antigen related to their subclasses. Selective subclass deficiencies are not generally harmful to individuals but in some specific conditions do cause to increased susceptibility of some specific class of pathogens. That harmful effect can be caused whole isotype or subclass deficiency because of some deletions of Ig loci (Vidarsson et al., 2014)

### 1.3 Anti-HLA Antibodies

Anti-HLA antibodies or antibodies against human leukocyte antigens are antibodies that produced specific to HLA and play a major role in graft survival, especially solid organ transplantation (Maccarone et al., 2005) Exposure of HLA antigens in blood transfusion, pregnancy or transplantation can lead the formation of antibodies specific for HLA antigens. This phenomenon called as a sensitization. The presence of those anti-HLA antibodies is harmful in especially organ transplantation when HLA antigens of donor match with the recipient's antibody. The quantity of HLA antibodies can be detected with testing serum from individuals in order to presence of HLA antibodies. In this measurement, an estimate of the amount of sensitization is measured with calculation of panel reactive antibodies (PRA) that is the number of cells that interacts positively with the serum divided by the number of cells in the test panel, times 100, equals the percent PRA. The percent PRA most likely determine what percentage antibodies of patients react with donor HLA (Adelman et al, 2002).

In transplantation process, recipient blood vessels are anastomosed to the vessels belong to the donor on the donor graft; recipient blood goes through the graft and is reacted with endothelial cell lining the donor vessels. Any preformed HLA antibodies in the recipient can attack the cells by fixation of complement and activation of complement system since endothelial cells can express both class I and II antigens. This phenomenon is named as hyperacute rejection and can be avoided with the development of more sensitive antibody detection method (Mehra et al, 2010).

There are two phases of evaluating HLA antibodies presence; screening and identification or detection of specificity. Screening of evaluating presence of anti-HLA antibodies (also named as lymphocytotoxic antibodies) is the first medical procedure after immunizing cases such as transfusion, transplant rejection, pregnancy and any change during immunosuppression therapy. Identification or detection of anti-HLA antibody specificity is performed for the determination of the HLA phenotypes of the test cells which the antibody is reactive. For instances, if all of the test cells that reacted positively with the serum have HLA-A1 in their HLA phenotypes, the antibody specificity of the patient is considered to be anti-A1. Some antibodies can be reactive with epitopes which is common among several HLA

antigens. These antigen groups are named as CREGs (cross-reactive groups). Identifications of the antibody specificities of patients for transplantation candidates are remarkable factor for the selection of suitable organ donor. Transplantation of an organ to the previously sensitized with an HLA antigen is undesirable practice in organ transplantation procedure (Adelman et al., 2002). There are two distinct anti-HLA antibody screening methods that have been commonly used for HLA antibody screening such as Cell based (cytotoxic), solid phase anti-HLA antibody screening methods.

### 1.3.1 Cell based (cytotoxic) anti-HLA antibody screening methods

For several years, the basic and common technique in HLA antibody screening was complement dependent cytotoxicity (CDC) assay. The CDC technique based on living T cells for class I antibody identification and living B cells for class I and II antibody identification. The CDC technique is relatively time-consuming, inconvenient and also requires testing a few panel donors to contain all major features or depending on commercially available frozen cell trays or immortalized lymphoblastic cell lines (LCLs). In these tests, only complement fixing antibodies can be detected. In spite of CDC assays being time-consuming and inconvenient, they have advantage of easy determination of antibodies of IgG and IgM isotype with adding dithiothreitol (DTT) (Howell et al., 2010).

Cell donors are chosen in a population by chance in order to contain variable HLA types and their lymphocytes from panels of cells. The HLA typings of these cell donors are the representative of the general distribution in same population from cadaveric donor (Figure 1.4). Only difference between serologic typing and the basic method of cell based HLA antibody screening is mixing cell donor lymphocytes and recipient serum in all wells along with complement and vital dye (Chandraker et al., 2012).

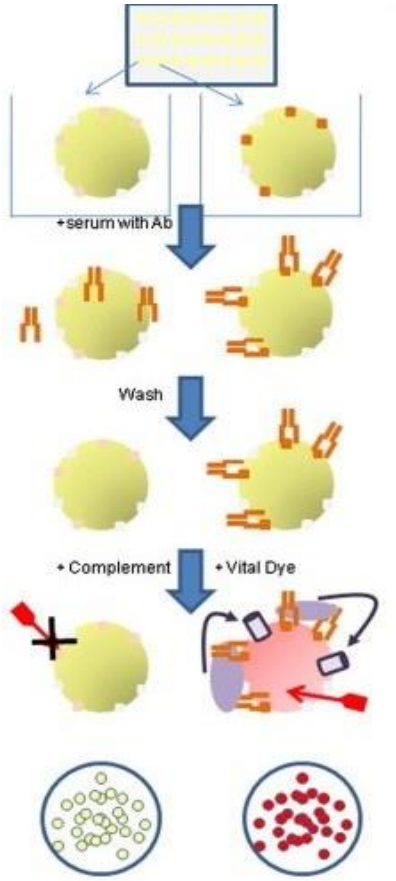



**Figure 1.4** Schematic demonstration of cell-based HLA antibody screening method (adapted from Tinckam, 2009)

### 1.3.2 Solid phase anti-HLA antibody screening methods

It has been reported that antibody-mediated damage can occur without any detectable antibody with the usage of cell based (cytotoxic) antibody screening method. For this reason, more sensitive assays have been developed. In order to analyze HLA antibody and non-HLA antibody separately as well as class I and II, developed solid phase methods have been created. Instead of usage of lymphocyte targets with both HLA and non-HLA antibody, solid based screening methods use soluble or recombinant HLA molecules (Figure 1.5). In those techniques, after purified molecules bind on ELISA platforms or beads, they interact with only HLA antibody in the added recipient serum (Tinckam, 2009). Generally, there are two different solid based anti-HLA antibody screening methods such as ELISA and flow cytometry/X-map techniques.

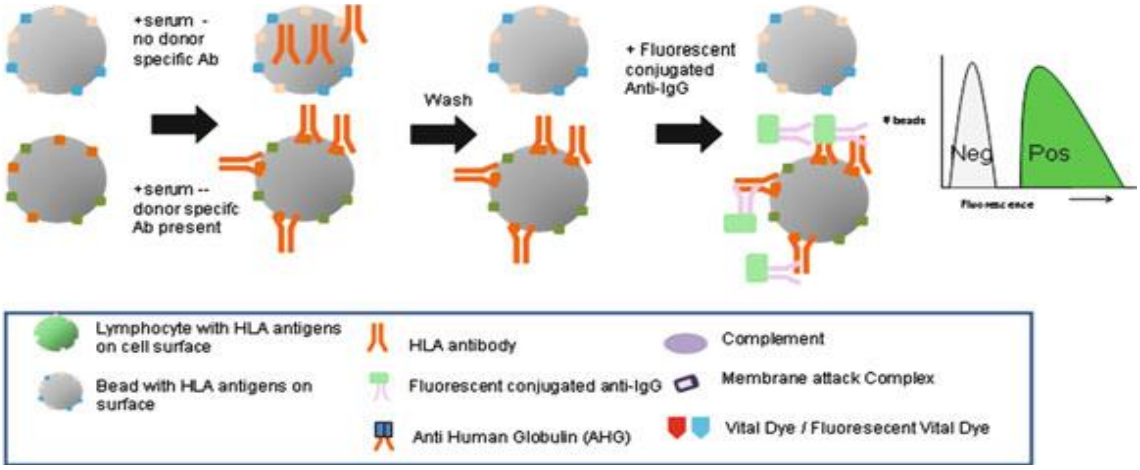



**Figure 1.5** General schematic of solid phase HLA screening method (adapted from Chandraker et al., 2012)

### 1.3.2.1 Anti-HLA antibody screening with ELISA

In ELISA (enzyme-linked immunosorbent assay) method for HLA antibody screening, multiwall plates which have purified HLA antigens bound to individual wells. After sample serum is added and washed to eliminate unbound unwanted antibody, reactive HLA antibody can be detected using labelled anti-human antibodies (Torpey et al., 2010)

ELISA PRA assays utilize soluble HLA antigens bound onto wells of an ELISA plate. Test sera are added to the individual wells, antibodies can bind to the related antigens when the sera contains HLA antibody. Antibody-antigen complexes can be detected with horseradish peroxidase-conjugated anti-human IgG antibody. With the enzyme-substrate interaction, substrate can be catalyzed into a colored product which provides measurement of light with absorbance ratio. The measurement is made with ELISA plate reader and analyzed with software in computer (Rudmann, 2005).

### 1.3.2.2 Anti-HLA antibody screening with flow cytometry/xMAP (Luminex) techniques

Flow cytometry/xMAP (Luminex) techniques depend on soluble and recombined HLA molecules on polystyrene particles and these assays are the gold standard for both antibody screening and identification. The xMAP (Luminex) assay has two distinct recording systems. First system is a laser that measure changes in 100 individual polystyrene particles. Those particles are colored with numerous amounts

of two different fluorochromes that make particles unique. HLA antigens or a single antigen interact with each bead set. For antibody binding, test serum is added on to beads. Second anti-human antibody with a reporter molecule is also added into the reaction mix, fluorescence from reporter molecule is measured by the laser. Therefore, semi-quantitative antibody level in the test serum is determined (Howell et al., 2010).

HLA antibody screening and identification with flow cytometry depends on availability of tools in laboratory. In flow PRA method, there are two different techniques. First technique is a house method in which whole lymphocytes are used as an antigen and second technique is commercial kits which contain beads with coated and recombined HLA antigens. Commercial flow PRA kits are demonstrated to be more sensitive than cell-based HLA antibody screening method (Hajeer, 2006).

### 1.4 Autoimmunity

An autoimmune response or autoimmunity can be defined as an attack of the immune system to the host itself. For all healthy individuals, immune system is tolerant to self but reactive foreign molecules defined as non-self such as bacteria and viruses. The ability of immune system in separating self from non-self is thought to be the crucial factor in immune system response. Autoimmunity phenomenon defines a precise damage to the mechanism which immune system regulates their activation. Interestingly, the effector mechanism of autoimmunity is not so different from normal immune response; they use same molecules such as antibodies and as well as cell-mediated immunity components. There is no single mechanism that has been determined for the diversity of autoimmune responses or the autoantibodies production (Figure 1.6) (Pollard, 2006).

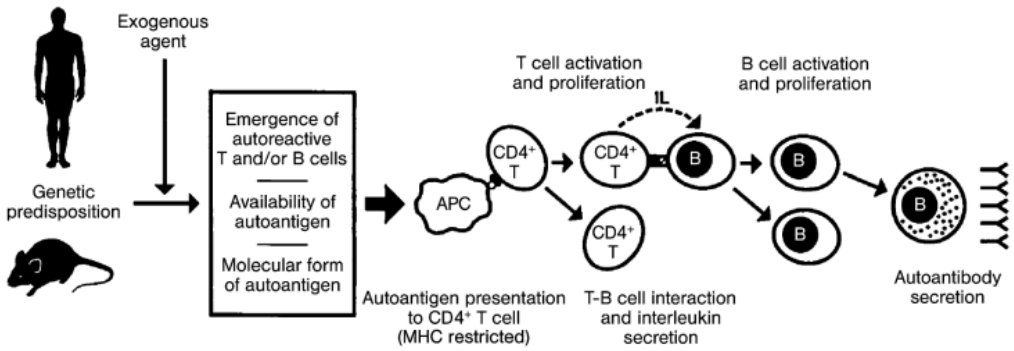


**Figure 1.6** Hypothetical pathway of autoantibody formation in autoimmunity (adapted from Pollard, 2006)

Since decades, the pathogenesis of the autoimmune diseases has been defined as a group of disorders with involvement of multi-organ or single-organ system. Highlight of these clinical features is enhanced activity of immune system against self with abnormalities in immune system components. The immune system normally defend the body against non-self-pathogens by using T and B lymphocytes as a tool, that direct antigen receptors and innate immune cells activated with a signal from pathogen and damaged molecules. This defense against pathogens must occur without leading any harm to self. For this reason, self-reactive T and B cells are discriminated in the thymus and bone marrow with the process called 'negative selection'. Negative selection process however can be defected and self-reactive lymphocytes can go through the periphery but tolerance mechanism must keep these cells under control. If regulatory and effector component of immune system are broken, self-reactive T and B cells can activate and cause autoimmunity (Smilek and Clair, 2015).

### 1.4.1 Autoimmune diseases

In civilized countries, autoimmune diseases are the major cause of death and morbidity and this situation leads to spend lot of money for health care. The pathogenesis and key factors of autoimmunity have not been known strictly. In order to enlighten these factors, important questions such as origin of autoantibodies, and their association with autoantigens must be solved (Gershwin and Shoenfeld, 2000).

When autoimmune diseases are classified, there are two branches of autoimmune disorders with immunity against self-antigens and without detectable anti-self response. Rheumatoid arthritis (RA), systemic lupus erythematosus, multiple sclerosis, type-1 diabetes, and celiac disease belong to the autoimmune diseases group of relation with autoantibody production and rarely reactive T cells. Other group includes psoriasis, inflammatory bowel disease, or ankylosing spondylitis; adaptive immune system is effective their pathogenesis (Smilek and Clair, 2015).

The etiology of many autoimmune disorders is unknown. On the other hand, microbial infection can be the trigger or initiation factor for autoimmunity induction.

There are several mechanisms to clarify this relation, including molecular mimicry, bystander activation and tissue damage leading to initiation of autoimmunity. In molecular mimicry, a microbial antigen or epitope mimics a self-antigen/epitope which T cells can be re-stimulated with endogenous self-antigens following microbial infection and so T cells can target cells or tissues expressing self antigens resulting in tissue damage. Bystander activation process can be occurred via random induction of potentially self-reactive T cells causing tissue damage. Tissue damage leading to initiation of autoimmunity mechanism is the result of microbial infection via the release of endogenous self-antigens that can be presented by APCs (antigen presenting cells) resulting in activation of self-reactive T cells (Shoenfeld et al., 2015)

### 1.4.2 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that can cause inflammation affecting multiple tissues and organs including skin, joint and kidney (Sherer et al., 2004). Variable clinical features of SLE are correlated with the complexity of genetic, hormonal and environment factors and diversity of autoantibodies (Lisnevskaia et al., 2014). Primary physiological mechanisms of SLE are polyclonal B-cell activation and autoantibody production against cytoplasmic, cell surface and nuclear antigens (Marchini et al., 2013)

Measurement of SLE disease activity is the problem for scientists and physicians for treating patients. Nowadays, there have been some valid and sensitive indices such as BILAG (British Isles Lupus Assessment Group), ECLAM (European Consensus Lupus Activity Measurement), SLAM (Systemic Lupus Activity Measure), SLEDAI (Systemic Lupus Erythematosus Disease Activity Index), and LAI (Lupus Activity Index). Until antinuclear antibody (ANA) was introduced, there is no valid test for diagnosis. Common clinical symptoms are malaise, over-whelming fatigue, fever and weight loss. Even, there are some similarities in some organ manifestations including skin diseases and arthritis, other systems or organs can be present together with other features (Tsokos et al., 2007).

It is a common fact that a number of environmental molecules or factors of SLE can corrupt autoreactivity by changing epigenetic mechanism. Generally, epigenetic mechanisms are DNA methylation and histone modifications which include

methylation of cytosine base of the DNA and deacetylation, ubiquination and trimethylation of histone tails, in order. Those epigenetic mechanisms can block gene transcriptions with numerous silencing of the expression of certain unnecessary and harmful genes. Beside epigenetics, there are numerous environmental factor that can create conditions that lead patients to SLE such as ultraviolet light, Vitamin-D deficiency, smoking, alcohol, occupationally and non-occupationally-related chemicals (Mak and Tay, 2014).

Although hormonal, environmental, genetic and epigenetic factors are considered to cause SLE, pathogenesis of this disorder needs to be clarified a little more. Normally, mature B cells produce antibodies to protect cells against exogenous pathogens. Auto-reactive B and T cells are generally eliminated from circulatory system during a negative selection step in their maturation phase. SLE is thought as a B cell disease because many lupus patients have high ratio of reactive autoantibodies against nuclear antigens related to their targets and to complement factors, which form circulating immune complexes (CICs). CIC accumulation in target organs and tissues induce and trigger an inflammatory process resulting in symptoms of SLE. SLE activity is correlated with ratio of autoantibodies, especially IgG and anti-dsDNA. Production of these autoantibodies in high level in SLE patients is the result of many abnormal immunological events in both adaptive and innate immune system components. Genetic and environmental factors could affect the loss of tolerance of T and B cells (Dema, 2014).

### 1.4.3 Systemic Scleroderma

Systemic Scleroderma (Sclerosis-SSc) is a chronic autoimmune disorder effecting multiple systems and organs, characterized with microangiopathy and fibrosis of the internal organs and skin. There are two important subtypes of SSc such as SSc with limited cutaneous involvement and SSc with diffuse cutaneous involvement (Lambova, 2014).

Scleroderma is an autoimmune and also a vascular disease with injury of blood vessels resulting in abnormal blood vessel function and fibrotic or scarring disorder involving multiple systems. Therefore, thickness and hardness increase with accumulation of collagen and several proteins in skin, also lungs, heart and gastrointestinal tract. The correlation of vascular assets of SSc and fibrotic assets of

SSc with autoimmune and inflammatory events make the treatment and understanding of pathogenesis difficult (Thompson and Ensz, 2003).

SSc can be also characterized with Raynaud's phenomenon and other vascular abnormalities, immune system activation seen by SSc-specific autoantibodies, in particular anti-centromere proteins (CENP), anti-topoisomerase I (topo I, also called Scl-70) and anti-RNA polymerase III (RNAP) and disorder in fibroblast function leading skin thickness and fibrosis. SSc is quite life effecting disease seen in especially women with age of around 45, causing social disability, high cost and increased mortality with lung disease and pulmonary arterial hypertension (Hudson and Fritzler, 2014)

### 1.4.4 Major Clinical Parameters and Autoantibodies for Systemic Scleroderma and Systemic Lupus Erythematosus

#### 1.4.4.1 Pleuritis

Pleuritis also known as pleurisy defines pleura inflammation with surroundings of lungs. Many factors can play role in pleuritis but viral infection that spreads from the lungs to plural cavity is most common. Inflamed pleural layers touch each other in breathe process so pleuritic chest pain can occur. Also, in systemic lupus erythematosus pleuritis is one of symptoms (Husain, 2012)

#### 1.4.4.2 Pericarditis

Pericarditis is the inflammation of the pericardium characterized with chest pain. There are several factors for pericarditis but mainly autoimmune (SLE) and microbial infection. Based on duration and time of presentation, there are two forms of pericarditis; acute and chronic form (Gorbach et al., 2004).

#### 1.4.4.3 Nephritis

Nephritis is the inflammation of the kidneys involved in glomeruli, tubules or interstitial tissue around the glomeruli and tubules. If this inflammation is caused by systemic lupus erythematosus, it is called as lupus nephritis (Schiffer et al., 2015).

#### 1.4.4.4 Pulmonary Hypertension

Pulmonary hypertension indicates different conditions generally occur with increases in pulmonary arterial pressure. Basically, pulmonary hypertension occurs with elevations of blood pressure exactly in pulmonary artery, pulmonary vein, or pulmonary capillaries and the symptoms are shortness of a breath, dizziness, fainting, and leg swelling (Hill and Farber, 2008).

#### 1.4.4.5 Antinuclear Antibody (ANA)

Antinuclear antibody (ANA) is a type of autoantibody produced against cellular proteins and nuclear acids. Although the name indicates produced against 'nuclear' antigen, ANAs are partially or completely located on cell cytoplasm. The common produced ANAs react with DNA-protein or RNA-protein complexes. ANAs are produced with high titer and have high affinity IgG antibodies which are produced with T-cell dependent process induced by the autoantigen. Antinuclear antibody testing is the best indicator for diagnosis of SLE and is the most-ordered tests in clinics (Petrelli, 2008).

#### 1.4.4.6 Anti-double stranded DNA antibody (anti-dsDNA)

Anti-double stranded DNA (anti-dsDNA ) antibody is the autoantibodies directed to double stranded DNA structure. Anti-dsDNA antibody can be produced in 40% to 80% of people who have SLE. These autoantibodies provide monitoring the treatment of lupus besides diagnosis of SLE since anti-dsDNA level can be increase or decrease with the activity of disorder. Also, people with anti-dsDNA antibody have increased risk of having kidney lupus disorder or vasculitis with their disease (Thomas Jr., 2014).

#### 1.4.4.7 Anti-RO/SSA antibody

Anti-RO/SSA autoantibodies are the most common anti-extractable nuclear antigens and are generally related with SLE and Sjögren's syndrome. The double name of anti-RO/SSA autoantibodies came from that two different research groups has defined it simultaneously (Lahita et al., 2010). These autoantibodies are related with common type of human RNA in a complex as hYRNA. Anti-RO antibody positivity is the indicator of a number of dermatologic conditions. Also, anti-RO/SSA antibodies are related with hematologic abnormalities, especially thrombocytopenia (Lotze and Thomson, 2011).

#### 1.4.4.8 Anti-ribonucleoprotein antibody (anti-RNP)

Anti-ribonucleoprotein (anti-RNP) antibody is related to antigens in small nuclear ribonucleoprotein particles. Anti-RNP antibodies can be found in rheumatoid arthritis, Sjögren's syndrome, systemic sclerosis (SSc) and SLE. Diagnostically, anti-RNP antibody test is not considered as suitable for diagnosis of SLE because of low sensitivity and moderate specificity (Wallace and Hahn, 2012).

#### 1.4.4.9 Anti-Smith antibody (anti-Sm)

Anti-Smith (anti-Sm) antibodies can react to several antigens in small ribonucleoprotein particles that have a role in splicing of precursor messenger RNA. There are variable methods for detection of anti-Sm antibodies such as immunodiffusion, ELISA, counterimmunoelectrophoresis and multiplex bead assays. Although about 30% of patients who have SLE are positive for anti-Sm antibodies, these autoantibodies have important diagnostic value since they can be found in other rheumatic disease such as scleroderma, rheumatoid arthritis (Wallace and Hahn, 2012).

#### 1.4.4.10 Anti-ribosomal protein P antibody

Anti-ribosomal protein P autoantibodies produced specifically in SLE disease. Three highly conserved ribosomal phosphoproteins are the molecular target of these autoantibodies. Indicated prevalence of anti-P antibodies among SLE patients can vary from 6% to 46%. In addition to association between SLE and anti-P autoantibody, some clinical association between hepatitis, nephritis and anti-P antibody have also been reported (Haddouk et al., 2009).

#### 1.4.4.11 Anti-centromere antibody

This antibody is produced against centromere protein that is a tool in diagnosis of CREST syndrome. In spite of abundance in CREST syndrome, about 10% of scleroderma patients can have these autoantibodies. These antibodies are present early in the course of disease and are especially cutaneous involvement (Wild, 2005).

#### 1.4.4.12 Anti-Scl-70 antibody

Anti-Scl-70 (also known as anti-topoisomerase-I) antibody specifically related with diffuse cutaneous disease. Anti-Scl-70 positivity indicates higher prevalence of arthritis, tendon fraction rubs, severe pulmonary fibrosis, severe heart disease and SSc. Also, patients with anti-Scl-70 antibody have higher risk for microvasculature, digital tip ulcers, gangrene and digital tuft resorption. The target molecule for anti-Scl-70 is topoisomerase antigen which is obtained from a 70 kD extractable immunoreactive fragment (Varga et al., 2012).

#### 1.4.4.13 Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a common systemic autoimmune disorder manifested with thrombophilic situation and by obstetrical symptoms. The association between antiphospholipid syndrome and autoimmune disorder (especially in SLE) is primarily close. Up to 15% of SLE patients can have APS and also 50% of the patients with APS are SLE patients. The female ratio according to male is 2:1 (World Health Organization, 2006). The presence of antiphospholipid antibodies (aPL) is the primer criteria for the diagnosis of APS and other two typical autoantibodies are the lupus anti-coagulant (LA) and anti-cardiolipin antibodies (aCL). aPL autoantibodies are a specific group of antibodies produced against phospholipid binding proteins. aCL are measured with ELISA. The LA antibodies is a misnomer that it might be produced in patients without lupus and it is actually prothrombotic autoantibody (Mason and Pusey, 2007).

## 1.5 Aim of the Study

Anti-HLA antibodies are related with acute and chronic rejection in all solid transplant recipients. According to newest theory, there is a significant similarity between alloimmune response in the post-transplant period and the development of autoimmunity against self-antigens. Although, the role anti-HLA antibodies in the pathogenesis of autoimmune disorders remain unclear, some theories have been suggested. One of those theories is antibody production against self-antigens that caused by microchimerism. However, there is few studies that have been investigated the role of anti-HLA antibodies in autoimmune disorders in the literature.

In our study, anti-HLA antibodies presence was investigated in patients with SLE, SSc which were selected as a model autoimmune disorder, and also in healthy

controls. Also, demographic and clinical data of individuals were studied and tried to establish correlation between clinical data and anti-HLA antibody presence. Also, anti-HLA antibody identification experiment was maintained to find a relationship between specific anti-HLA antibody type and autoimmune disorder.

# 2. MATERIALS & METHODS

## 2.1 Materials

### 2.1.1 Patients and Controls

We included 77 SLE and 46 SSc patients who were being treated at the Trakya University Medical School, Department of Rheumatology. Also, 53 healthy controls were included to this study. All patients and controls were signed written informed consent form and were given a copy of same form. The study was approved by the local ethics committee at Trakya University Medical Faculty and it conformed to the provisions of the 1995 Declaration of Helsinki (as revised in Edinburgh 2000).

### 2.1.2 Commercial Kits

All commercial kits that were used in this study are listed with their supplier companies in the table below.

**Table 2.1** Commercial kits used in this study.

| *Commercial Kit* | *Supplier Company* |
|---|---|
| Life Screen Deluxe Anti-HLA Class I and Class II Screening kit | Lifecodes |
| Life Screen Deluxe Anti-HLA Class I and Class II identification kit | Lifecodes |

### 2.1.3 Equipment

Laboratory equipments which were used in this study are shown in Table 2.2 with their suppliers.

**Table 2.2** Laboratory equipment used in this study.

| *Equipment* | *Supplier Company* |
|---|---|
| Centrifuge | Thermo Scientific |
| Pipettes | 10 µl, 20 µl, , 200 µl, 1000 µl Thermoand 100 µl, 1000 µl Ependorf |
| Vortex | Biosan |
| Vacuuum Pump | Millipore |
| Serum Seperator tubes | Beacon Diagnostics |
| Fridge (-20$^{o}$ C) | Arcelik |
| LuminexFluoroanalyzer | Lifecodes |
| UPS | Imtek |
| Printer | Lexmark |
| Filtered plate (96 wells) | Millipore |
| Vacuum Filter | Millipore |
| Eppendorf 1.5 ml tubes | ATQ |
| Falcons (15 ml, 50 ml) | ATQ |
| Pipette tips (10 µl, 20 µl, , 100 µl, 200 µl, 1000 µl) | ATQ |
| Sterile Gloves | Bend |
| Minispin Centrifuge | Hangzhou |
| Fridge (-80$^{o}$ C) | Haier |
| Sheath | Beacon Diagnostics |

### 2.1.4 Softwares

All softwares which were used in this study for data analysis are shown in Table 2.2 with their suppliers.

**Table 2.3** Softwares used in this study.

| *Software* | *Supplier Company* |
|---|---|
| Quicktype Match 2.6 xMAP software | Lifecodes |
| SPSS | IBM |

## 2.2 Methods

### 2.2.1 Patient Selection

In this study, we included 77 SLE and 46 SSc patients who were being treated at the Trakya University Medical School, Department of Rheumatology. 1997 American college of Rheumatology (ACR) criteria were used for the diagnosis of SLE. Patients who were suitable at least four criteria of 1997 American college of Rheumatology were counted to be diagnosed as SLE. SSc patients were also diagnosed according to ACR criteria. The inclusion criteria of SLE and SSc patients were being older than age of 30, being fulfilled according to ACR criteria and patients must be classified according to European Consensus Lupus Activity Measurement index. The exclusion criteria of SLE and SSc patients were being younger than age of 30, using anti-CD20 therapy, receiving transplants and having a diagnosis of another autoimmune disease. The inclusion criteria of healthy controls were older than age of 30 and not being diagnosed as an autoimmunity disease.

### 2.2.2 Collecting of Demographic and Clinical Data of Patients and Controls

All demographic and clinical data of both patient and controls were kindly provided from Trakya University Medical Faculty hospital files by Rheumatology Department. Also, all patients were fully examined physically by same department. Laboratory data taken from Rheumatology department included whole blood counts, biochemical tests, antinuclear antibody (ANA), anti-double-stranded DNA (anti-dsDNA) and extractable nuclear antigen (ENA). Moreover, SLE Disease Activity Index (SLEDAI) of the SLE patients was kindly requested from same department and it has been calculated for each SLE patient at the time of initial diagnosis.

### 2.2.3 Serum Separation

Serum separation process was started with blood taken procedure. Two milliliters of blood were taken from both patients and controls with sterile injector and were drawn into serum separator tubes. Tubes were centrifuged at 2000 x g for 10 minutes. After centrifugation process, serum which was the upper part of three layer of centrifuged blood was taken with micropipette into 2 ml Eppendorf tubes. Then, Eppendorf tubes were labelled, numbered and stored at -20$^{o}$ C until Anti-HLA

Antibody Screening studies begin. After experiment was done, sample serum was stored at -80$^{o}$C permanently.

### 2.2.4 Anti-HLA Antibody Screening

Anti-HLA antibody screening studies were carried by Lifocodes anti-HLA Class I and Class II Screening kits (Lifecodes, Immucor, Norcorss, GA, USA). First of all, 300 µl sterile distilled water were added into wells of Millipore filtered plates in order to make wells wet. After 2-5 minutes waiting period, plate was vacuumed at the vacuum pump briefly. At the same time, Lifescreen deluxe beads were centrifuged briefly at 1500 x g for 15-30 seconds and then mixed with vortex. After that, mix components which were given at Table 2.4 were added each wells respectively.

**Table 2.4** Screening protocol mix components.

| *Component* | *Amount* |
|---|---|
| Wash Buffer | 40 µl |
| Serum Sample | 12,5 µl |
| Class I-II Beads | 5 ul |

Plate was sealed and incubated for 30 min at room temperature avoiding light in shaker. During incubation, conjugant mix was prepared for every sample with amount given at Table 2.5 and was stored in the dark until usage.

**Table 2.5** Conjugant mix components.

| *Component* | *Amount* |
|---|---|
| Wash Buffer | 45 µl |
| Conjugant | 5 µl |

After incubation of samples, 100 µl wash buffer was added into wells, mixed and vacuumed at the vacuum pump briefly. Then, 300 µl wash buffer was added into wells, mixed and vacuumed again and this step was repeated three times. 50 µl of prepared conjugant mix were then added into wells. Plate was sealed incubated for 30 min at room temperature avoiding light in shaker. At the end of the incubation,

150 µl wash buffer was also added into wells and mixed. Plate was loaded into Luminex after necessary wash, calibration was done and all samples were analyzed at xMAP software (Luminex Co, Hertogenbosch, The Netherlands).

### 2.2.6 Anti-HLA antibody identification

Anti-HLA antibody identification studies were carried by Lifecodes anti-HLA Class I and Class II Identification kits (Lifecodes, Immucor, Norcorss, GA, USA). For this experiment, 17 anti-HLA positive SSc patients, 37 anti-HLA positive SLE patients and 4 anti-HLA positive healthy controls were included. First of all, 300 µl sterile distilled water was added into wells of Millipore filtered plates in order to make wells wet. After 2-5 minutes waiting period, plate was vacuumed at the vacuum pump briefly. At the same time, Lifescreen deluxe beads were centrifuged briefly at 1500 x g for 15-30 seconds and then mixed with vortex. After that, mix components which were given at Table 2.4 were added each wells respectively.

**Table 2.6** Identification protocol mix components.

| *Component* | *Amount* |
|---|---|
| Wash Buffer | 40 µl |
| Serum Sample | 12,5 µl |
| Class I-II Beads | 5 ul |

Plate was sealed and incubated for 30 min at room temperature avoiding light in shaker. During incubation, conjugant mix was prepared for every sample with amount given at Table 2.5 and was stored in the dark until usage.

**Table 2.7** Conjugant mix components.

| *Component* | *Amount* |
|---|---|
| Wash Buffer | 45 µl |
| Conjugant | 5 µl |

After incubation of samples, 100 µl wash buffer was added into wells, mixed and vacuumed at the vacuum pump briefly. Then, 300 µl wash buffer was added into wells, mixed and vacuumed again and this step was repeated three times. 50 µl of

prepared conjugant mix were then added into wells. Plate was sealed incubated for 30 min at room temperature avoiding light in shaker. At the end of the incubation, 150 µl wash buffer was also added into wells and mixed. Plate was loaded into Luminex after necessary wash, calibration was done and all samples were analyzed at xMAP software (Luminex Co, Hertogenbosch, The Netherlands).

### 2.2.5 Analyze of the samples and statistical analysis

Analysis of the samples for anti-HLA antibodies screening and identification was done with xMAP software analysis tool. Samples with high control values according to xMAP software were investigated twice in case eliminating false negative and false positive results. Each sample was run twice with positive control serum, negative control serum and non-template control (NTC). A typical analysis image gained with screenshot in this software was given in Figure 2.1.

LM-HLA Class I/II Deluxe Screen Results

Save New Results

Test Date: 04.06.2014 Report By: Admin
Lot Number: 3000875 3000855-I Expiration Date: 15.04.2014
Assignment Cutoffs: 1, 1 Supplemental Assignment Cutoffs: 2, 2
Adj Val Cutoffs: 0.00, 0.00 Supplemental Adj Val Cutoffs: 0.00, 0.00

| | | | Class I Results | | | | | | | | Class II Results | | | | | | Pos Ctl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CI-01 | CI-02 | CI-03 | CI-04 | CI-05 | CI-06 | CI-07 | Assign | CII-01 | CII-02 | CII-03 | CII-04 | CII-05 | Assign | CONs |
| Sample ID | 04062014-1 | MFI | 9150 | 9722 | 13485 | 11409 | 11250 | 10355 | 14820 | | 14952 | 16381.5 | 9759 | 17931.5 | 3398 | | 19873 |
| Patient | POZT KONT | Adj 1 | 80.15 | 86.02 | 120.42 | 101.51 | 100.06 | 91.54 | 132.7 | | 133.48 | 146.02 | 84.72 | 161.5 | 26.34 | | 108.5 |
| Draw Date | | Adj 2 | 40.21 | 43.73 | 61.64 | 51.6 | 50.85 | 46.02 | 68.1 | | 67.77 | 74.1 | 42.31 | 83.01 | 11.84 | | 208 |
| | | Adj 3 | 35.7 | 38.68 | 54.54 | 45.74 | 45.14 | 41.03 | 60.12 | | 59.89 | 65.73 | 37.81 | 73.44 | 11.01 | | 237 |
| | | Score | 3 | 3 | 3 | 3 | 3 | 3 | 3 | Positive | 3 | 3 | 3 | 3 | 3 | Positive | |
| Sample ID | 04062014-2 | MFI | 136 | 113 | 61 | 90 | 75.5 | 118 | 60 | | 157.5 | 157 | 141 | 35 | 148 | | 18890 |
| Patient | NEGT KONT | Adj 1 | -3.18 | -2.75 | -3.42 | -2.98 | -3.07 | -3.03 | -3.44 | | -3.17 | -3.81 | -4.18 | -3.51 | -3.89 | | 136 |
| Draw Date | | Adj 2 | -2.45 | -1.91 | -2.6 | -2.37 | -2.5 | -2.61 | -2.56 | | -2.58 | -3.13 | -3.23 | -2.85 | -3.06 | | 102.5 |
| | | Adj 3 | -1.81 | -1.42 | -1.86 | -1.67 | -1.72 | -1.71 | -1.92 | | -1.92 | -2.12 | -2.22 | -1.94 | -2.13 | | 123 |
| | | Score | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Negative | 0 | 0 | 0 | 0 | 0 | Negative | |
| Sample ID | 04062014-3 | MFI | 153 | 100.5 | 127 | 103 | 84.5 | 108 | 108 | | 164 | 199 | 174.5 | 104 | 171 | | 12833 |
| Patient | | Adj 1 | -3.32 | -3.02 | -3.16 | -3.07 | -3.15 | -3.29 | -3.28 | | -3.41 | -3.85 | -4.25 | -3.19 | -4.02 | | 179 |
| Draw Date | | Adj 2 | -2.13 | -1.93 | -1.83 | -2.14 | -2.33 | -2.6 | -1.99 | | -2.36 | -2.52 | -2.73 | -2.08 | -2.66 | | 93 |
| | | Adj 3 | -2.48 | -2.05 | -2 | -2.11 | -2.09 | -2.36 | -2.1 | | -2.73 | -2.83 | -2.87 | -1.93 | -2.84 | | 351 |
| | | Score | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Negative | 0 | 0 | 0 | 0 | 0 | Negative | |
| Sample ID | 04062014-4 | MFI | 2060 | 867 | 1207.5 | 1633.5 | 1735.5 | 2023 | 1460.5 | | 693 | 613.5 | 852 | 617.5 | 1200 | | 16335.5 |
| Patient | | Adj 1 | -3.64 | -3.36 | -3.55 | -3.22 | -3.17 | -3.37 | -3.5 | | -4.14 | -4.8 | -5 | -3.61 | -4.67 | | 3829 |
| Draw Date | | Adj 2 | -3.19 | -2.76 | -2.85 | -2.79 | -2.74 | -3.18 | -2.73 | | -3.92 | -4.48 | -4.36 | -3.02 | -4.16 | | 3514.5 |
| | | Adj 3 | -2.43 | -2.14 | -2.08 | -2.02 | -1.93 | -2.2 | -2.07 | | -3.04 | -3.25 | -3.17 | -2.08 | -3.05 | | 4303 |
| | | Score | 0 | 0 | 0 | 0 | 0 | 0 | 0 | Negative | 0 | 0 | 0 | 0 | 0 | Negative | |
| Sample ID | 04062014-5 | MFI | 516.5 | 88.5 | 950 | 293 | 507 | 318 | 553 | | 285.5 | 425.5 | 374 | 145 | 215 | | 16211 |

**Figure 2.1** An image of a typical anti-HLA antibodies screening analysis in xMAP software.

In Figure 2.1, mainly there were two different sections that represent Class I and Class II screening results. Each section contains seven different bead results that obtain fluorometric measurement from beads. Cl-01 and Cll-01 beads represent IgG measurement and other six beads from each section represent IgM results. According to adjustment of MFI (mean fluorescence intensity) from each bead, software gave a

score changing from 0 to 3. 0 point means negativity and 3 point means positivity. Also, 1 and 2 points define low positivity. With those analysis rules, all patients and healthy controls were analyzed and their positivity and negativity for anti-HLA antibodies were determined.

An example image of anti-HLA identification analysis is demonstrated in Figure 2.2. In the first step of anti-HLA antibodies identification analysis, MFI cutoff value of the beads was adjusted into 500-1000 according to adjusted value of beads considering raw MFI. Beads which have raw MFI above cutoff value were considered as positive, beads under the cutoff value were considered as negative. Then, software selected reactive antibodies for each HLA subtype from selective epitopes in each positive bead. Thus, reactive antibodies and PRA ratios was determined with this analysis.

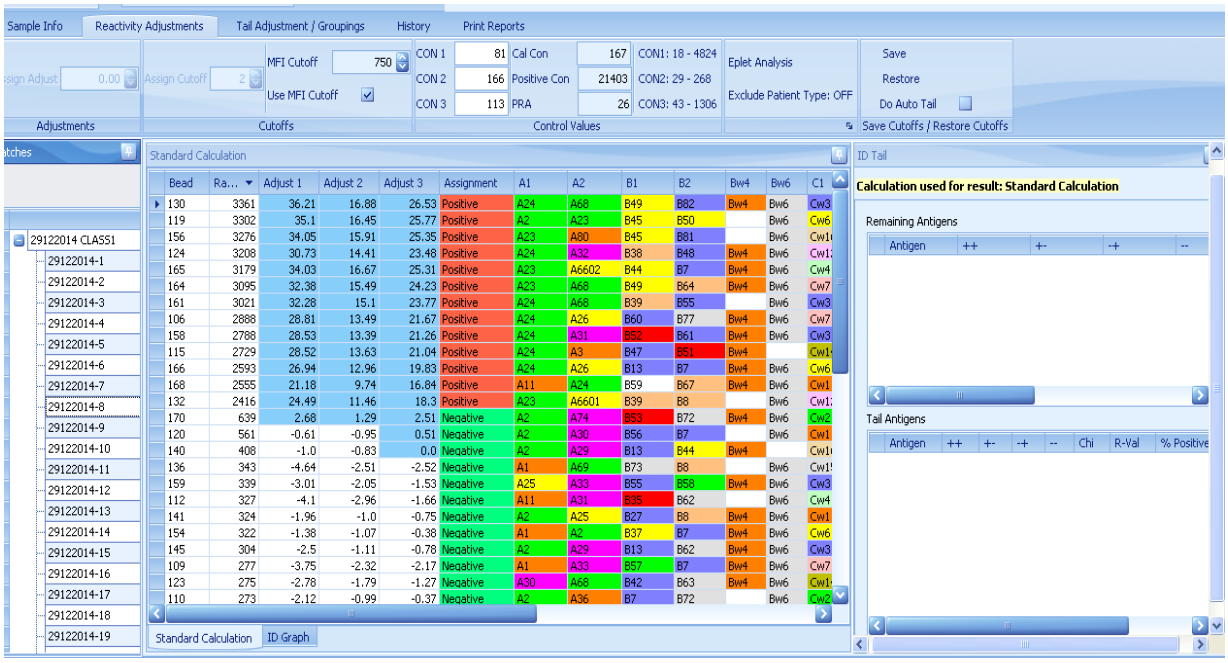



**Figure 2.2** An image of a typical anti-HLA antibodies identification analysis in xMAP software.

Reactive antibodies positive beads above MFI cutoff value were selected according to CREGs groups in Table 2.8. Then, class I antibodies were classified for each SLE and SSc patients and controls according to CREGs and frequency of CREGs were determined. Anti-HLA class-II antibodies were classified without CREGs for all groups together as there is no known CREGs classification for Class II antibodies.

**Table 2.8** CREGs list of reactive antibodies against HLA antigens shared same CREG (adapted from Duqesnoy et al., 2003)

| CREG name | Reactive antibodies against HLA antigens shared same CREG |
|---|---|
| **INTL3** | A11+Cw3+Bw6 |
| **INTL2** | A32+A23+A24+A9+Bw4 |
| **5C** | B51+B52+B35+B53+B62+B63+B71+B72+B75+B76+B77+B57+B58+B18 |
| **28C** | B35+B18+B45+B50+B62+B75+B76+B71+B72+B46+B41+B60+B61+B7+B42+B54+B55+B56+B67+B39+B8+B64+B65 |
| **2C1** | A2+A28+A68+A69 |
| **B4** | B51+B52+B53+B44+B49+B57+B58+B63+B77+B37+B13+B47+B27+B38+B59 |
| **22C** | B7+B22+B54+B55+B56+B27+B17+B57+B58+B46 |
| **2C2** | A2+A28+A68+A69+A9+A23+A24 |
| **12C** | B44+B45+B49+B50+B13+B41+B47+B60+B37+B61 |
| **8C** | B8+B14+B64+B65+B18+B51+B38+B39 |
| **21C** | B49+B50+B51+B52+B35+B62+B63+B75+B76+B77 |
| **1C2** | A1+A36+A9+A23+A24+A11 |
| **27C** | B7+B27+B13+B40+B60+B61+B47 |
| **1C3** | A25+A26+A34+A66+A11+A1+A36+A29 |
| **1C1** | A1+A36+A3+A11 |
| **B6** | B35+B18+B45+B50+B62+B75+B76+B71+B72+B46+B41+B60+B61+B7+B42+B54+B55+B56+B67+B39+B8+B64+B65 |

For statistical analysis, categorical data was evaluated with chi-square test, and when needed Fisher's exact test was used. The distribution of continuous data was normal, so, unpaired t-test was used for comparison. Coefficient Interval (CI) was 95% and $p<.05$ value was accepted as significant unless otherwise stated.

## 3. RESULTS

### 3.1 Demographic Data of Patients and Controls

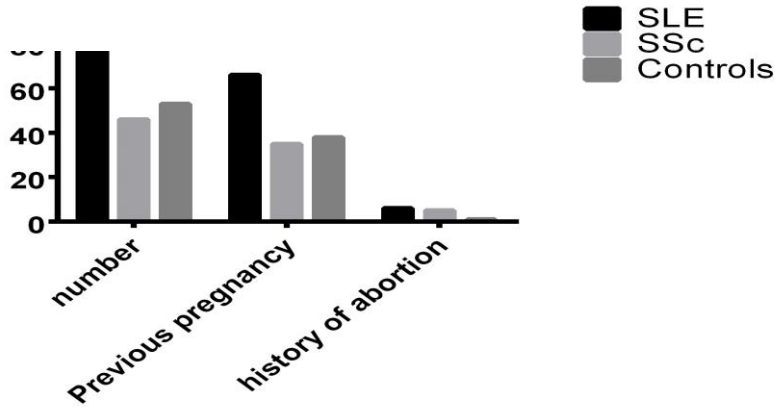
All demographic data of patients and control were kindly provided from hospital files of Trakya University by Rheumatology Department with permission. Four different categories that all patients were evaluated as a demographic data such as, age, previous pregnancy, history of abortion were indicated. Demographic data of SLE and SSc patients and healthy control were given separately for different categories in Table 3.1 and also graphics were given in Figure 3.1.

**Table 3.1** Demographic data of SLE, SSc and healthy controls.

| | **SLE** | **SSc** | **Controls** |
|---|---|---|---|
| **Number (F/M)** | 77 (73/4) | 46 (44/2) | 53 |
| **Age (mean)** | 39.8 | 48,1 | 43,2 |
| **Previous Pregnancy n, (%)** | 66 (85,7) | 35 (76,1) | 38 (71,7) |
| **History of abortion n, (%)** | 6 (7,8) | 5 (10,9) | 1 (1,9) |

F, Female; M, Male; SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; %, ratio of patient into selected groups.

**a.**



**b.**

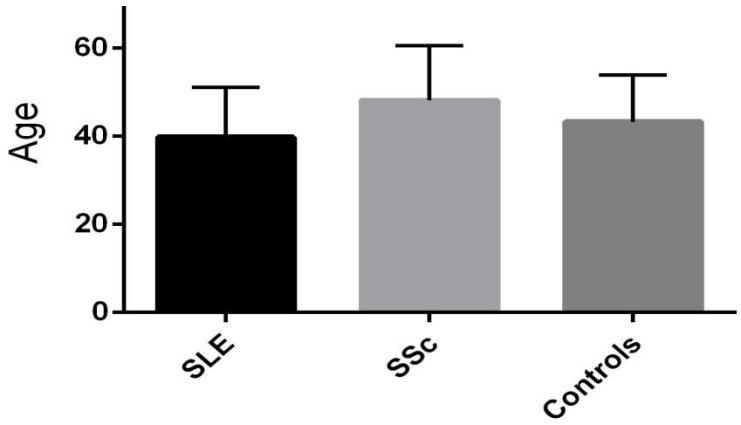



**Figure 3.1.** Demographic data of the SLE, SSc patients and healthy controls. All demographic data of patients and control were kindly provided from hospital files of Trakya University by Rheumatology Department with their permission. Demographic data was collected from informed consent form of the subjects. Bar graphs of data were drawn with GraphPad Prizm software. **a.** number of patients who had previous pregnancies and history of abortion of were used for systemic lupus erythematosus patient (black bar),systemic sclerosis (scleroderma) patients (light grey bar) and healthy control group(dark grey). **b.** age mean with error values for systemic lupus erythematosus patient (black bar),systemic sclerosis (scleroderma) patients (light grey bar) and healthy control group(dark grey) was used.SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; controls, healthy control group.

According to both Table 3.1 and Figure 3.1, number of female SLE and SSc patients was higher than number male patients. Also, number of SLE and SSc patients who had an abortion history was also slightly higher than control patient with no abortion history. Pregnancy rate was slightly elevated on SLE group rather than SSc and control group.

### 3.2 Clinical Data of Patients and Controls

Clinical features of SLE and SSc patients kindly requested from hospital files of Trakya University by Rheumatology Department. Involved organs, administered therapies and laboratory findings were recorded. Complete physical examination was done toall patients by Rheumatology Department. Laboratory data such as whole blood counts, biochemical tests, antinuclear antibody (ANA),anti-double-stranded DNA (anti-dsDNA) and extractable nuclear antigen (ENA) and those entire tests

were done by Trakya University Central Laboratory. Clinical data of SLE and SSc patients and healthy control were given separately for different categories in Table 3.2.

**Table 3.2** Clinical data of SLE and SSc patients and healthy control that were recorded from hospital files.

| | **SLE** | **SSc** | **Controls** |
|---|---|---|---|
| **Photosensitivity, n (%)** | 46 (59.7) | - | 53 |
| **Pleuritis, n (%)** | 16 (20.8) | 1 (2.2) | - |
| **Pericarditis, n (%)** | 10 (13) | 4 (8.7) | - |
| **Nephritis, n (%)** | 27 (35.1) | 1 (2.2) | - |
| **Arthritis, n (%)** | 59 (76.6) | 17 (37) | - |
| **CNS involvement, n (%)** | 15 (19.5) | - | - |
| **Pulmonary hypertension, n (%)** | 1 (1.3) | 16 (34.8) | - |
| **Pulmonary fibrosis, n (%)** | 1 (1.3) | 13 (28.3) | - |
| **Thrombocytopenia, n (%)** | 10 (13) | 1 (2.2) | - |
| **Hemolytic anemia, n (%)** | 5 (6.5) | - | - |
| **Antinuclear antibody, n (%)** | 76 (98.7) | 42 (91.3) | - |
| **Anti-dsDNA, n (%)** | 38 (49.4) | 1 (2.2) | - |
| **Anti-Ro, n (%)** | 32 (41.6) | 5 (10.9) | 1 (1.9) |
| **Anti-RNP, n (%)** | 23 (29.9) | 6 (46) | - |
| **Anti-Sm, n (%)** | 19 (24.7) | - | - |
| **Anti-Ribosomal P, n (%)** | 7 (9.1) | - | - |
| **Anti-centromere, n (%)** | 1 (1.3) | 20 (43.5) | - |
| **Anti-Scl-70, n (%)** | 0 | 14 (30.4) | - |
| **APS, n (%)** | 11 (14.3) | 2 (4.4) | - |
| **Digital ulcers, n (%)** | 0 | 18 (39.1) | - |
| **Active SLE (SLEDAI >6)** | 34 (44.2) | - | - |
| **Cyclophosphamide usage, n (%)** | 21 (27.3) | 4 (8.7) | - |

n, number of the patient; SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; %, ratio of patient into selected groups; CNS, central nervous system; anti-dsDNA, anti-double-stranded DNA antibodies; anti-RNP, anti-ribonucleoprotein antibodies; APS, antiphospholipid syndrome; anti-Sm, anti-Smith antibodies; SLEDAI, SLE Disease Activity Index.

As it is represented in Table 3.2, classic parameters of both SLE and SSc showed their characteristic frequency specific for each disease. Most of parameters were not investigated for healthy controls. Typical parameters for SLE patients such as photosensitivity, thrombocytopenia, anti-RNP positivity, anti-Ro positivity, Anti-nuclear antibody (ANA) etc. had slightly higher frequency than SSc patients. On the other hand, SSc parameters frequency such as digital ulcers, anti-centromere, pulmonary hypertension, and pulmonary fibrosis were higher in SSc patients.

### 3.3 Anti-HLA antibody screening

For understanding the role of anti-HLA antibodies in SLE and SSc pathogenesis, anti-HLA antibodies presence analysis was done. Anti-HLA antibody screening was maintained with panel reactive antibody testing that was based on bead specific florescence measurement technique. All SLE and SSc patients and healthy controls serum was measured with xMAP technology in Luminex device. After analysis with xMAP software, software gave a score changing from 0 to 3. 0 point means negativity and 3 point means positivity with adjustment of MFI (mean fluorescence intensity) from each bead. Also, 1 and 2 points define low positivity. With those analysis rules, all patients and healthy controls were analyzed and their positivity and negativity for anti-HLA antibodies were determined. With integration of those data, anti-HLA antibody positivity of SLE, SSc patients and controls were determined. Low and high positivity was considered as a positivity concept which had 3, 2, 1 points in any bead; negativity was determined 0 points for all seven beads. The frequencies of anti-HLA antibodies positivity for SLE, SSc patients and healthy controls were given in Table 3.3. Also, frequencies of class I and II anti-HLA antibodies positivity for each group were given separately in Table 3.4. Also, graphics based on data in Table 3.3 and Table 3.4, percentage of patients and controls according to total number in anti-HLA antibodies positivity were given in Figure 3.2.

**Table 3.3** Frequencies of anti-HLA antibodies positivity for SLE, SSc patients and healthy controls.

| | SLE | SSc | Controls |
|---|---|---|---|
| **Anti-HLA antibody, n (%)** | 38 (49.3) | 24 (52.1) | 4 (7.5) |

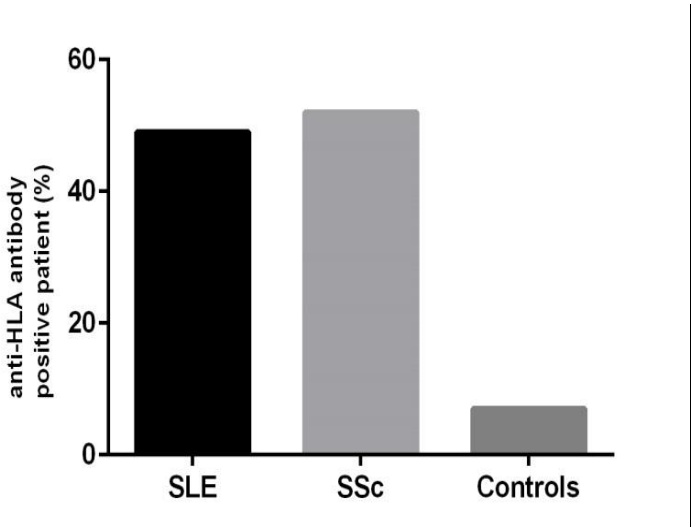
n, number of the patient; SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; %, ratio of patient into selected groups

**Table 3.4** Frequencies of class I and II anti-HLA antibodies positivity for SLE, SSc patients and healthy controls.

| | SLE | SSc | Controls |
|---|---|---|---|
| **Anti-HLA class I Antibody, n (%)** | 21 (27.3) | 12 (26.1) | 1 (1.9) |
| **Anti-HLA class II Antibody, n (%)** | 32 (41,6) | 19 (41,3) | 3 (5,7) |

n, number of the patient; SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; %, ratio of patient into selected groups

**a.**



**b.**

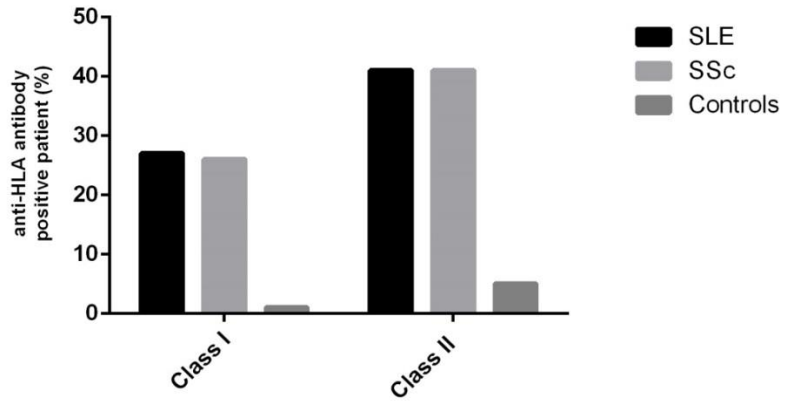



**Figure 3.2.** Frequency of anti-HLA antibody positivity in SLE and SSc patient are higher than healthy controls. Serum of individuals from SLE, SSc patients and healthy controls were studied with panel reactive antibody testing that was based on bead specific florescence measurement technique. After analysis in xMAP software, number of anti-HLA antibody positive patients and healthy controls were determined and percentage of positive patients for each group was evaluated. Bar graphs of data were drawn with GraphPad Prizm software. **a.** percentage of anti-HLA antibody positive patients for three different groups. **b.** percentage of anti-HLA antibody positive patients for both Class I and Class II were showed separately (all $P<0.001$).

As in Table 3.3 and Figure 3.2, SLE and SSc patients had significantly increased considering anti-HLA antibody both class I and class II comparison to healthy control groups (all $P<0.001$). When SLE and SSc patients were compared to each other, there was no significant difference. Analysis with xMAP software gave a score changing from 0 to 3. 0 point means negativity and 3 point means high positivity with adjustment of MFI (mean fluorescence intensity) from each bead. Also, 1 and 2 points define low positivity. According to this analysis, graphic based on number of patients and controls in low or high positivity of anti-HLA antibodies were given in Figure 3.3.

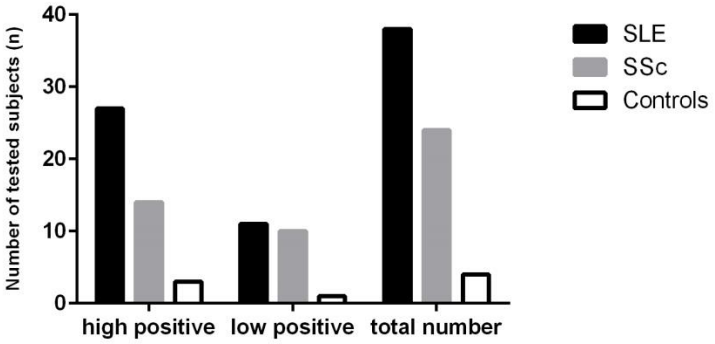



**Figure 3.3.** Among SLE and SSc patients, high positivity of anti-HLA antibodies are higher than low positivity. After working with serum of individuals, analysis was done with xMAP software according to manufacturer's instructions. xMAP software gave a score changing from 0 to 3. 0 point means negativity and 3 point means high positivity with adjustment of MFI (mean fluorescence intensity) from each bead. Also, 1 and 2 points define low positivity. Bar graphs of data were drawn with GraphPad Prizm software. SLE patients (black bar), SSc patients (grey bar) and healthy controls (white bar)

As it was shown in Figure 3.3, patients with high positivity for anti-HLA antibodies in both SLE and SSc group was found higher than low positivity individuals in same groups. However, in healthy controls there was no significant difference between low and high positivity for anti-HLA positivity because of lacking sufficient sample size.

### 3.4. Anti-HLA antibody identification

For the further analysis of anti-HLA antibodies in pathogenesis of SLE and SSc diseases, anti-HLA antibody identification experiment was done with patients who have positive anti-HLA antibody screening. For this reason, 17 SSc patients (6 of them both Class I and II positive, 4 of them Class-I positive, 7 of them Class-II positive), 37 SLE patients (16 of them both Class I and II positive, 4 of them Class-I positive, 13 of them Class-II positive), 4 healthy control (1 of them Class-I positive, 3 of them Class-II positive) were added to this experiment. In SLE patient group, 21 of 21 patients who were tested for class-I anti-HLA antibody positivity in identification experiment were found positive; 20 patients were found positive for class-II anti-HLA antibody and 12 patients were negative. In SSc group, all of 10 patient who were tested for class-I anti-HLA antibody positivity in identification experiment were found positive; 12 patients in total of 13 patients were found positive for class-II anti-HLA antibody and 1 patient was negative. In Table 3.5, arithmetic mean of PRA ratio for SLE and SSc patient groups in anti-HLA antibody

identification experiment were given. Also, column chart for arithmetic mean of PRA ratio for SLE and SSc patient groups in anti-HLA antibody was demonstrated in Figure 3.4. Healthy control group PRA ratio data was not given due to data insufficiency.

**Table 3.5** Arithmetic mean of PRA ratio for SLE and SSc patient groups in anti-HLA antibody identification experiment

| | **SLE** | **SSc** |
|---|---|---|
| **Anti-HLA class I Antibody PRA ratio (%)** | 41.8 | 27.4 |
| **Anti-HLA class II Antibody PRA ratio (%)** | 45.8 | 40.3 |

SLE, systemic lupus erythematosus patient; SSc, Systemic sclerosis (scleroderma) patients; %, PRA ratio

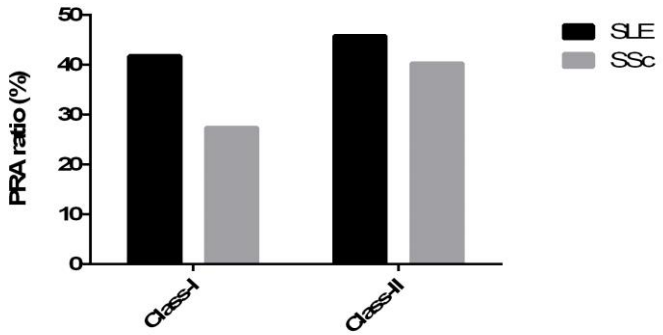
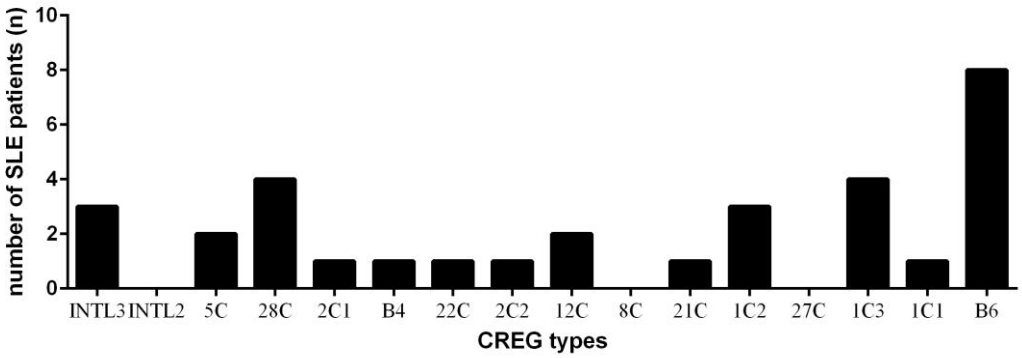


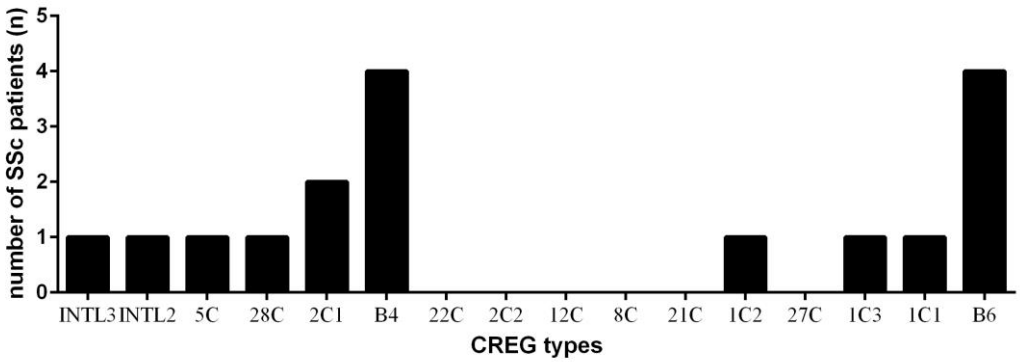

**Figure 3.4** PRA ratio of SLE patient group is higher than SSc patient group in Class-I anti-HLA antibody. Serum of individuals from anti-HLA antibody positive SLE, SSc patients and healthy controls were studied with anti-HLA antibody identification testing that was based on bead specific florescence measurement technique. In the first step of anti-HLA antibodies identification analysis, MFI cutoff value of the beads was adjusted into 500-1000 according to adjusted value of beads considering raw MFI. Beads which have raw MFI above cutoff value were considered as positive, beads under the cutoff value were considered as negative. Then, arithmetic mean value of PRA ratios belongs each specific group were determined. Bar graphs of data were drawn with GraphPad Prizm software.

As seen in Table 3.5 and Figure 3.4, anti-HLA class I antibody PRA ratio in SLE group was slightly increased according to SSc group. In addition, there was no

significant difference in anti-HLA class II antibody PRA ratio between SLE and SSc patient groups.

Besides PRA ratio analysis, CREG classification analysis was done in anti-HLA antibody identification study. For this step, reactive antibodies against HLA antigens in every positive SLE, SSc patients and healthy controls were classified according to their CREGs group as in Table 2.8. Totally, frequencies of all CREGs for all PRA positive SLE, SSc patients and healthy controls were demonstrated as a column chart in Figure 3.5. As seen in Figure 3.5, most common CREGs for class I anti-HLA antibodies against class I HLA antigens were B6 and B4.



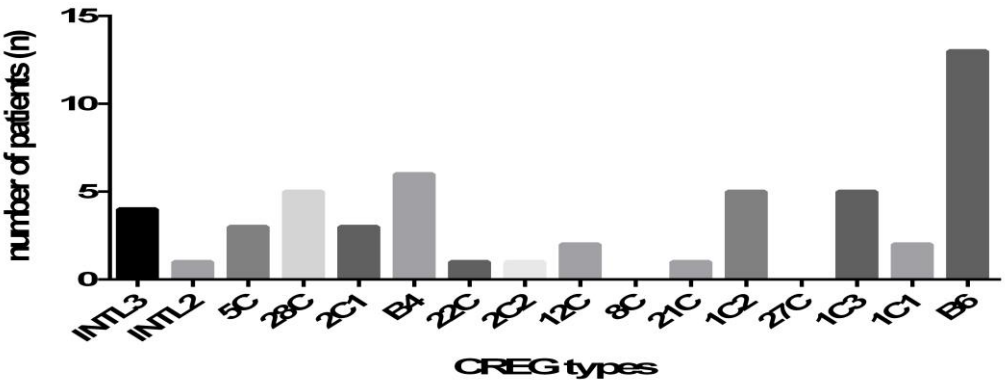



**Figure 3.5** Among CREG types, B6 and B4 have higher frequency in total anti-HLA antibody positive SLE, SSc patient and healthy controls. Reactive antibodies positive beads above MFI cutoff value were selected according to CREGs groups in Table 2.8. Then, class I antibodies were classified for each SLE and SSc patients and controls according to CREGs and frequency of CREGs were determined. Anti-HLA class-II antibodies were classified without CREGs for all groups together as there is no known CREGs classification for Class II antibodies. Bar graphs of data were drawn with GraphPad Prizm software.

For analysis of anti-HLA class-II antibody identification study, most common antibodies against HLA class-II antigens were determined. Accordingly, most common anti-HLA class-II antibodies were DQ7, DQ8, DQ9, DQ2; respectively.

## 4. DISCUSSION & CONCLUSION

In this study, the relationship between anti-HLA antibodies and autoimmunity were generally investigated. SLE and SSc were selected as a model autoimmune disorders and anti-HLA antibodies presence was compared among SLE, SSc patients and healthy controls. We evaluated that the frequencies of both class I and II anti-HLA antibodies were increased in both diseases when compared to controls. Also, anti-HLA antibodies frequencies were similar in SLE and SSc patients. Our findings may help to establish the relationship between autoimmune pathogenesis and the anti-HLA antibodies.

Generally, anti-HLA antibodies can cause acute and chronic rejection in solid transplantation so early allograft rejection happens (Zeevi et al, 2006). According to new approach, it has been indicated that there may be a similarity between alloimmune responses occur during transplantation and autoimmunity for self-antigen (Meng et al., 2009; Fukami et al., 2009). Microchimerism is a well-known phenomenon effecting after solid transplantation, pregnancy and blood transfusion. The analogy between autoimmune diseases and alloimmune response in transplantation may explain role of microchimerism related with fetal or maternal origin (Nelson et al., 1998; Mosca et al., 2003). It was also indicated that females can have autoimmune diseases as a result of an immune reaction between fetal and maternal cells (Kekow et al., 2013). Our findings with demographic and clinical data of individuals also support this finding with correlation between previous pregnancy and anti-HLA antibody positivity (Table 3.1, Figure 3.2). As in our findings, the increased frequency of anti-HLA antibodies in autoimmune diseases like SLE and SSc can support the role of microchimerism.

Anti-class I antibodies can induce the upregulation of inflammatory chemokines, growth factors and cytokines so inflammatory cells can accumulate in organs (Nath et al., 2010). This accumulation can lead tissue damage and this induction may be the

key of similarity between alloimmune responses occur during transplantation and autoimmunity for self-antigen. According to our findings, anti-HLA class I antibodies had higher frequency in both SLE and SSc patients compared to healthy controls (Figure 3.2). Also there have been some studies establishing relationship between anti-HLA antibodies and autoimmunity. In their study, Fukami and co-workers indicated that anti-HLA antibodies against donor antigens in transplantation also trigger autoimmune response (2009). Also, Yamagiwa and co-workers in their study found in autoimmune hepatitis patients that the frequency of anti-HLA antibody increased in autoimmune hepatitis patients compared to patients with non-autoimmune liver disease and healthy controls (2014).

In addition, there are several evidences that emphasize the relationship HLA and autoimmunity. The studies focus on interaction between some HLA allele subtypes with anti-HLA antibodies and autoimmunity. Especially, individuals with HLA-DR and HLA-DQ subtypes have increased risk of predisposition to autoimmune diseases (Zanelli et al., 2000; Yamagiwa et al., 2014) These studies correlates with our findings that most common reactive antibodies in Class-II are DQ epitopes.

In conclusion, anti-HLA class I and II antibodies were determined as a significantly increased in patients of SLE and SSc that were selected as model autoimmune diseases compared to healthy controls in which microchimerism is presumed to play a role. In order to focus the role of microchimerism in this relationship, more targeted studies should be done.

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