A MULTIFACTORIAL ANALYSIS OF ASSOCIATION BETWEEN CYTOKINE LEVELS AND PATIENTS WITH AUTOANTIBODIES ANTI-RO/LA FROM NORTHERN REGION OF MALAYSIA

ANISAH BINTI ABDUL ZUBIR

UNIVERSITI SAINS MALAYSIA

2019

A MULTIFACTORIAL ANALYSIS OF ASSOCIATION BETWEEN CYTOKINE LEVELS AND PATIENTS WITH AUTOANTIBODIES ANTI-RO/LA FROM NORTHERN REGION OF MALAYSIA

by

ANISAH BINTI ABDUL ZUBIR

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

August 2019

ACKNOWLEDGEMENT

First of all, the highest gratitude and all praise to Allah SWT for His guidance, a lot of mercy and strength that was given to me to complete this research project.

I would like to thankful to my supervisor, Dr. Nor Effa Syazuli Zulkafli for all the supervison, encouragement and support through the learning process of this project. Deepest thanks also to my co-supervisor, Dr. Siti Mardhiana Mohamad for the knowledge sharing throughout the research process.

I also would like to acknowledge my deepest gratitude to all the staff and members of Regenerative Medicine Laboratory, AMDI USM that always give their help when needed. Special thanks to Kementerian Kesihatan Malaysia for their scholarships and study leaves. Thanks to staffs of Microbiology Laboratory, Hospital Tuanku Fauziah, Perlis and Northern Regional Blood Center, Hospital Sultanah Bahiyah II, Kedah for their facillities in completing this research.

I would like to express my deepest thanks to my beloved husband, Mohd Syazwan Mohd Ariff and my only son, Muhammad Umar Sufi who inspired, encouraged, understanding and fully supported me morally and spiritually. To my late mother, Norhayati Abdullah a strong and gentle soul, your spirit is always with me. To my father, Abdul Zubir Abdul Ghani thanks for always supporting me to believe in myself. To my family, especially my sister, Asmak that's always there for me and provide endless love. Genuine thanks to my family in law for all the kindful help. Thanks to all my friends that always give support when I am facing difficulties while doing this project. Lastly, I would also express lots of thanks to anyone who directly or indirectly involve in finishing this project.

Thank you.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

%	percentage
kDa	kilo Dalton
pg/ml	picogram per milliliter
mm^2	millimeter squared
mm	millimeter
min	minute
mL	milliliter
ºC	degree Celsius
M	moles
g	gram
µg/ml	microgram per milliliter
µL	microliter
M_1V_1	concentration/ volume

LIST OF ABBREVIATIONS

AARD	Anti-nuclear antibody associated rheumatoid disease
AECC	American-European Consensus Criteria
AIDs	Autoimmune diseases
ACR	American College of Rheumatology
Anti-CL	Anti-cardiolipin
Anti-CCP	Anti-cyclic citrullinated peptide
Anti-DNA	Anti-deoxyribonucleic acid
Anti-Ro	Anti-SSA/Ro
Anti-La	Anti-SSB/La
Anti-U1RNP	Anti-U1 ribonucleoprotein
AECA	Anti-endothelial cell antibodies
ANA	Anti-nuclear antibody
APCs	Antigen-presenting cells
BAFF	B-cell-activating factor
BCR	B cell receptor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CTD	Connective tissue diseas
Cu^{+1}	Cuprous ion $^{+1}$
CRP	C-reactive protein
DM	Dermatomyositis

EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
ENA	Extractable nuclear antigen
ER	Estrogen receptors
ERα	Estrogen receptor α
ERß	Estrogen receptor ß
EULAR	European League Against Rheumatism
EP	Eppendorf
FITC	Fluorescein Isothiocyanate
FDA	Food and Drug Administration
GD	Graves' disease
Gp	Glycoprotein
HRP	Horseradish Peroxidase
HEp-2	Human epithelial type 2
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITP	Immune thrombocytopenic purpura
IIM	Idiopathic inflammatory myopathies
IFA	Indirect fluorescence assays
ILCs	Innate lymphoid cells
IR	Incidence Rate
IFN	Interferon
IFN-γ	Interferon-γ
IL-2	Intereukin-2
IL-6	Interleukin-6

IL-10	Interleukin-10
IL-35	Interleukin-35
LIA	Line immunoassay
MBP	Myelin basic protein
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
MREC	Medical Research and Ethics Committee
MS	Multiple sclerosis
NK	Natural killer
NFκB	Nuclear factor kappa-light-chain
NaOH	Sodium hydroxide
NON-AID	Non-autoimmune related
NSAIDs	Nonsteroidal anti-inflammatory drugs
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PRRs	Pattern-recognition receptors
PM	Polymyositis
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
SICCA	Sjogren's International Collaborative Clinical Alliance
SD	Standard deviation
SS	Sjogren's syndrome
SSc	Systemic sclerosis
SLE	Systemic lupus erythematosus
SLEDAI	Systemic lupus disease erythematosus disease activity index

SLICC	Systemic Lupus International Collaborating Clinics
SCLE	Subacute cutaneous lupus erythematosus
TFH	T follicular helper
Th	T helper
TID	Type 1 diabetes
TGF-β	Transforming growth factor-β
TNF-α	Tumour necrosis factor-α
TLR	Toll-like receptors
Tregs	T regulatory
UCTD	Undifferentiated connective tissue disease
WR	Working reagent

ANALISIS MULTIFAKTOR DARIPADA PERKAITAN ANTARA ARAS SITOKIN DAN PESAKIT DENGAN AUTOANTIBODI ANTI-RO/LA DARI WILAYAH UTARA MALAYSIA

ABSTRAK

Penyakit tisu penghubung (CTDs) adalah salah satu penyakit autoimun (AIDs) yang biasa di negara dan sentiasa dikaitkan dengan tahap autoantibodi anti-Ro dan anti-La. Kajian semasa dijalankan untuk menentukan analisis multifaktorial perkaitan antara sitokin pro- dan anti radang, interleukin (IL)-6 & IL-10 masing-masing dalam sera wanita positif anti-Ro atau anti-La atau kedua-duanya di utara Malaysia. Faktor-faktor seperti jenis penggunaan ubat dan status menopaus dianalisis dari sampel yang didiagnosis dengan CTD mengikut tahun perkembangan penyakit. Sampel serum dikumpulkan dari hospital kerajaan di wilayah utara Malaysia dan sampel ini telah disaring untuk CTD menggunakan enzim immunoassay (EIA). Kehadiran corak antibodi anti-nuklear (ANA) diuji dengan ujian imunofluoresensi tidak langsung (IFA). Sampel serum positif corak IFA speckled digunakan untuk ujian anti-Ro dan anti-La dengan kaedah EIA. Tahap IL-6 dan IL-10 diukur menggunakan ELISA. Penemuan kami menunjukkan bahawa purata usia positif anti-Ro atau anti-La dalam pesakit wanita utara Malaysia adalah 38.1 tahun (SD ± 15.01) (n = 106). Perempuan melayu mewakili frekuensi positif antibodi anti-Ro dan anti-La tertinggi berbanding kaum Cina, India dan kaum lain di Malaysia Utara (72.6% berbanding 17.0%, 7.5%, 2.8%). Tiada perkaitan yang ketara di antara tahap sitokin dengan jenis ubat dan status hormon ($p>0.05$). Walau bagaimanapun, keputusan menunjukkan bahawa 97.4% pesakit yang didiagnosis dengan autoimun-CTD diuruskan dengan ubat berasaskan steroid. Analisis menunjukkan bahawa tahap IL-6

dan IL-10 ketara lebih tinggi semasa tahun pertama perkembangan penyakit dan kedua-dua sitokin dilaporkan mempunyai tahap tinggi dalam pesakit autoimmune-CTD menopaus. Tahap purata IL-6 adalah lebih tinggi (70.56 ± 115.59 pg / ml) daripada IL-10 (32.96 ± 28.36 pg / ml) dalam positif anti-Ro & La berbanding dengan kumpulan kawalan (20.48 ± 15.19 dan 17.10 ± 12.08 pg / ml), masing-masing ($p < 0.05$). Keputusan kami menunjukkan bahawa IL-6 berkorelasi dengan kedua-dua kumpulan (anti-Ro) dan (anti-Ro & La) dengan nilai $p=0.045$ dan 0.023 masing-masing. Sebaliknya, IL-10 hanya berkorelasi dalam kumpulan (anti-Ro) ($p < 0.001$). Tahap sitokin pro-radang IL-6 telah menunjukkan tahap yang lebih tinggi berbanding dengan sitokin anti-radang IL-10. Data-data ini mencadangkan bahawa tahap IL-6 boleh secara langsung menyumbang kepada pembentukan autoantibodi pada wanita dengan anti-Ro / La. Data-data ini boleh dikemukakan untuk semakan semula protokol dalam menentukan AID atau simptom di Malaysia. Kajian ini juga boleh memberi laluan kepada manipulasi penggunaan perencat kepada IL-6 sebagai molekul sasaran terapeutik dalam menguruskan CTD di kalangan penduduk Malaysia.

A MULTIFACTORIAL ANALYSIS OF ASSOCIATION BETWEEN CYTOKINE LEVELS AND PATIENTS WITH AUTOANTIBODIES ANTI-RO/LA FROM NORTHERN REGION OF MALAYSIA

ABSTRACT

Connective tissue diseases (CTDs) are one of the common autoimmune diseases (AIDs) in many countries and always associated with high levels of autoantibody anti-Ro and anti-La. The current study was conducted to determine the multifactorial analysis of association between pro- and anti- inflammatory cytokines, interleukin (IL)-6 & IL-10 respectively in sera of female positive anti-Ro or anti-La or both in northern Malaysia. Multiple factors including medication and menopause status were analyzed from samples diagnosed with CTD at different disease onset. Serum samples were collected from government hospitals in northern region Malaysia. Serum samples were screened for CTD using enzyme immunoassay (EIA). Presence of anti-nuclear antibody (ANA) patterns was tested by indirect immunofluorescence assay (IFA). Speckled IFA pattern was tested for anti-Ro and anti-La by EIA. Levels of IL-6 and IL-10 were measured using ELISA. Our findings showed that the mean age of positive anti-Ro or anti-La in female of Northern Malaysia was 38.1 years (SD±15.01) (*n=106*). Malay female represented the highest frequency of positive anti-Ro and anti-la autoantibodies compared to Chinese, Indian and other races in North Malaysia (72.6% vs. 17.0%, 7.5%, 2.8%). There are no significant associations between cytokine levels with medicines taken and hormonal status ($p>0.05$). However, result indicated that 97.4% of patients diagnosed with autoimmune-CTD were managed with steroid-based therapy. Analysis showed that mean levels of IL-6 and IL-10 were higher during the first year of disease

progressions and both cytokines were reported to have high levels in menopause autoimmune-CTD patients. The mean levels of IL-6 was significantly higher (70.56 ± 115.59 pg/ml) than IL-10 (32.96 ± 28.36 pg/ml) in positive anti-Ro & La compared to control group (20.48 ± 15.19 and 17.10 ± 12.08 pg/ml), respectively ($p<0.05$). Our result indicated that IL-6 was significantly correlated with both group (anti-Ro) and (anti-Ro & La) with p-value of 0.045 and 0.023 respectively. In contrast, IL-10 was significantly correlated only in group (anti-Ro) ($p<0.001$). Level of pro-inflammatory cytokine IL-6 was shown to have higher levels compared to anti-inflammatory cytokine IL-10. These data suggest that IL-6 levels may directly contribute to the formation of autoantibodies in female with anti-Ro/La. These data can be put forth to establish revised protocol in determining AID or symptoms in Malaysia. This study may also pave way on manipulation of IL-6 inhibitors as therapeutic target molecules in managing CTD among Malaysian population.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Autoimmune diseases (AIDs) which develop when the immune system attacks its own cells and tissues and leads to the production of autoantibodies directed againts self proteins (Abbas, Lichtman & Pillai, 2016). AIDs are the third most common diseases in United States (US) after cancer and cardiovascular disease (NIH, 2000). There are more than 80 types of AIDs such as type 1 diabetes (TID), multiple sclerosis (MS), sjogren's syndrome (SS), rheumatoid arthritis (RA), graves' disease (GD) and systemic lupus erythematosus (SLE) (Simmonds & Gough, 2007). Prevalence of AIDs is about 5-8% in general population with predominantly in females (78%) (Wang, Wang & Gershwin, 2015). AIDs varies according to ethnicity and geographical area (Mackay & Rose, 2006). For instance, Southern European countries have a lower event of AIDs compared to North European and North American countries (Oliver & Silman, 2009) . A previous study demonstrated that Asian and non-Caucasian SLE patients are more prone to severe SLE with poor outcomes (Crosslin & Wiginton, 2009). The aetiology of AIDs is not entirely elucidated, but evidences suggest that both genetics and environmental factors play key roles (Rosenblum, Remedios & Abbas, 2015). Besides that, dysregulation of the protective immune responses cuases AIDs. Usually, self-reactive T cells are suppressed in a normal state. When the balance between self-reactive and regulatory T cells is disturbed, the risk for AIDs onset increases (Kuwabara *et al.*, 2017).

Malaysia is one of the developing countries in South East Asia comprised of three major ethnic groups which is Malay, Chinese, and Indian. A recent study has reported that the prevalence of SLE pateints in Malaysia was 85.9% (359/418) in

females compared to that of males (14.1%) in which majority of them were Malays followed by Chinese, Indians and others (Shaharir *et al.*, 2019). Anisah *et al.*, (2015) reported that the prevalence of positive anti-nuclear antibody (ANA) test that were used in AIDs detection in North Malaysia region was 6.3% (210/3328) with predominantly in females compared to males (75.8% vs. 24.4%). Prevalence of positive ANA test was higher in Malay compared to Chinese, Indian and other races in Malaysia (52.6% vs. 37.3%, 7.2%, 2.9%, respectively). The most frequently observed ANA indirect immunofluorescence assay (IFA) pattern was speckled with anti-Ro being the highest autoantibodies detected (Anisah *et al.*, 2015). On the other hand, SLE occurrence with positive ANA test in US population was 13.8% with higher percentage of risk among females and African Americans (Satoh *et al.*, 2012).

Basically, cytokine are protein that are secreted by many different cell types play crucial role in inflammatory and regulation of immune responses. However, there are some adverse effects if it is overproduced. The execessive secretion of inflammatory cytokines may lead to several acute and chronic inflammatory responses and virtually affect all organ sites and systems (Moudgil & Choubey, 2011). The imbalance between production of proinflammatory cytokines such as Interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and anti-inflammatory cytokines such as interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) contributes to immune dysfunction and thus, contribute to the pathogenesis of certain AIDs (Roescher, Tak & Illei, 2010). Previous studies analysed the pattern of cytokines production in labial salivary gland of sjogren's syndrome patients, peripheral blood lymphocytes and circulating serum samples from human (Garcic-Carrasco *et al.*, 2001; Furuzawa-Carballeda *et al.*, 2014).

The presence of autoantibodies which acts as serological indicator of autoimmunity and is often observed in systemic autoimmune diseases such as RA, SS, SLE and mixed connective tissue diseases (MCTD) (Gershwin, Meroni & Shoenfeld, 2007). Various antigens specific for autoantibodies serve as biomarkers that can be used for the diagnosis of AIDs (Satoh *et al.*, 2012). Anti-SSA/Ro (Anti-Ro) is the most prominent antigen specific for autoantibodies associated with many autoimmune diseases including SLE, SS/SLE overlap syndrome, subacute cutaneous lupus erythematosus (SCLE), neonatal lupus and primary biliary cirrhosis (Gershwin, Meroni & Shoenfeld, 2007). Meanwhile, anti-SSB/La (Anti-La) is often related to SS (Franceschini & Cavazzana, 2005). However, anti-Ro and anti-La specific autoantibodies may present in both tear and sera of SS patients (Toker, Yavuz & Direskeneli, 2004). Several studies have shown that autoantibodies productions were associated with elevated levels of inflammatory cytokines (Anaya *et al*., 2002; Mozo *et al*., 2014).

1.2 Justification of study

It was reported that many cytokines are involved in immune regulation activity of various AIDs (Zvezdanović *et al.*, 2006). The cause of sex bias in AIDs is more likely to be multifactorial, including differences in the sex chromosomes, sex hormones and their receptors (Oliver & Silman, 2009). Thus, this study was conducted to determine the multifactorial analysis of association between the potential pro- inflammatory (IL-6) and anti-inflammatory (IL-10) cytokines production in female patients with anti-Ro and anti-La specific autoantibodies from Northern Malaysia. This study may provide data on the anti- and pro-inflammatory roles of IL-6 and IL-10 in serum samples positive anti-Ro and positive both anti-Ro

and La autoantibodies. It is crucial to investigate the importance of balanced production of these pro- and anti-inflammatory cytokines and their roles in pathogenesis of inflammatory diseases. Multiple factors including medication and menopause status and their interrelation with both cytokines secretion in samples diagnosed with connective tissue disease (CTD) at different disease onset stages were also anaylsed in this study. Since reports on the correlation between cytokines profiling and autoantibodies during disease progression in different ethnic groups from North Malaysia is limited, this study was conducted to fill such knowledge gap. Findings from this study have emphasised the important role of cytokines in development of autoantibodies and disease progression and thus may serve as potential pharmacological target drug in future. Figure 1.1 show the schematic workflow of the current study.

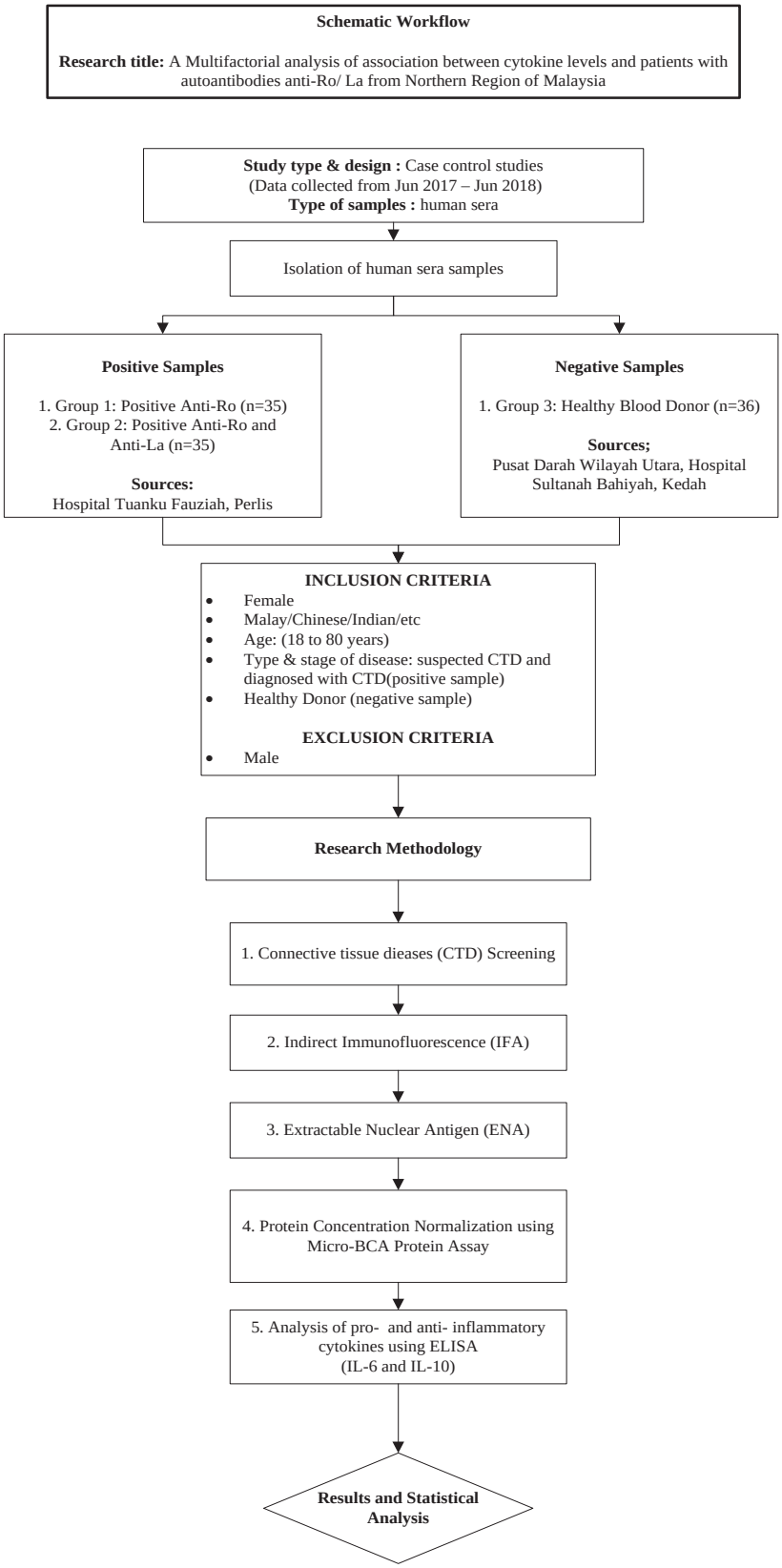

Figure 1.1 Schematic workflow of the current study

1.3 Research Objective

The main objective of this study is to investigate the multifactorial analysis of association between cytokine levels and patients with autoantibodies anti-Ro and both (anti-Ro and La) from Northern Region of Malaysia. The specific objectives of the study were listed below.

Objective 1: To determine the levels of pro- and anti-inflammatory cytokines in female positive anti-Ro and positive both (anti-Ro and La) autoantibodies in Northern Malaysia.

Objective 2: To investigate the multifactorial analysis of female positive anti-Ro and positive both (anti-Ro and La) autoantibodies in Northern Malaysia.

Objective 3: To compare the cytokines level between female positive anti-Ro and positive both (anti-Ro and La) autoantibodies in Northern Malaysia with healthy blood donor.

Objective 4: To determine the association between cytokine levels and positive anti-Ro and positive both (anti-Ro and La) autoantibodies in female of Northern Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 General on Immune system and its regulation

2.1.1 Innate and adaptive immunity

The physiological function of immune system is to provide protection against infections and tissue injuries. The host defense mechanism is classified into innate and adaptive immunity. The innate immunity provides an initial protection against microbial invasion where the first line of defenses is provided by epithelial barriers of skin and mucosal tissues. Epithelial cells locally produced antimicrobial substances called defensins and cathelicidins that kill bacteria and thus serve as chemical barrier againts infection. Other mechanisms such as phagocytes, natural killer (NK) cells and other innate lymphoid cells (ILCs) and the complement system eliminate microbes (Abbas, Lichtman & Pillai, 2016). Figure 2.1 illustrated the principal mechanism of innate and adaptive immunity.

Initially, the invading microbes are recognised by the innate immunitive through germ line-encoded pattern-recognition receptors (PRRs). These receptors recognise and bind to microbial patterns known as pathogen associated molecular patterns (PAMPs) on the surfaces of microbes (Akira, Uematsu & Takeuchi, 2006). Toll-like receptors (TLRs) is member of PRRs family and is described as type I transmembrane proteins with leucine-rich repeats to optimise host protection (Janssens & Beyaert, 2003; Kawai & Akira, 2010). TLRs facilitate the recognition of PAMPs and intracellular Toll-interleukin 1 receptor domains necessary for downstream signal transduction (Kawai & Akira, 2010).

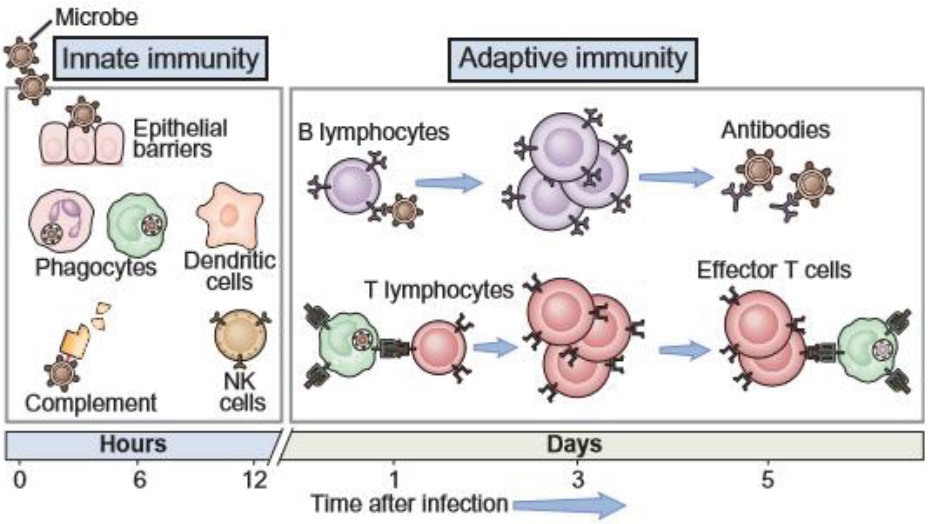

Figure 2.1 Principal mechanism of innate and adaptive immunity

Phagocytes of the innate immunity which include monocytes, macrophages and neutrophils are responsible to recognise, ingest and destroy the invaded microbes. Macrophages and neutrophils express surface receptor called lectin receptor that binds microbes. This receptor binding activated several enzymes in the phagolysosomes such as phagocyte oxidase and nitric oxide synthase (iNOS). Other enzymes involved are the lysosomal protease which break down microbial proteins and subsequently killed the microbes without destroying the phagocytes (Greenberg & Grinstein, 2002).

Meanwhile, other cellular components of innate immune system such as eosinophils, basophils and mast cells are known to eliminate parasitic infections (Abbas, Lichtman & Pillai, 2016). Moreover, NK cells (cytolytic effector lymphocytes) are capable to induce death of tumor cells and virus infected cells in the absence of specific immune response (Vivier *et al*., 2011). NK cells secrete cytokines such as interferon-γ (IFN-γ) in various physiological and pathological conditions. IFN-γ stimulate dendritic cells and macrophages to produce two types of signals that can activate T lymphocytes. The mechanism occurs in which dendritic cells increase their expresion of surface molecules called costimulators. Subsequently, the costimulators which bind to receptors on naive T cells and together with antigen recognition to activate the T cells (Abbas, Lichtman & Pillai, 2016). Secondly, dendritic cells and macrophages secrete cytokines such as IL-1 and IL-6 which stimulate the differentiation of naïve T cells into effector cells of cell-mediated adaptive immunity. NK cells also produce TNF-α and IL-10 that act on other immune cells like dendritic cells to enhance the immune response (Vivier *et al.*, 2011).

The immune system has developed a highly specialized system for capturing and displaying antigens to lymphocytes. Microbes enter through an epithelial barrier and are captured by antigen-presenting cells (APC) in the tissue forming dendritic cell-associated antigen, subsequently enter lymphatic vessels or blood vessels. The microbes and antigens are transported to peripheral lymphoid organs (lymph nodes) (Abbas, Lichtman & Pillai, 2016). APC are displayed for recognition by T lymphocytes by major histocompatibility complex (MHC) molecules (Greenberg & Grinstein, 2002). MHC molecules contain peptide-binding clefts derived from protein antigens and recognized antigen-spesific T lymphocytes. Figure 2.2 illustrated the capture and display of microbial antigens. Thus, the innate immunity that recognises the invading pathogens which results in subsequent activation of the adaptive immune system.

Adaptive immunity is mediated by lymphocytes stimulated by microbial antigens. The adaptive immunity comprises of humoral immunity and cell-mediated immunity. B lymphocytes eliminate extracellular microbes by secreting antibodies thus provide humoral immunity, whereas T lymphocytes help to remove eradicate intracellular microbes in cell-mediated immunity (Abbas, Lichtman & Pillai, 2016).

The development of B cells is initiated in bone marrow and is completed in peripheral lymphoid tissues (spleen) while T cells develop in thymus (Delves & Oitt, 2000; Hoffman, Lakkis & Chalasani, 2016). These “naive” B and T lymphocytes then migrate to secondary lymphoid organs (lymph nodes and spleen), where they become activated.

Antibodies (immunoglobulins, Igs) are glycoprotein molecules present on the surface of B cells (surface Igs) (Abbas, Lichtman & Pillai, 2016). Antibodies exist in

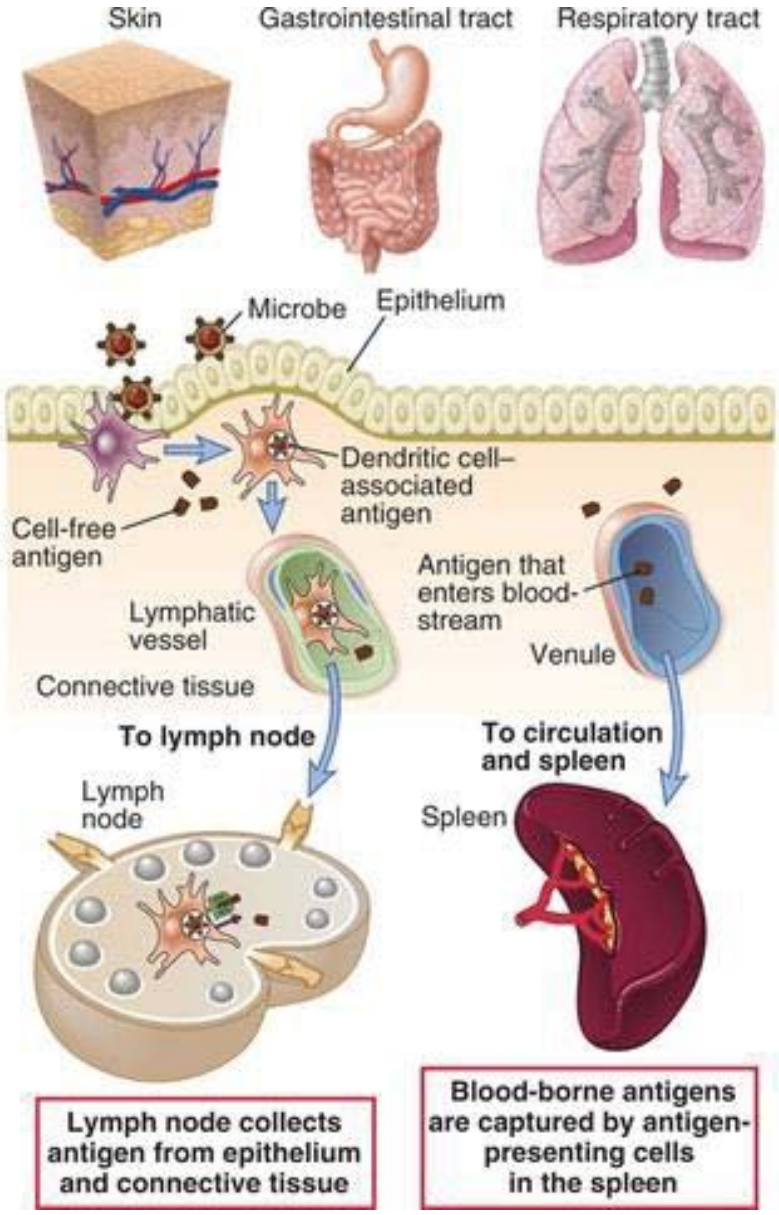

Figure 2.2 Capture and display of microbial antigens

two forms, first as membrane bound antigen receptors on matured B cells known as B-cell receptor (BCR) (Hoffman, Lakkis & Chalasani, 2016). Secondly, antibodies are presented as secreted protein into the extracellular space where they bind and neutralise their target antigens (Hoffman, Lakkis & Chalasani, 2016). BCR and the antibodies secreted by matured B cells are able to recognize the conformations of macromolecules including proteins, lipids, carbohydrates and nucleic acids hence increase its ability to capture diverse formation of microbes (Abbas, Lichtman & Pillai, 2016).

Antibodies consist of two identical heavy chains and two identical light chains that are hold together by disulphide bonds as illustrated in figure 2.3, (Delves & Oitt, 2000). The N-terminal regions of each chain possesses a variable domain with high specificity to binds antigens. The C-terminals regions of the heavy chains are constantly exist in five classes of Igs (IgG, IgA, IgM, IgD, and IgE). The regions (designated Fc) are important for the effector functions of the secreted antibodies to eliminate pathogens or cause tissue injuries (Hoffman, Lakkis & Chalasani, 2016).

The process of T-lymphocytes maturation are depends on the specificity of different types of T cells recognize to peptides bound expressed by different class of MHC molecules (Abbas, Lichtman & Pillai, 2016). The immature T cells also known as pro-T cells do not express CD4 and CD8 ($CD4^-$ $CD8^-$). Then, the pro-T cells than develop into pre-T cells which the TCR β chain is first expressed. The complete T cells receptor is expressed in double positive T cells ($CD4^+CD8^+$) immature T cells. Mature T cells develop into single positive T cells ($CD4^+$ T cells) and ($CD8^+$ T cells) through positive selection (Abbas, Lichtman & Pillai, 2016). Figure 2.4 showed the steps in maturation of T-lymphocytes to $CD4^+$ and $CD8^+$ T lymphoctye respectively.

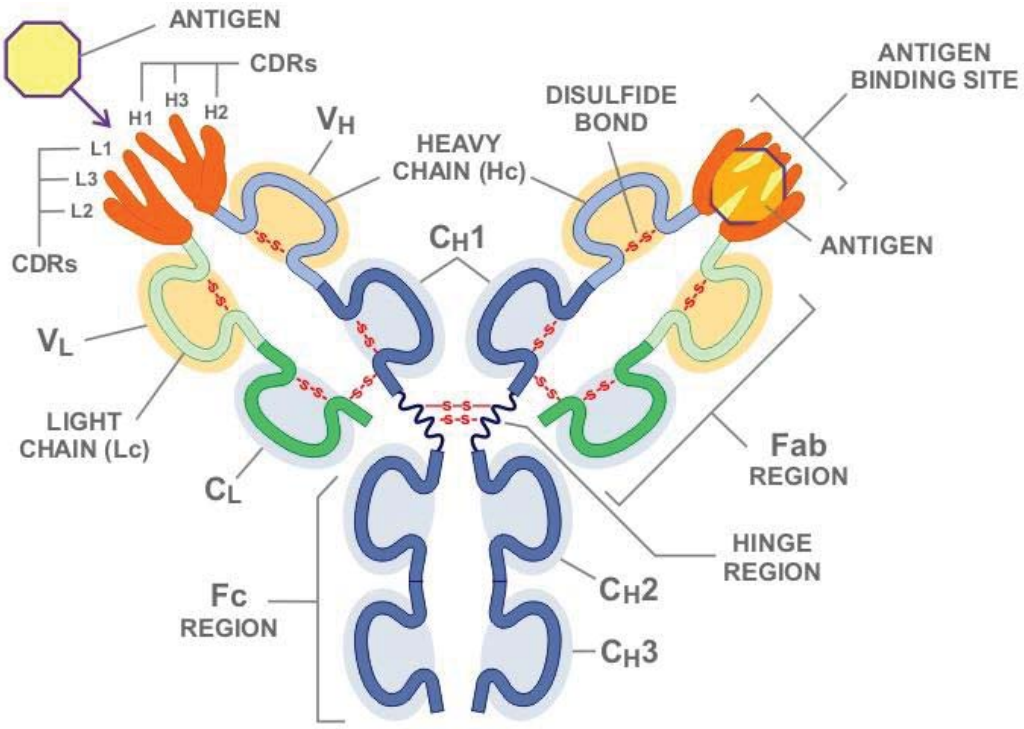

Figure 2.3 Structure of antibodies

CDR: complementarity-determining regions

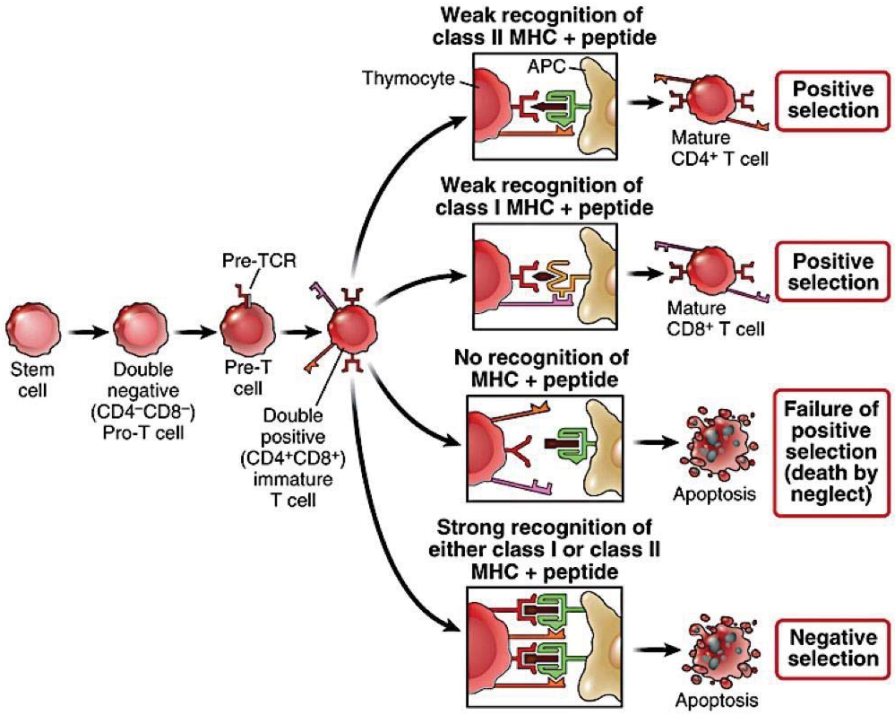

Figure 2.4 Steps in the maturation of T- lymphocytes

The $CD4^+$ T cells recognise antigens presented on MHC class II molecules by antigen presenting cells including dendritic cells, macrophages and B cells which in turn results in activation of the $CD4^+$ T cells into effector cells. Thereafter, these cells differentiate into different T helper (Th) cell subset, including Th1, Th2 and Th17 helper T cells that produce dictinct cytokines profiles and response to the specific type of microbes. For example Th1 secrete IFN-γ cytokine and express CD40 ligands which activate macrophage to kill phagocytosed microbes (House & Descotes, 2007). Meanwhile, mature $CD8^+$ T cells recognise antigens presented on MHC Class I molecules, and capable of becoming cytotoxic T lymphocytes on activation to kill virus infected cells (Delves & Oitt, 2000). Immature T-lymphocytes that strongly recognized self-antigens undergo negative selection to prevent lymphocytes that could react in a harmful way to its own protein (Abbas, Lichtman & Pillai, 2016).

2.1.2 The importance of immune regulation

A remarkable characteristic of normal immune system is to only react towards an enormous variety of microbes without affecting the individual's own (self) antigens. This unresponsiveness to self-antigens is termed as immune tolerance (Abbas, Lichtman & Pillai, 2016). A complex system of immuno-competent cells with proper regulatory function is important to minimise clonal expansion of auto reactive cells to prevent tissue and organ damages (Szodoray *et al.*, 2009). Immunological self-tolerance is an adaptive (or acquired) process to avoid self-destruction by auto-reactive T and B cells (Sakaguchi, Wing & Yamaguchi, 2009). There are two principal mechanism of central tolerance in T lymphocyte. Firstly, death of immature T cells in the thymus through negative selection (Abbas, Lichtman

& Pillai, 2016). Negative selection are a strong recognition of immatue T cells specific to self antigen that displayed peptide bound to self MHC triggers program cell death (apoptosis). Secondly are the development of $CD4^+$ regulatory T cells. Some immature T cells that do not die by apoptosis will develop into regulatory T cells and enter peripheral tissues (Abbas, Lichtman & Pillai, 2016).

Peripheral tolerance also prevent T cell response to self antigen and there are three major mechanism take part in peripheral tolerance. First of all, anergy are referred as long-lived unresponsivenes of T cells to self antigens. This event occur when antigen recognition towards T cells happen without adequate costimulation (B7 protein) since there are no microbial presentation (Sakaguchi, Wing & Yamaguchi, 2009). Second mechanism are the immune suppresion by regulatory T cells which include $CD4^+$ T cells that express high level of CD25 (IL-2α receptor). IL-2 are importance cytokine involved in proliferatation of T cells and play a responses by maintaining funcitional of T regulatory (Tregs) (Sakaguchi, Wing & Yamaguchi, 2009). Besides, Treg cells are regarded as suppressors, since they are involved in maintaining tolerance to self-antigens and produce anti-inflammatory cytokines such as IL-10 and TGF-β (Jin & Yu, 2013). Tregs are comprised of two subgroups, natural Tregs (nTregs) and inducible Tregs (iTregs) (Gol-Ara *et al.*, 2012). For instance, subsets of Tregs are Tr1 regulatory cells, $CD8^+$ Tregs, nTregs, Th3 cells and natural killer like T (NKT) cells (Gol-Ara *et al.*, 2012). The third mechanism of peripheral tolerance in T cells are apoptosis of mature lymphocyte caused by the engagement of death receptor ligand that result in deletion (Abbas, Lichtman & Pillai, 2016). In the case of B cells, central tolerance occur in the bone marrow, where immature B cells that recognise self-antigens switch their specificity by undergoing receptor editing and thereby express a new Ig light chain. If editing fails,

immature B cells that strongly recognise self-antigens will receive death signals and die through apoptosis (deletion) (Abbas, Lichtman & Pillai, 2016). Figure 2.5 illustrated the mechanism of central and peripehral tolerance of B and T cells to self antigens.

2.1.3 Loss of immune regulation

One of the key feature of the immune system is its ability to distinguish 'self' molecules from 'non-self' molecules (Abbas, Lichtman & Pillai, 2016). Despite the immune system's efficiency in eliminating auto reactive B and T cells through central and peripheral tolerance, sometimes the immune system fails to distinguish between self and non-self, molecules which results in condition known as autoimmunity. While the recognition of foreign antigens is vital to initiate immune responses againts pathogens, the failure to recognise self-antigens results in AID as illustrated in Figure 2.6. AIDs results from multiple genetic alterations that are likely to be influenced by environmental factors (Elkon & Casali, 2008). Besides, presence of human leukocyte antigen (HLA) genes was shown to be a risk factors that enhance the development of AIDs including SS (Hernández-Molina *et al.*, 2015).

Moreover, an infections may activate APC causes induction of costimulator (B7 and CD28). Thus, APCs present self antigens to self-reactive T cells hereby attack self tissue triggering the development of AIDs (Abbas, Lichtman & Pillai, 2016). Furthermore, some microbial infections produce peptide antigens that cross reacts with self-antigens, a term called molecular mimicry that may promote autoimmunity (Abbas, Lichtman & Pillai, 2016).

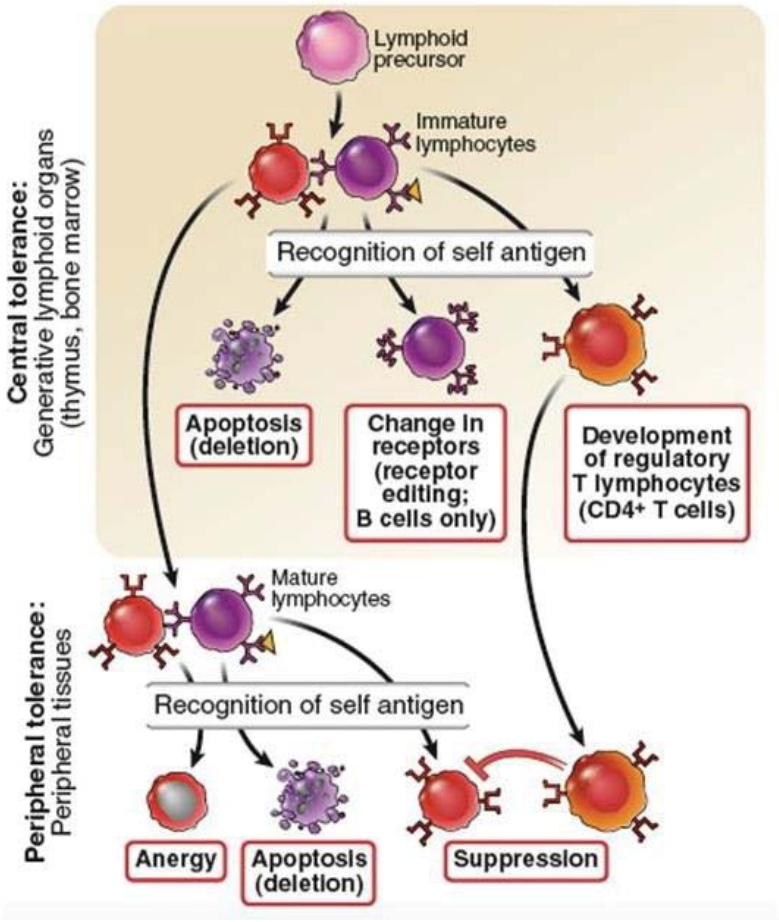

Figure 2.5 Central and peripheral tolerance to self-antigen.

Central tolerance: Immature lymphocytes specific for self-antigens may encounter these antigens in the generative (central) lymphoid organs and are deleted; B lymphocytes change their specificity (receptor editing); and some T lymphocytes develop into regulatory T cells. Some self-reactive lymphocytes may complete their maturation and enter peripheral tissues. Peripheral tolerance: Mature self-reactive lymphocytes may be inactivated or deleted by encounter with self-antigens in peripheral tissues or suppressed by regulatory T cells. Figure adapted from (Abbas, Lichtman & Pillai, 2016)

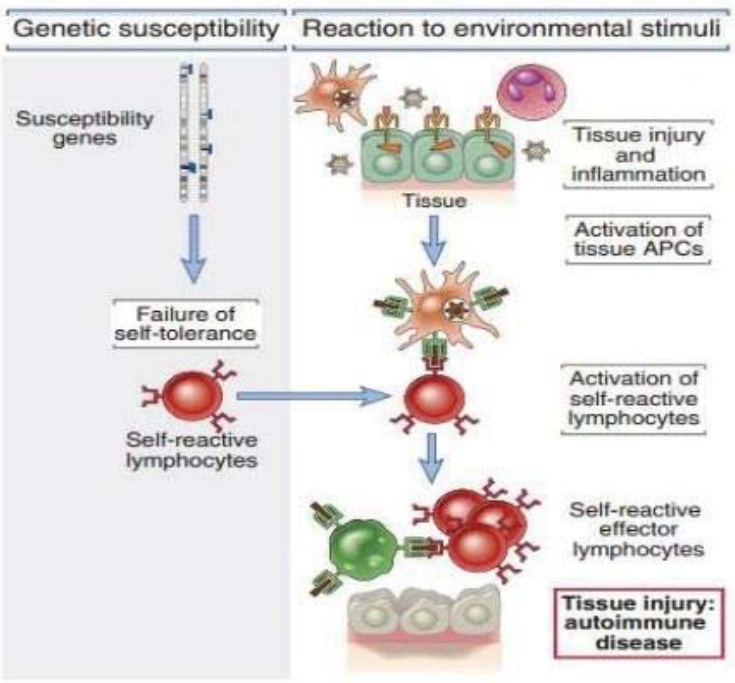

Figure 2.6 Postulated mechanisms of autoimmunity.

In this proposed model of an organ-specific T cell-mediated autoimmune disease, various genetic loci may confer susceptibility to autoimmunity, in part by influencing the maintenance of self-tolerance. Environmental triggers, such as infections and other inflammatory stimuli, promote the influx of lymphocytes into tissues and the activation of antigen-presenting cells (APCs) and subsequently of self-reactive T cells, resulting in tissue injury. Figure adapted from (Abbas, Lichtman & Pillai, 2016).

2.2 Pathogenesis of autoimmune diseases (AIDs)

2.2.1 Autoantibodies production

Autoantibodies have been defined as immunoglobulins reacting againts self molecules (Gershwin, Meroni & Shoenfeld, 2007). Autoantibody production against cellular components is the serologic indicator of autoimmunity and is commonly seen in systemic AIDs (Satoh *et al.*, 2012). AIDs generally develops with multiple and high titre of autoantibodies and detected in healthy individuals (Brandt *et al.*, 2015). Collectively, ANA prevalence in the US was higher among middle age of females with anti-Ro being the most common specific autoantibodies (Satoh *et al.*, 2012). Previous study was performed in Thailand (1987) found that the frequency of ANA positive among Thailand patients with different CTD found to be 11.6% (Sucheela *et al.*, 1987).

Nucleus is one of the main structure in the intracellular targets of the autoimmune responses response (Gershwin, Meroni & Shoenfeld, 2007). Basically, immunologist normally will referred to the initial positive of ANA testing (titre of antibodies) from human sera using IFA method. Besides, this fluoresecence microscopy will also provide information on staining pattern with autoantigen specificities displayed by distinct sera as described in Table 2.1.

Table 2.1 Antinuclear antibody staining patterns at IFA and specificities of major types of ANA producing these patterns.

IIF Pattern in Hep2 cells	Autoantigen Specificities
Rim-like	Nuclear pore complexes (gp210,p62, Tpr)
	Lamins A, B, C
	Inner nuclear membrane (LAP1, LAP2, LBR and MAN1)
Homogenous	Chromatin
	ds DNA
	Histone
	Scl70
Centromere	CENP-A, B, C
Speckled	n RNP
	Sm
	PCNA
	La (SSB)
	Ro (SSA)
	Ku
	RNAP II
Nucleolar	Fibrillarin
	U3 RNP
	NOR90
	B23
	RNAP I-III
	Th/To
	PM-Scl
Nuclear Dot	Sp100
	PML
	SUMO

Adapted from (Gershwin, Meroni & Shoenfeld, 2007)

2.2.2 Anti-Ro and Anti-La antibodies

The presence of anti-Ro and/or anti-La specific autoantibodies are reffered classification criteria in SS diagnosis proposed by American College of Rheumatology (ACR) (Takagi *et al.*, 2014). The frequency of anti-Ro and anti-La specific autoantibodies present in sera of SS patients were estimated to be 96% and 87% respectively (Harley *et al.*, 1986). Anti- Ro and anti-La specific autoantibodies were reported to be detected in salivary and lacrimal glands extracts from SS patients (Franceschini & Cavazzana, 2005).

In 1979, Ro and La have been described to be antigenically identical to SSA and SSB respectively. Therefore, these antigens are also known as SSA/Ro and SSB/La (Alspaugh & Maddison, 1979). The Ro/La system is regarded as heterogeneous antigenic complex which the Ro antigen is comprised of two different Ro proteins of 52 and 60kDa and the latter binds small cytoplasmic ribonucleic acid (RNA) known as hY-RNAs (Franceschini & Cavazzana, 2005). Meanwhile, La antigen which was formerly named as Ha is a 48kDa phosphorylated protein present in nucleus and cytoplasm and it was shown to be able to bind various RNA molecules including RNA polymerase III (Franceschini & Cavazzana, 2005). The binding of 60kDa Ro antigen with small hY-RNAs is also associated with La at various sites, as shown in Figure 2.3.

Autoantibodies specific to anti-Ro is often detected in AIDs such as SLE, SS/SLE overlapping syndrome and subacute cutaneous LE (SCLE) (Franceschini & Cavazzana, 2005). In contrary, anti-La is mostly found in SS patients (Franceschini & Cavazzana, 2005). Furthermore, anti-Ro specific antibodies were detected alone in human sera whereas the presence of anti-La specific antibodies were accompanied by

anti-Ro specific antibodies (Gottenberg *et al.*, 2003)**.** In Malaysia, the prevalence of test positive results for anti- Ro and anti- La in SLE patients was reported to be 36% and 8%, respectively. The occurence of anti-Ro in western countries was similar to that of in Malaysia (Wang, Ooi & Wang, 1996).

A previous study have shown that cultured epithelial cells from SS patients constitutively produced exosomes that contain major autoantigens Ro, La and Sm. The mechanism may involve a pathway where intracellular autoantigens are presented to the immune system (Gershwin, Meroni & Shoenfeld, 2007). Besides, there are correlation between anti-Ro specific antibodies with photosensitive rashes ($p<0.05$) and malar rashes ($p<0.001$) in patients with MCTD (Szodoray *et al.*, 2012).

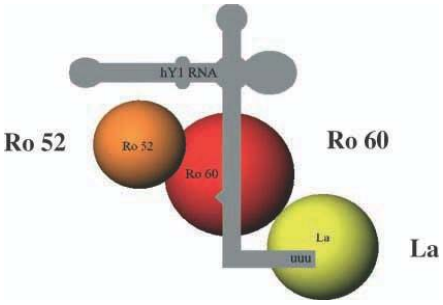

Figure 2.7 This figure illustrates the Ro/La RNA complex
Figure was adapted from (Franceschini & Cavazzana, 2005)

2.2.3 AIDs predominance in females

Women are predominantly affected by various types of AIDs worldwide. This corresponds to a study conducted in Caucasian SLE patients where most of them were females with mean age of 38.6 years old (McCarthy *et al.*, 2014). In

addition, previous study showed that primary SS was mostly diagnosed in women with average age of 56.2 years old (Qin *et al.*, 2014). The sex hormones (oestrogen, androgens and prolactin) play some vital roles in enhancing susceptibility towards AIDs. Oestrogen in females activates B cells which then results in increased levels of antibodies and autoantibodies production whereas androgens suppress maturation of B cell and production of antibodies (Brandt *et al.*, 2015). Furthermore, oestrogen and prolactin cooperatively to increase autoantibody levels especially Ro and La specific autoantibodies. Prolactin is capable to alter immune functions via prolactin receptors expressed on mast cells, macrophages, T and B cells and thus enhance the production of autoantibodies (Brandt *et al.*, 2015). The X chromosomes in females contain immune-associated genes that capable to establish immune tolerance. This genetic factor may have contributed to the enhanced risk of AIDs in female since more X chromosomes are present in females (XX) compared to males (XY) (Oliver & Silman, 2009). Besides, SS in women is associated with other AIDs which present primarily in women such as RA, thyroiditis and Raynaud's phenomenon (Brandt *et al.*, 2015).

2.3 The role of cytokines in pathogenesis of AIDs

2.3.1 The structures, functions and origin of cytokines

Cytokines are large group of secreted proteins with diverse structures and functions. They regulate and coordinate activities of the cells of innate and adaptive immunity (Abbas, Lichtman & Pillai, 2016). All cytokines are pleiotropic and they are capable to interact with various cellular targets through specific receptors expressed on the surface of cells (House & Descotes, 2007). They function as a