THE ROLE OF OESTROGEN IN ANTIBODY PRODUCTION BY IL-27 STIMULATED-B CELLS

FARHANA BINTI MUHAMMAD YUSOFF

UNIVERSITI SAINS MALAYSIA

2020

THE ROLE OF OESTROGEN IN ANTIBODY PRODUCTION BY IL-27 STIMULATED-B CELLS

by

FARHANA BINTI MUHAMMAD YUSOFF

Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

October 2020

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and Most Merciful. All praises to Allah for giving me opportunity, endless guidance and help in finishing this thesis entitle 'The role of oestrogen in antibody production by IL-27 stimulated-B cells'. A special thanks and gratitude to my supervisor, Dr Norhanani Mohd Redzwan for the opportunities, guidance, patience, and trust for me throughout this research under the Short Term Grant (304/PPSP/61313178). Her enthusiasm and knowledge in science especially in Immunology field was such an inspiration and motivates me to complete this research and becoming a better researcher. Special thanks to my co-supervisor, Dr Wan Zuraida Wan Abdul Hamid for her help and the knowledge throughout this research. I would like to thanks some laboratory technologist from Immunology Diagnostic Laboratory, Mr Jamaruddin Mat Asan, Mr Azlan and Miss Fazilah for their guidance and knowledge to help me to finish my laboratory part of this study. My utmost gratitude to my research teammates, Suhana, Ellene, Azrini, Husna, Farah, Sarah, Fatihah, Dr Diana, and Hatmal for the friendship, helps, knowledge and for the best support system during this Master journey. Special dedication to Immunology Department lecturers and staffs for the input, involvement and the vast knowledge throughout this research. Thank you and gratitude to my husband, Abdul Raffi and my whole family, especially my parents, Muhammad Yusoff Abdullah and Na'aimah Ag Sulaiman for being the strongest support system including the financial support and encouragement. To my son, Muhammad Fathi, the journey was colourful and memorable with you around during thesis writing and towards the end of completing this thesis. I could not possibly come to this end without all of you. Thank you very much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

α	alpha
ß	beta
ºC	degree celcius
γ	gamma
µg	micro gram
µl	micro litre
ml	millilitre
min	minute
>	more than
<	less than
ng	nano gram
nm	nano meter
nM	nano molar
pg	pico gram
%	percent
rpm	rotation per minute

LIST OF ABBREVIATIONS

ACR	American College of Rheumatology
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CNS	central nervous system
C3	complement 3
C4	complement 4
CO_2	carbon dioxide
DC	dendritic cell
DNA	deoxyribonucleic acid
EBI3	epstein-barr virus–induced gene 3
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
et al.	and other
FBS	fetal bovine serum
H_2O_2	hydrogen peroxide

HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia
IFN-γ	interferon gamma
IL	interleukin
IL-27Rα	interleukin 27 receptor alpha
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgM	immunoglobulin M
MHCI	major histocompatibility complex class I
MHCII	major histocompatibility complex class II
NKC	natural killer cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
rIL-27	recombinant IL-27
RPMI	Roswell Park Memorial Institute
SD	standard deviation
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index

STAT1	signal transducer and activator of transcription 1
STAT3	signal transducer and activator of transcription 3
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
USM	Universiti Sains Malaysia

PERANAN ESTROGEN DALAM PENGHASILAN ANTIBODI OLEH SEL B YANG DISTIMULASI OLEH IL-27

ABSTRAK

Sistemik lupus eritematosus (SLE) adalah penyakit autoimun yang melibatkan organ-organ dan menyebabkan kerosakan tisu serta menghasilkan ciri-ciri klinikal yang pelbagai. Tahap serum interleukin (IL)-27 telah dilaporkan meningkat dalam pesakit SLE berbanding penderma yang sihat. Tambahan pula, tahap serum IL-27 memainkan peranan dalam perkembangan sel B dan penghasilan auto-antibodi. Kebanyakan pesakit SLE adalah wanita yang dalam linkungan umur yang berpotensi untuk melahirkan anak. Oleh itu, hormon estrogen telah dicadangkan memainkan peranan penting dalam penyakit SLE. Oleh yang demikian, tujuan kajian ini adalah untuk membandingkan tahap serum IL-27 dalam pesakit SLE dan penderma yang sihat. Selain itu, kajian ini juga bertujuan untuk mengkaji potensi kesan estrogen khususnya terhadap penghasilan antibodi oleh sel B yang telah distimulasi oleh IL-27 dalam kondisi pesakit SLE dan penderma yang sihat. Teknik pengasingan serum telah dilakukan ke atas 39 orang pesakit SLE dan 39 orang penderma yang sihat dengan menggunakan teknik pengemparan. Tahap serum daripada kumpulan pesakit SLE dan penderma yang sihat telah diukur dengan menggunakan teknik ELISA dan data dianalisa menggunakan GraphPad Prism, versi 5.01. Perbandingan di antara kedua-dua kumpulan tersebut juga dianalisa menggunakan ujian Mann Whitney. Untuk mengkaji peranan hormon estrogen dalam penghasilan antibodi oleh sel B yang telah distimulasi oleh IL-27, 20 ml darah telah diambil daripada 3 individu pesakit SLE dan 3 individu penderma yang sihat. Sel B telah diasingkan daripada sel mononuklear darah daripada setiap

kumpulan menggunakan teknik pengasingan magnetik. Sel B yang tulen seterusnya telah distimulasi dengan anti-IgM, CD40 ligand, rekombinan IL-27 dan kemudian dirawat dengan 1000 nM 17β-oestradiol (estrogen) dan dikultur selama 48 jam di dalam 37ºC inkubator karbon dioksida. Supernatan daripada sel yang telah dikultur diambil dan ELISA IgG telah dilaksanakan untuk kedua-dua kumpulan. Tahap serum IL-27 dalam pesakit SLE lebih tinggi berbanding penderma yang sihat. Dalam penderma yang sihat dan pesakit SLE, keputusan menunjukkan penghasilan antibodi adalah rendah apabila sel B yang distimulasikan oleh IL-27 dirawat dengan estrogen berbanding dengan kondisi yang tidak dirawat dengan estrogen. Kesimpulannya, dalam kondisi normal dan SLE, kehadiran estrogen dengan sel B yang distimulasi oleh IL-27 mempunyai kesan terhadap penghasilan antibodi dan mempunyai fungsi yang penting dalam patogenesis SLE.

THE ROLE OF OESTROGEN IN ANTIBODY PRODUCTION BY IL-27 STIMULATED-B CELLS

ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease involving multiple organs and this disease can lead to tissue damage and diverse clinical manifestations. Serum level of interleukin (IL)-27 was reported to be elevated in SLE patients compared to healthy donors. In addition, serum level of IL-27 plays a role in B cell development and autoantibody production. Since most SLE patients are women of child-bearing age, oestrogen has been suggested to play an important role in SLE pathogenesis. Thus, this study aimed to compare the serum level of IL-27 from SLE patients and healthy donors, and also to investigate the potential effects of oestrogen particularly on the antibody production by IL-27 stimulated-B cells in normal and SLE condition. Serum was isolated from 39 healthy donors and 39 SLE patients using centrifugation technique. Serum level of IL-27 from both healthy donors and patient groups were measured using ELISA. The data were analysed using GraphPad Prism, version 5.01. Comparison between SLE patients and healthy donors groups was done using Mann Whitney test. To investigate the role of oestrogen in antibody production by IL-27 stimulated-B cells, 20 ml of blood was collected from 3 healthy donors and 3 SLE patients. B cells were isolated from peripheral blood mononuclear cells (PBMCs) of both groups using magnetic separation technique. The purity of B cells was checked using flow cytometry. Purified B cells were stimulated with anti-IgM, CD40 ligand, and recombinant IL-27, then treated with 1000 nM of 17β-oestradiol (oestrogen) before cultured for 48 hours in 37ºC in CO_2 incubator. The supernatants of the cultured cells were

collected to measure total IgG ELISA for both healthy donors and SLE patients. The serum level of IL-27 in SLE patients was higher compared to healthy donors. In healthy donors and SLE patients, total IgG concentration was lower when IL-27 stimulated-B cells treated treated with 17β-oestradiol compared with untreated condition. In conclusion, in normal and SLE condition, oestrogen does have an effect on IL-27 stimulated-B cells through the antibody production and has a critical role in SLE pathogenesis.

CHAPTER 1

INTRODUCTION

1.1 The immune system

Immune system is a critical defense system in human body. Immune system comprised of the entire organs, vessel systems, individual cells and proteins which is explicitly coordinated to react against infection (Abbas *et al.*, 2015). The immune protection from threats is crucial without activating abnormal response to self-antigens that can lead to autoimmunity (Mangino *et al.*, 2017). Human immune system varied between individuals but relatively stable over time for each person (Brodin and Davis, 2017).

1.2 The organ of the immune system

Several organs are involved in immune system which comprise of primary and secondary lymphoid organs. The examples of primary lymphoid organs are bone marrow and thymus. Bone marrow is a tissue located in the bone and produces most of the defence cells and also proliferate in there (Abbas *et al.*, 2015). Immune cells produced in the bone marrow migrate to the bloodstream as well as organ and tissue, where they mature and become specialised cells. Meanwhile, the thymus (thymus gland) is located above the heart which is behind the breast bone, where T lymphocytes (T stands for thymus) differentiate and mature in the thymus and later move throughout the whole body to constantly do its defence role (Kurd and Robey, 2016; Abbas *et al.*, 2015).

Meanwhile, secondary lymphoid organs consist of the lymph nodes, spleen, tonsils and mucosal-associated lymphoid tissue (Randall *et al.*, 2008; Drayton *et al.*, 2006). Lymph nodes are small kidney-shaped in size and located throughout the body (Moore Jr and Bertram, 2018; Nurken and Marzhan, 2016). They are structurally organised with separated regions (cortex, paracortex and medulla) which consists of different immune cells in each region (D'Rozario *et al.*, 2018). Meanwhile, spleen is located beneath the diaphragm at the upper left abdomen, and structurally organised into red and white pulp. Red pulp region is rich in macrophage and erythrocyte for surveillance role from blood-borne pathogens, meanwhile, white pulp is enriched with supports for antigen-specific immune response (Golub *et al.*, 2018; Steiniger *et al.*, 2011). In addition, tonsil is located at the wall of oral pharynx which its role is mainly to defend the body from ingested or inhaled pathogens (Hosokawa *et al.*, 2017). The mucosal-associated lymphoid tissue provides protection at mucosal surfaces for example, in lungs, reproductive tract, and gastrointestinal tract (Drayton *et al.*, 2006). The presence of mucous membrane in the nose and the urinary bladder or vagina is the first line of defence to prevent the bacteria and viruses from attaching and harming the body (Zhang *et al.*, 2016b)

1.3 The cells of the immune system

First and second line of defence is crucial in the immune system. Various immune cells are designed to be part of the immune response upon exposure to extracellular or intracellular pathogens. The first line of defence or known as the innate immune response involves neutrophils, basophils, macrophages, dendritic cells (DCs), and monocytes (Simon *et al.*, 2015). These cells will interact with cells in the adaptive

immune system which are T and B cells as well as natural killer (NK) cells. In immune system, majority of cells are derived from progenitor's stem cell in the bone marrow. T and B cells that already mature will circulate in the bloodstream and some will assemble in specialised lymphoid tissue (Todd, 2010). During adaptive immune response, more cells are activated upon pathogens exposure. Importantly, this type of immunity has a vital function to distinguish foreign from self (Ketelhuth and Hansson, 2016).

1.3.1 T cell development

Cortex and medulla are the sites where T cell developed in the thymus. T cells progenitors migrated into thymus via blood vessels. The α and β loci of T cell receptor (TCR) rearrangement occur at the outer region of thymic cortex in order to respond to a wide range of foreign antigens (Kurd and Robey, 2016). During the development, T cells go through multiple differentiation steps based on the expression of CD4 and CD8 cell-surface marker. T cells with lack of receptor expression which is the early T cells are termed as double negative. First, thymocytes started as $CD4^-CD8^-$ double negative (DN). After that, they become $CD4^+CD8^+$ double positive (DP), then mature to become single positive (SP) $CD4^+$ or $CD8^+$ T cells (Zuniga-Pflucker, 2004). During DN stage, thymocytes further divide into four stages; DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). DN2 is the pivotal stage where the thymocytes undergo normal T lineage specification (Li *et al.*, 2010a).

The signalling facilitated by the interaction of TCR with self-peptide major histocompatibility complex (MHC) ligands affects the fate of DP thymocytes. Thymocytes with TCR expression that bind to self-peptide MHC I complexes develop

into $CD8^+$ T cells meanwhile, those with TCR expression that bind to self-peptide MHC II complexes develop into $CD4^+$ T cells (Germain, 2002). Positive selection is essential for thymocyte survival either to become CD4 or CD8 T cells. Besides, thymocytes that recognise self-antigen mediate negative selection (Klein *et al.*, 2014). Naïve T cells recirculate in the bloodstream and secondary lymphoid organs (Palmer *et al.*, 2015). Competent thymocytes then move out from thymus as functional mature T cells (Kurd and Robey, 2016).

1.3.2 B cell development

B cells derived from hematopoietic stem cells in the bone marrow with organised selection and maturation process. The migration of hematopoietic cells occur from inner bone surface to central area of bone marrow cavity (Nagasawa, 2006). Developed B cells migrate into the blood before reaching the peripheral lymphoid organs (Pieper *et al.*, 2013). Development of B cells is an ordered process resulting in sequential expression and assembly of B cell receptor (BCR). BCR composed of heavy and light chain of ligand-reorganizing immunoglobulin (Ig) in non-covalent association with Igα and Igβ transmembrane protein (Pieper *et al.*, 2013; Monroe *et al.*, 2003).

B cell development is organised throughout the process functional rearrangement of Ig gene segment. A random process of gene assortment to recombine heavy and light chain can result in more than 4 million different combinations (Dunn-Walters, 2015). Heavy chain (V_H, D_H, and J_H) with light chain (V_L-J_L) gene segment rearrange to produce B cell lineage expressing antibodies that recognize more than 50 trillion different antigens (Pieper *et al.*, 2013). The first stage in the rearrangement process, pro-

B cells rearrange D and J segments of heavy chain. Then, second rearrangement process occurs by joining the upstream of V region to rearrange DJ segment. Heavy chain gene segment with functional rearrangement leads to the next phase entry which is the pre-B cell stage (Pieper *et al.*, 2013). Only functional heavy chain effectively binds to surrogate light chain and kappa or lambda light chain will survive and further develop (Dunn-Walters, 2015). The combination of μ chain forms IgM molecule expressed on the surface of the cell to become immature B cells and ready to migrate from the bone marrow. The immature B cells migrate into spleen where they finalise early developmental process then, differentiate into naïve, marginal zone (MZ) and follicular B cells (Pieper *et al.*, 2013). Antibody affinity maturation takes place during B cells differentiation in germinal centre (GC) (Zhang *et al.*, 2018b; Victora and Nussenzweig, 2012). T follicular regulatory (Tfr) cells regulate the response in the GC upon receiving survival signals from Tfh cells. Response in the GC occurs before further process of proliferation to produce high affinity antibody secreting plasma cells or memory B cells (Figure 1.1) (Stebegg *et al.*, 2018). Besides, cytokine such as IL-27 was reported to directly enhances GC B cells and potentially caused lupus in *Sanroque* mice (Vijayan *et al.*, 2016).

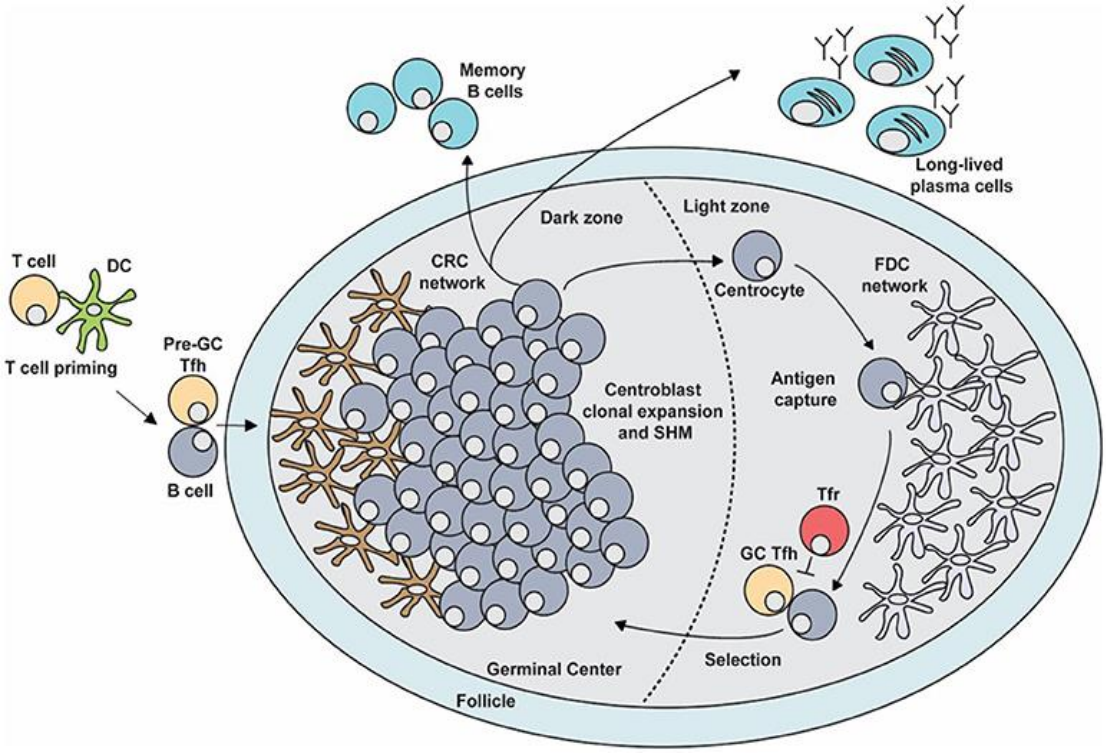

Figure 1.1: Germinal centre (GC). This specialised microenviroment is divided into two distinct compartments which are dark zone and light zone. GC is formed within B cell follicles of secondary lymphoid tissues upon infection and immunisation. Adapted from Stebegg *et al*., 2018.

During development, B cells are screened at multiple check points in order to avoid auto-reactivity. In addition, mature B cells consist of three major subsets; B1 cells, follicular B cells, and MZ B cells (Nutt *et al.,* 2015). Mature B cells recirculate between secondary lymphoid organs in order to identify foreign antigen (Nutt *et al.*, 2015).

1.4 The immune response

Human has a sophisticated immune response against evolutionised pathogens that can cause infection and any dysregulated auto-reactivity leading to autoimmune disease. Immune response comprised of two systems which are innate and adaptive immune responses. The innate immune response is immediate and nonspecific meanwhile, adaptive immune response is specific and also known as acquired immunity. Although innate and adaptive immune responses are usually described as contradictory with different mechanism of action, however, they need to interact with each other with the DC presented the antigenic peptide into MHC proteins to T cell until the antibody and memory are produced (Yatim and Lakkis, 2015). This interaction between innate and adaptive immune system is crucial in order to possess effective immune response (Chaplin, 2010). Infections are recognized by innate immune system to give the immediate defence responses with recognition system linked to adaptive immunity for generation of long lasting defence (Iwasaki and Medzhitov, 2015). Innate and adaptive immune response against pathogens activates complement system which improve the capability of antibody and phagocytic cells to abolish microbes and damaged cells. Complement system activation promotes inflammation, lysis and opsonisation of the antigen (Dunkelberger and Song, 2010).

1.4.1 Innate immune response

Innate immune response is a rapid and non-specific response towards pathogens. Innate immunity facilitated by pattern-recognition receptor (PRR) that detect pathogen-associated molecular pattern (PAMP) such as cell wall of certain bacteria, virus, fungi and unrecognised nucleic acid. PRR is expressed on the plasma membrane of macrophages, DCs and other types of host cells. Induction of inflammatory response and innate host defence is the result of detection of PAMP by PRR (Iwasaki and Medzhitov, 2015).

Epithelial cells with chemical and biological agents such as lysozyme and phospholipase ensure the effectiveness of first line of defence. Macrophages and neutrophils are examples of professional phagocytes where they eliminate the pathogens via phagocytosis. Other than destroying pathogens, phagocytes also act as antigen presenting cells (APCs) that can alert the adaptive immunity to respond to the invaders. DCs and monocytes are the examples of professional APC. They act upon the pathogens by cellular uptake and then taken up the proteins of the pathogens. After that, the APC will present the protein from the pathogens on its surface by major histocompatibility complex Class II (MHCII) to alert the adaptive immunity. Other examples of phagocytes are eosinophils, basophils and mast cells. Furthermore, the non-self particles captured by non-professional phagocytes for example, the epithelial cells, are still needed to be eliminated by macrophages that redirect phagocytosis process. Particles that engulfed by non-professional phagocytes are modified by macrophages before undergo redirect phagocytosis and induce their inflammatory response (Han *et al.*, 2016).

In addition, NK cells, the cytotoxic cells are also one of the innate immunity components. NK cells kill the infected cells by inducing cell death. NK receptor distinguish MHC Class I (MHC-1) molecules to recognise between infected and healthy cells and attack any cells with infection, malignancy or any form of stress from cells (Parham and Guethlein, 2018). However, if the infection persists, adaptive immunity will take action to fight the infection.

1.4.2 Adaptive immune response

Adaptive immune response takes place when innate immune response unable to eliminate pathogens. Adaptive immunity is specific towards invading pathogens and long lasting mainly due to the generation of memory. Thus, the immune response will be rapidly activated when encounter the same pathogens later in life. Adaptive immunity permits recognition of millions of different molecular structures that mediated by surface receptors. The specific surface receptors lead to specific recognition by TCR and BCR structurally and functionally (Ketelhuth and Hansson, 2016).

Professional APC such as DC engulfs invaded pathogens and present part of the pathogens to the surface via MHC Class II. The activated DCs migrate to local lymph nodes and then present the part of pathogen protein to T and B cells (Appenheimer and Evans, 2018). T and B cells that are specific will recognise the pathogen's protein and bind to the antigen presented on the DCs surface. This specificity of T and B cells occurs by random recombination of gene segment during development stage (Kurd and Robey, 2016; Dunn-Walters, 2015). Upon binding with the antigens, T and B cells become

activated and differentiate into effector cell type which activates phagocytic cells such as macrophage to destroy the pathogens.

1.4.3 T cell activation

Activation of naïve T cells will occur upon interaction with APC for examples, macrophages, DCs, and B cells. Specifically, T cells become activated by a combination of antigen-specific signals from TCR and antigen-independent signalling from co-signalling receptors. Co-signalling receptor consists of costimulatory and co-inhibitory receptors expressed on T cell surface (Hui *et al.*, 2017). Two simultaneous signals are needed to activate T cell (Pollard, 2007). The first one is by the antigen presentation in complex with MHC by APC and being recognised by antigen-specific receptor on the surface of T cell. The second signal is by costimulatory signals which is crucial for T cell to become activated, for example, CD28 ligand on T cells with CD80/CD86 ligand on the APC that interact for initial activation of T cell (Pollard, 2007). In addition, cytokines release by APCs are also essential for improvement of T cell activation and influence T cell differentiation to become effector cell types (Pennock *et al.*, 2013). The activated T cell increase the APC activity via interaction of CD40-CD40 ligand to release cytokines such as interferon gamma (IFN-γ) and GM-CSF (Hernandez *et al.*, 2007). However, before the activation occurs, specific effector functions are required for a condusive metabolic condition, which is glucose metabolism to regulate the T cell activation (Palmer *et al.*, 2015). This is to ensure T cells have ATP-generating processes and high metabolic flux to support their functional needs especially in combating infection (MacIver *et al.*, 2013).

1.4.4 B cell activation

B cell becomes activated when BCR recognises and binds to its complementary antigen. B cells activation occurs at secondary lymphoid organs for example at spleen and lymph nodes. B cells can be activated either with T-independent (TI) response which activation is without T cell interaction or with T-dependent (TD) response which requires $CD4^+$ T cells interaction. B1 cells, MZ B cells, and follicular B cells are able to respond towards antigens by TI response (Nutt *et al.*, 2015).

The TD response is a two-step process in which both steps provide instant and persistent protection. The first is called extra-follicular response that involve B cell receiving signal from antigen receptor-dependent that lead to B lymphoblast development (involve with recombination of Ig class-switch) and short-lived plasmablast that produce antibody (De *et al.*, 2018). This process exhibits little somatic hypermutation, thus, the antibody produced for the antigen have a tendency to be moderate and fixed. The second step of TD response occurs by the effect of specialised T follicular helper cell. Some activated B cell re-enter B cell follicle and some also proliferates vigorously to form a GC (Nutt *et al.*, 2015). In addition, the activated T cell also produces cytokine to promote B cells differentiation and specify the subtype of antibody secreted (De *et al.*, 2018).

Immediately after B cell activation either by TI or TD, B cell undergoes differentiation to become antibody-secreting cells known as plasma cells. These plasma cells produce high titres of antibody specific to the antigen, which are released into circulation to neutralise the invading antigen (De *et al.*, 2018). However, plasma cell

does not respond to secondary infection as they have low BCR surface expression. The antibody remains in the circulation for several months. Meanwhile, B cell activation via TD produces memory B cells that remain in the tissue and circulation for a longer period. These memory B cells multiply extensively but do not secrete antibody. Contrary to plasma cell, memory cell maintain BCR expression on its surface and rapidly produce the antibody after encounter with the same antigen later in life (Nutt *et al.*, 2015). Figure 1.2 shows the summary of developmental stages of B cells and their differentiation until maturation stage.

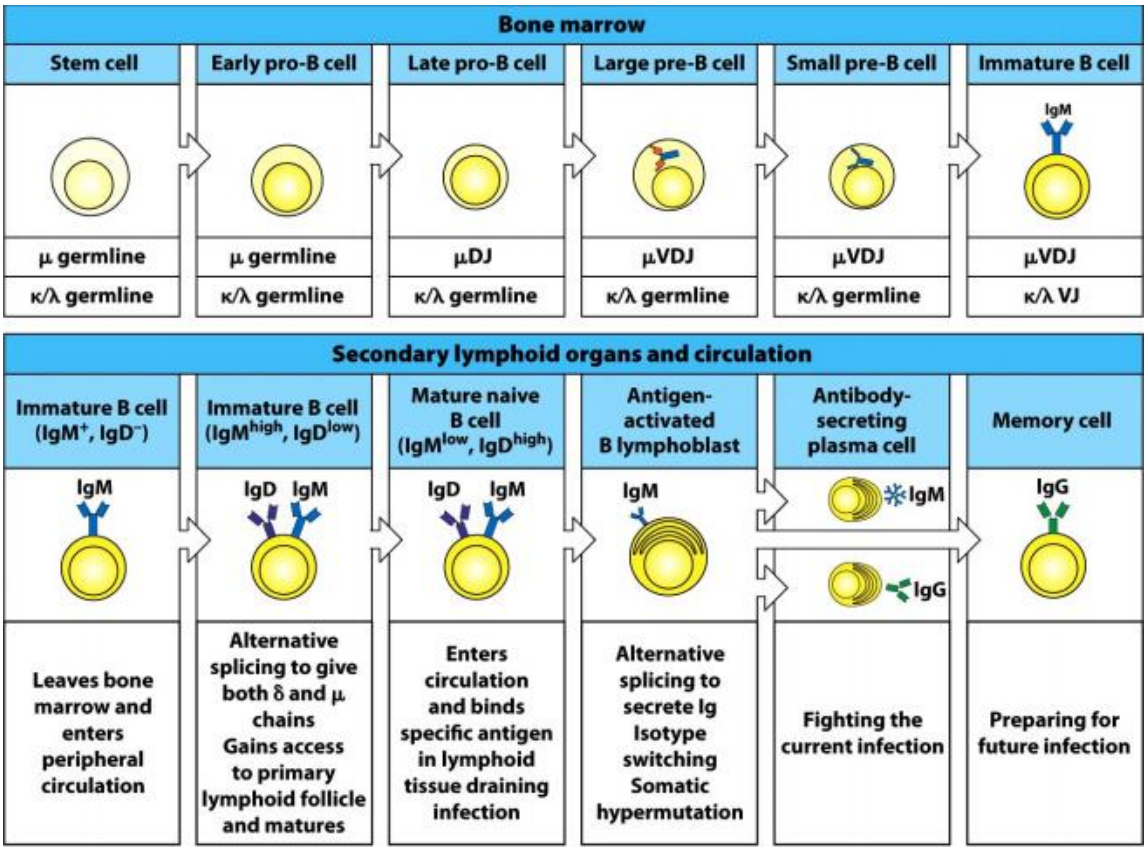

Figure 1.2: Developmental stages of B cells. The developmental stage of B cells from bone marrow together with differentiation and maturation stage in secondary lymphoid organs and circulation. Adapted from (Parham, 2009).

1.5 The cytokine

Cytokine is a cell-signalling protein molecule that activates and regulates immune responses (Schirmer *et al.*, 2018). Other names of cytokine include chemokine, interleukin (IL) (Zhang and An, 2007), interferon (IFN), granulocyte colony-stimulating factor (G-CSF), tumour-necrosis factor (TNF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Holdsworth and Gan, 2015). These polypeptide glycoproteins with low molecular weight are synthesised by different immune cells such as T cells, B cells, stromal cells neutrophils and macrophage (Ferreira *et al.*, 2018; Jandl and King, 2016). Cytokine is essential in immune response by inducing local inflammation protection and response of systemic acute phase both in innate and adaptive immunity (Holdsworth and Gan, 2015).

Cytokine can be divided into functional classes such as primary lymphocytes growth factors, pro-inflammatory or anti-inflammatory factors and functioning mainly in immune response to antigen (Dinarello, 2007). However, some cytokines exert multiple actions (called pleiotropic) that leads to the ability to activate multiple signalling pathways that contribute to different functions (Ozaki and Leonard, 2002).

1.6 Tolerance and autoimmunity

1.6.1 Tolerogenesis

The immune system is able to differentiate between self and non-self-antigen. The system has several checkpoints to prevent cells with self-responsive antigen receptors that can lead to autoimmunity. The first selective checkpoint occurs in primary lymphoid organs during lymphocytes development process which is termed as central tolerance

(Anderson *et al.*, 2017). Second selective checkpoint is implemented once the cells enter the circulation and termed as peripheral tolerance (Anderson *et al.*, 2017).

1.6.2 Central T cell tolerance

In the thymus, clonal deletion is one of the important mechanisms of T cell tolerance. Cortex region is where thymocytes undergo positive selection, while the deletion occurs during the transition phase from double negative (DN) to double positive (DP) that occurs in the thymic cortex (Xing and Hogquist, 2012). Tissue-specific antigens are usually exclusively expressed in the thymic medulla. In addition, the development of T cell that includes clonal deletion is depending on the connections with other cells (e.g. macrophages, epithelial cells, and DC) in the thymic microenviroment. During thymic tolerance, medullary thymic epithelial cells (mTECs) play a crucial role and a nuclear regulatory protein called autoimmune regulator (AIRE) gene will be expressed (Yamano *et al.*, 2015; Xing and Hogquist, 2012).

During this stage of T cell tolerance, AIRE, which is highly expressed in thymic medulla specifically in mTECs, plays its role as the master regulator and important during this stage of tolerance (Proekt *et al.*, 2017; Abramson and Husebye, 2016; Chan and Anderson, 2015; Yamano *et al.*, 2015). This gene helps to remove the thymocytes with self-reactivity from T cell repertoire via negative selection (Cheng and Anderson, 2018).

Like mTECs, medullary and cortical DCs are also have a vital role in central T cell tolerance by promoting the apoptosis to self-tolerance thymocytes (Yamano *et al.*, 2015; Xing and Hogquist, 2012). Based on ‘affinity model’ of selection, thymocytes

with low affinity of self-peptide-MHC will induce positive selection, whereas those with high affinity experience negative selection (Starr *et al.*, 2003).

1.6.3 Peripheral T cell tolerance

Although the mechanism of central tolerance is efficient, not all self-reactive T cells can be removed and some will escape into periphery. Therefore, peripheral tolerance exists to control lymphocytes tolerance outside of the thymus (Xing and Hogquist, 2012). In peripheral T cell tolerance, some of the escaped precursors that display TCR with high affinity towards self are being eliminated either by negative selection or restrained by regulatory T (Treg) cells. Treg cells differentiate and functioned with the control of X-chromosome-encoded Forkhead box P3 (Foxp3) transcription factor which expressed on the self-reactive T cells that escape negative selection (Feng *et al.*, 2015).

Besides, anergy which is a condition of unresponsiveness, and self-reactive T cells deletion can take place in the periphery (Xing and Hogquist, 2012). T cells become activated with the presence of TCR signalling and costimulatory signal pathway or secondary signal (Xing and Hogquist, 2012). Negative second signals provided by costimulatory pathways to prevent T cell response, facilitate tolerance of T cell and inhibit autoimmunity (Xing and Hogquist, 2012). Ligation of TCR and programmed death 1 (PD-1) receptor control self-reactivity and limit the first activation phase and self-reactive T cell expansion (Xing and Hogquist, 2012).

In addition, peripheral DC is also an important regulator of inducing and maintaining tolerance. Tolerogenic DCs can express PD-1 ligands to regulate the decision between T cell tolerance and activation (Xing and Hogquist, 2012).

1.6.4 Central B cell tolerance

Effective selection system of immune system is needed to prevent the migration of mature autoreactive B cells into circulating B cells pool (Pieper *et al.*, 2013). During central B cell tolerance, the immature B cell that recognise high affinity self-antigen in the bone marrow will be deleted or activate the receptor editing to alter their specificity. Whether to be deleted or undergo receptor editing is dependent to the BCR signalling. High affinity autoantigen that bind with strong BCR signal will continue to deletion or receptor editing. Meanwhile, the binding with intermediate affinity will allow B cells to endure and survive before entering the periphery (Tobón *et al.*, 2013).

Major mechanism of central B cell tolerance is by receptor editing. Unlike T cells, B cells have other chance to escape negative regulation which the receptors undergo editing their specificities together with additional antibody gene rearrangements. In the bone marrow, immature B cells that encounter self-antigens will return to pre-B stage. Then, they will continue to rearrangement process to generate new B cells that no longer self-reactive. Then, this new novel rearrangement of immature B cells will move into periphery and become mature circulating B cells (Tobón *et al.*, 2013).

Immature B cells at about 30% - 35% experience receptor editing. Meanwhile, the remaining of autoreactive immature B cells will undergo other selection processes, which are clonal deletion, ignorance or anergy (Pelanda and Torres, 2012). These mechanisms lead to the autoreactive clones to be deleted and prevent self-reactivity. Besides, apoptosis will occur when immature B cell reacts with high avidity self-

antigen. In contrast, low avidity B cells interact that with self-antigen will induce anergy or unresponsiveness to stimulation and permitted the migration to peripheral (Gururajan *et al.,* 2014). Although clonal deletion is not the primary mode of negative selection, it acts as default mechanism when receptor editing fails (Pelanda and Torres, 2012). Increased reactivity level known to be associated with the defective negative selection by reduction of receptor editing or impaired apoptosis (Nemazee, 2017).

1.6.5 Peripheral B cell tolerance

Survived immature B cells from central tolerance leave the bone marrow to become transitional B cells in the periphery. B cells go through negative and positive selection as they mature in the periphery (Stadanlick and Cancro, 2008). These B cells undergo tolerance based on their specificity of BCR and the strength of signal. The selection occurs based on the interaction between BCR-mediated signals and B cell survival factor called B lymphocyte stimulator/ activator (BAFF) or also known as BLyS signalling bind to BAFF receptor (BAFF-R) (Gururajan *et al.*, 2014; Pieper *et al.*, 2013; Scholz *et al.*, 2013). Deficiency of BAFF results in the reduction of the number of peripheral B cell and reduced the ability to support the humoral immune response. Meanwhile, overexpression of BAFF is associated with human autoimmunity and lead to defect in tolerance of transitional B cell during peripheral tolerance. Thus, B cells that are usually be deleted during negative selection would survive due to overexpression of BAFF signalling and enter periphery (Gururajan *et al.*, 2014).

If autoantigens being recognised by mature B cells in peripheral tissues in the absence of specific helper T cell response, the function of the cell will be inactivated by

anergy or die by apoptosis (Tobón *et al.*, 2013). Peripheral B cells survive by excluding B cells that consist of autoreactive BCR transgene from splenic follicles will die rapidly when competing with normal B cells (Stadanlick and Cancro, 2008). Some self-reactive B cells in the periphery are not deleted but they would not differentiate to plasma cells even with the interaction with self-antigens due to clonal ignorance (Tsubata, 2017).

1.7 Mechanisms to tolerance disruption

Human body is responsible to maintain all of the above mechanisms for tolerance towards self-antigens. The breakdown or defective of the mechanisms can cause autoimmunity. T and B cells bound to high avidity self-antigen will undergo tolerance process during central tolerance. However, if the central tolerance fails to avoid lymphocytes with self-antigens, the elimination process will proceed in the periphery to avoid autoimmunity, termed as peripheral tolerance. There are many factors that tolerance can be disrupted, leading to autoimmunity. Some of the factors causing autoimmunity are detailed below.

1.7.1 Increase exposure to antigen

Self-antigen exposure or expression level is too low to induce tolerance may occur for several reasons. One of it is defective expression of antigens in the thymus which prevent negative selection of self-reactive T cells. As described above, AIRE gene helps to remove the thymocytes with self-reactivity from T cell repertoire via negative selection (Cheng and Anderson, 2018). The increase exposure to antigen may lead to tolerance disruption and cause autoimmunity. Thus, AIRE can prevent autoimmunity by tissue-specific antigen (TSA) upregulation of expression in mTECs. These thymic TSAs

is recognised by self-reactive T cells that lead to clonal deletion and/or lineage of regulatory T cell diversion (Anderson and Su, 2016). Besides, AIRE gene is also identified to cause multi-organ system autoimmunity in human and plays a role in thymic tolerance.

1.7.2 Ineffective clearance of apoptosis cells

Autoimmunity is associated with ineffective or failed clearance of apoptotic cells. The process of programmed cell death or apoptosis that includes multiple steps which involve caspase activation after the cell is impaired by physiological and pathological stimuli (Jung and Suh, 2015). Regulation of apoptotic cell death naturally preserves the integrity of membrane and avoid the potential inflammatory and intracellular contents to be released (Nagata, 2018; Elliott and Ravichandran, 2010). Damaged and dead cells as well as debris are rapid and efficiently cleared by professional phagocytes in an immunologically silent manner (Mahajan *et al.*, 2016). However, if slow clearance of apoptotic cells occurs, it will lose the membrane integrity and proceed to secondary necrosis. DNA and intracellular antigens release from dying cells would provoke an inflammatory response, thus leading to autoimmune disorder (Elliott and Ravichandran, 2010). Apoptotic cells with defective clearance may contribute to the accumulation of apoptotic debris and the autoantigens from secondary necrotic cells may be released (Shao and Cohen, 2011). In addition, the autoantigens is produced from inefficiency of the DNA-containing neutrophil extracellular traps (NETs) degradation that lead to autoimmunity (Mahajan *et al.*, 2016).

1.7.3 Molecular mimicry

One of the multifactorial causes of autoimmunity in human is due to cross-reactive epitopes or known as molecular mimicry. Molecular mimicry is the sharing of epitopes among self and non-self antigen where the foreign antigen shares similar structural sequence with self-antigens (Rose, 2015; Cusick *et al.*, 2012) favour an activation of autoreactive T or B cells in susceptible individual (Rojas *et al.*, 2018a). Self-reactive T and B cells that undergo incomplete negative selection will lead to cross-reacting epitopes commonly occur between invaded microorganism and the host (Rose, 2017). Furthermore, the cross-reactive epitopes may occur due to the inflammation that is caused by foreign antigen. When T and B cells presented these foreign antigens together with MHC, it will activate $CD4^+$ and $CD8^+$ T cells. Clearance of invaded pathogens will occur and pro-inflammatory cytokine will be secreted. However, this pro-inflammatory response can occur to specific host tissues due to the sequence and similar structure between foreign and self-antigens known as molecular mimicry, leading to autoimmunity (Cusick *et al.*, 2012).

1.7.4 Marginal zone B cells and autoimmunity

Marginal zone (MZ) B cells carrying antigen into the follicles. They are a potent activator for T cell with rapid response than follicular B cells (Zhou *et al.*, 2011; Cinamon *et al.*, 2008; Attanavanich and Kearney, 2004). MZ B cells can also differentiate rapidly into plasma cells (Zhou *et al.*, 2011). In autoimmunity, MZ B cells are enhanced for autoreactive specificities via self-reactive germline-encoded BCR expression (Zhou *et al.*, 2011; Bendelac *et al.*, 2001). MZ B cells carry BCRs which are

specific for self-antigens and cannot be deleted by mechanism of self-tolerance, thus affecting in maintenance of homeostasis (Palm *et al.*, 2015).

1.7.5 Germinal centre dysfunction that leads to autoimmunity

Germinal centre (GC) that forms in secondary lymphoid organs is a dynamic microenvironment. GC functioned to produce somatically mutated high-affinity antibodies that is required for an effective humoral immune response (Domeier *et al.*, 2017; Gatto and Brink, 2010). Exaggerated chronic GC reactions may occur due to dysregulation of GC mechanisms in development and maintenance phase (Gatto and Brink, 2010). In addition, the production of autoantibodies also associated with the spontaneous GC formation (Domeier *et al.*, 2017; Gatto and Brink, 2010). In fact, defective of molecules that aid in recognition, uptake and the digestion of apoptotic cells can make human susceptible to autoimmune disease (Gatto and Brink, 2010; Vinuesa *et al.*, 2009).

1.8 Autoimmune disease

Autoimmune disease results from self-recognition by the immune system. It is a chronic, relapsing, and can be lethal, commonly characterized by defective of immune system lead to defective tolerance and over-expression of autoantibodies (Zhang and Lu, 2018). Diseases are termed as autoimmune when immune cells or antibodies recognize self-antigens; the autoreactive antibodies resulting tissue injury and affects the organ; and the autoantibodies transfers to another human (e.g. maternal autoantibodies transfers to embryo caused neonatal lupus) (Moutsopoulos *et al.*, 2018). Autoimmune disease can affect one organ which is known as organ-specific autoimmune disease and can also

affect many organs simultaneously and are known as systemic autoimmune disease (Moutsopoulos *et al.*, 2018). Autoimmune disease involves both innate and adaptive immune responses (Theofilopoulos *et al.*, 2017). Autoimmunity affects approximately eight percent of population globally and the incidence is increasing due to multifactorial reasons (Zharkova *et al.*, 2017). The examples of autoimmune diseases are systemic lupus erythematosus (SLE), multiple sclerosis, rheumatoid arthritis (RA), Lyme disease, Sjogren's syndrome, psoriasis, Graves' disease, Crohn's disease, and insulin-dependent Type 1 diabetes (Mahler, 2019; Pham and Mathis, 2019).

1.8.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that leads to autoantibody production and multi-organ damage which mainly afflicts women in reproductive years (Katsuyama *et al.*, 2018; Rider *et al.*, 2018; Zhang *et al.*, 2018a). 20 to 150 cases per 100,000 population was stated as the SLE prevalence (Cai *et al.*, 2017a). The highest estimation of prevalence and incidence of SLE were in North America with 241/100,000 and 23.2/100,000 person-years respectively (Stojan and Petri, 2018; Rees *et al.*, 2017). The lowest prevalence was in Northern Australia with 0 case in a sample of 847 people (Stojan and Petri, 2018; Rees *et al.*, 2017). In Malaysia which is a multi-racial country, SLE prevalence was higher in Chinese population, followed by Malays and generally low in Indians (Shaharir *et al.*, 2016; Jasmin *et al.*, 2013). However, the studies of SLE prevalence were not the latest and the trend would have changed in Malaysia and worldwide.

SLE is characterised by the breakdown of tolerance to self (Blanco *et al.*, 2016) and involves systemic organ, due to systemic inflammatory response that leads to autoantibodies production causing multiple organs damage (Akizuki *et al.*, 2019; Shaharir *et al.*, 2019). It commonly affects skin, joints, kidney, musculoskeletal, hematopoietic system, and central nervous system (CNS) (Akizuki *et al.*, 2019; Torell *et al.*, 2019; Goulielmos *et al.*, 2018; Katsuyama *et al.*, 2018; Phuti *et al.*, 2018). Severe organ damage such as renal failure and the involvement of CNS sometimes can be fatal (Akizuki *et al.*, 2019). Many SLE patients experience "flares" of inflammation in one or more organ system, scattered times with inactive periods (Phuti *et al.*, 2018). The precise mechanism of SLE remains unclear, however, the loss of immune tolerance is one of the main characteristics of SLE (Shao *et al.*, 2019). Even SLE is more frequent in women than men, it was reported that men have a more severe disease than women (Shaharir *et al.*, 2019). In SLE, the severity or the disease activity index was evaluated using SLE disease activity index (SLEDAI) score with SLEDAI ≥ 4 is considered as active patient, while SLEDAI < 4 is considered as inactive patient (von Spee-Mayer et al., 2016; Lee et al., 2014).

1.8.2 Pathogenesis of SLE and cytokines involved

Various immunological abnormalities related to SLE have been identified in human and animal models. For instance, loss of B cell tolerance (Yang *et al.*, 2015), T and B cell signalling with abnormal interactions, the hyperactivity of T and B cells (Jackson *et al.*, 2015; Moulton and Tsokos, 2015; Sanz, 2014; Tenbrock *et al.*, 2007), production of pathogenic autoantibodies due to polyclonal B cell activation (Zhang *et al.*, 2009), and the defective clearance of autoantigens and immune complexes (Malik *et al.*, 2016).