DETERMINATION OF LYMPHOCYTE SUBSETS IN PATIENTS WITH MAJOR DEPRESSIVE DISORDER (MDD)

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by

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LIST OF ABBREVIATIONS

α	level of significance
ACTH	adrenocorticotrophic hormone
AIDS	acquired immunodeficiency syndrome
AR	allergic rhinitis
AVP	Vasopressin
β	Beta
BCR	B cell receptor
BDI	Beck Depression Inventory
CD	cluster of differentiation
CRH	corticotropin releasing hormone
DASS	Depression, Anxiety and Stress Scale
DSM-V	Diagnostic and Statistical Manual of Mental Disorders Fifth Edition
DCs	dendritic cells
HIV	human immunodeficiency virus
HPA	hypothalamic pituitary adrenal
HUSM	Hospital Universiti Sains Malaysia
IFN	interferon
IDO	indoleamine 2,3 dioxygenase
IL	interleukin
IQR	interquartile range
MADRS	Montgomery-Asberg Depression Rating Scale
MDD	major depressive disorder

MHC	major histocompatibility complex
ml	millilitre
n	number of sample
NK	natural killer
NKCA	natural killer cell activity
PBS	phosphate buffer solution
rpm	rotation per minute
SD	standard deviation
SPSS	statistical package for the sosial sciences
TCR	T cell receptors
TNF	tumour necrosis factor
T_C	T cytotoxic
TGF-β	transforming growth factor beta
T_H	T helper
Tregs	regulatory T cells
µl	microliter
USA	United States of America
WHO	World Health Organization
%	percent
1-β	power of study
ºC	degree Celcius
γ	gamma

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PENENTUAN SUBSET LIMFOSIT PADA PESAKIT "MAJOR DEPRESSIVE DISORDER (MDD)"

ABSTRAK

"Major Depressive Disorder" telah dikaitkan dengan gangguan sistem imun. Walaupun banyak kajian mengenai pengaktifan tindak balas imun semula jadi pada masa ini menguasai cabang penyelidikan, namun gangguan dalam sistem imun adaptif terutamanya dalam peredaran subset limfosit jarang diterokai. Beberapa kajian menunjukkan bahawa tahap keterukan penyakit MDD adalah penting dalam mengesan sejauh mana perubahan imun pada pesakit MDD. Objektif kajian ini adalah untuk menentukan faktor penyumbang kepada penyakit MDD, symptom biasa pesakit MDD berdasarkan "Beck Depression Inventory (BDI)" dan "Montgomery-Asberg Depression Rating Scale (MADRS)", peratusan serta bilangan mutlak subset limfosit dalam pesakit MDD dan perbandingannya antara tahap keterukan penyakit ini. Kajian ini melibatkan 47 pesakit MDD yang diambil dari Kinik Psikatri, Hospital Universiti Sains Malaysia (HUSM) dan 47 individu sihat. Pesakit MDD didiagnosa mengikut kriteria "Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V)". Simptom-simptom dan tahap keterukan penyakit MDD dinilai dengan menggunakan skala BDI dan MADRS. Peratusan dan bilangan mutlak $CD4^+$ T sel, $CD8^+$ T sel, $CD4^+$ $CD25^+$ Treg sel, $CD4^+$ $CD25^+$ $Foxp3^+$ Treg sel, $CD16^+$ $CD56^+$ NK sel dan $CD19^+$ B sel ditentukan dengan menggunakan teknik "immunophenotyping". "Mann-Whitney" digunakan untuk membandingkan peratusan dan bilangan mutlak subset limfosit antara pesakit MDD dan individu sihat. "Kruskal-Wallis" digunakan untuk membandingkan peratusan dan bilangan

mutlak subset limfosit antara pesakit MDD dengan tahap keterukan yang berbeza. Hasil kajian menunjukkan bahawa mereka yang berkahwin, perokok, mempunyai tahap pendidikan yang rendah dan tinggal di kawasan luar bandar mempunyai risiko yang lebih tinggi untuk mendapat penyakit MDD. Berdasarkan BDI, simptom yang biasa dilaporkan oleh pesakit MDD adalah keletihan dan cepat marah. Walau bagaimanapun berdasarkan MADRS, pesakit MDD juga mempunyai ketegangan dalaman, masalah tumpuan, ketidakupayaan untuk merasa dan pemikiran pesimis. Kajian ini menunjukkan bahawa tiada perbezaan yang signifikan dalam peratusan dan bilangan mutlak sel $CD4^+$ T (p=0.148; p=0.190), sel $CD8^+$ T (p=0.316; p=0.783), sel $CD16^+CD56^+$ NK (p=0.731; p=0.530), dan sel $CD19^+$ B (p=0.136; p=0.148) antara pesakit MDD dan individu sihat. Walau bagaimanapun, kami mendapati peratusan dan bilangan mutlak sel $CD4^+$ $CD25^+$ Tregs (p<0.001) dan sel $CD4^+$ $CD25^+$ $Foxp3^+$ Treg (p=0.003; p=0.001) pada pesakit MDD lebih tinggi berbanding dengan individu sihat. Kajian ini juga menunjukkan tiada perbezaan yang signifikan dalam peratusan dan bilangan mutlak subset limfosit antara pesakit MDD biasa, sederhana dan teruk.

DETERMINATION OF LYMPHOCYTE SUBSETS IN PATIENTS WITH MAJOR DEPRESSIVE DISORDER (MDD)

ABSTRACT

Major depressive disorder (MDD) has been associated with dysregulation of immune system. While many studies on activation of innate immune response currently dominates the research area, the dysregulation in adaptive immune system especially in circulating lymphocyte subsets has rarely been explored. Some studies indicated that the severity of MDD is important with respect to the extent of the immune changes in MDD patients. The objective of this study was to determine the predisposing factors of MDD and the common symptoms in MDD patients based on Beck Depression Inventory (BDI) and Montgomery-Asberg Depression Rating Scale (MADRS), and to determine the percentage and absolute count of lymphocyte subsets in MDD patients and their comparison between different severity of the disease. This study involved 47 MDD patients recruited from Psychiatric Clinic, Hospital Universiti Sains Malaysia (HUSM) and 47 healthy controls. MDD patients were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) criteria. The symptoms and severity of MDD was assessed using BDI and MADRS scale. The percentage and absolute count of $CD4^+$ T cells, $CD8^+$ T cell, $CD4^+$ $CD25^+$ Tregs, $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, $CD16^+$ $CD56^+$ NK cells and $CD19^+$ B cells were determined by using immunophenotyping technique. Mann-Whitney test was used to compare the percentage and absolute count of lymphocyte subsets between MDD patients and healthy controls. Kruskal-Wallis test was used to compare the percentage and absolute count of lymphocyte subsets

between different severity of MDD. The results showed that those married, smoker, possess lower educational level and living in rural area have higher risk for MDD. Based on BDI, the most common symptoms reported by our MDD patients were fatigue and irritability. While based on MADRS, MDD patients also have inner tension, concentration difficulties, inability to feel and pessimistic thought. This study showed that there were no significant differences in the percentage and absolute count of $CD4^+$ T cells (p=0.148; p=0.190), $CD8^+$ T cells (p=0.316; p=0.783), $CD16^+$ $CD56^+$ NK cells (p=0.731; p=0.530), and $CD19^+$ B cells (p=0.136; p=0.148) between MDD patients and healthy controls. However, we found a significatly higher percentage and absolute count of $CD4^+$ $CD25^+$ Tregs (p<0.001) and $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells (p=0.003; p=0.001) in MDD patients compared with healthy controls. This study also showed that there were no significant differences in the percentage and absolute count of lymphocyte subsets between mild, moderate and severe MDD patients.

CHAPTER 1

INTRODUCTION

1.1 Major Depressive Disorder (MDD)

Major depressive disorder (MDD) is the most common mental disorder reported worldwide. MDD is characterised by the presence of psychological symptoms like sadness, loss of interest or pleasure, feelings of guilt or worthlessness and recurrent thoughts of death or suicide. These symptoms present together with somatic symptoms like sleep or appetite disturbance, significant weight loss, concentration difficulties, physical agitation or retardation and fatigue (Fava and Kendler, 2000). MDD can be long-lasting or recurrent and its condition significantly affects an individual's family and personal relationships, work or school life, and general health (Moussavi *et al.*, 2007).

1.1.1 Epidemiology of MDD

Major depressive disorder is a mental disorder that can affect all people regardless of age, geographic location, demographics, or social position (Firdaus Mukhtar and Tian P.S. Oei, 2011b). According to the World Health Organization (WHO), an estimated 350 million people of all ages suffer from depression (World Health Organization, 2016). It is projected that MDD will be the second leading cause of worldwide disability by the year 2020 and major contributor to the overall

global burden of disease (World Health Organization, 2016). In 2014, about 6.7% of the U.S population aged 18 or older had MDD (National Institute of Mental Health, 2016b).

Across the Asia Pacific region, the rates of current or 1-month MDD ranged from 1.3 to 5.5%, while the rates of MDD in the previous year ranged from 1.7 to 6.7% (Chiu, 2004). The lifetime occurrence of MDD in any country is between 8 to 10% (Malaysian Psychiatric Association, 2006). MDD is predicted to affect about 2.3 million people in Malaysia and at some point in their lives (Firdaus Mukhtar and Tian P.S. Oei, 2011b). The prevalence of MDD in general population in Malaysia is about 8 to 12% regardless of the geographical differences of the study setting (Ng, 2014). While in the primary care population, the prevalence ranged from 6.7 to 14.4% (Firdaus Mukhtar and Tian P. S. Oei, 2011).

Epidemiologic studies consistently shown that women were twice as likely as men to be classified as having MDD (Van de Velde *et al.*, 2010; Bromet *et al.*, 2011). In the south-east Asian region (SEAR), 7 to 12% of men are at a lifetime risk of MDD, while the incidence of MDD among women is 20 to 25% (Khan *et al.*, 2011). The mean age of onset for MDD is in the late 20s (Bromet *et al.*, 2011). In Asia Pacific region, the rates of MDD are highest in those aged 25 to 44 (Chiu, 2004). MDD is more common in urban than in rural population (Wang, 2004) and the prevalence is higher in groups with lower socioeconomic status (Lorant *et al.*, 2007; Meriam Omar Din and Noraini M. Noor, 2010; Shi *et al.*, 2014).

1.1.2 Aetiology of MDD

Major depressive disorder is a multifactorial and heterogeneous group of disorder involving both environmental and biological factors. Childhood trauma and stressful life events such as separation, loss of someone, sexual abuse and poverty are the most common causes of MDD (Kendler *et al.*, 2002; Shapero *et al.*, 2014). Besides that, MDD was also associated with history of chronic illness and social life events such as drug abused and alcohol consumption. Previous study showed that people with chronic illness was 2.7 times at risk to develop MDD, while those with recent alcohol consumption or drug abuse were 39.1 times at risk (Aris *et al.*, 2014). The study also reported that among those MDD patients presented with chronic illness, majority of them suffered from Hypertension and Diabetes Mellitus (Aris *et al.*, 2014).

Genetic factors also play a crucial role in the aetiology of MDD. Evidence from twin studies suggested that MDD has a concordance of 40% to 50%, while family studies indicated that first-degree relatives of depressed individuals are about three times as likely to develop MDD as the general population (Tsuang and Faraone, 1990). It has been found that genetic polymorphisms of serotonin transporter gene (5-HTTLPR) are involved in the development of MDD. Caspi *et al.* (2003) reported that individuals with one or two copies of the short allele of serotonin transporter gene (5-HTTLPR) experienced more depressive symptoms and have higher rates of MDD in response to stressful life events than individual who is homozygous for the long allele (Caspi *et al.*, 2003).

In addition, neuroimaging studies showed that MDD patients had increased volume of the lateral ventricles and adrenal gland and smaller volumes of the basal ganglia, thalamus, hippocampus, and frontal lobe compared to healthy controls (Koolschijn *et al.*, 2009). In conclusion, the onset of MDD involved a combination of genetic, psychologic and environmental factors.

1.1.3 Pathophysiology of MDD

The pathophysiology of MDD has not been clearly defined. However, several biological mechanism with a possible role in the pathophysiology of MDD have been identified. One of the major theories of MDD pathogenesis is the monoamine deficiency theory (Hasler, 2010). This theory suggested that the underlying pathophysiological basis of depression is the depletion of the neurotransmitter serotonin, norepinephrine or dopamine in the central nervous system (Dunlop and Nemeroff, 2007; Hasler, 2010). Most of the serotonergic, noradrenergic and dopaminergic neurons are located in midbrain and brainstem nuclei which project to large areas of the entire brain. This monoaminergic systems are involved in the regulation of a broad range of brain functions, including mood, attention, reward processing, sleep, appetite, and cognition (Belmaker and Agam, 2008). The deficiency of the neurotransmitters produces a number of physiological and behavioural alterations that resemble the symptoms of MDD, including low mood, decreased appetite, sleep disturbance, poor concentration and psychomotor alteration (Delgado, 2000). Almost every compound that inhibits monoamine reuptake, leading to an increased concentration of monoamines in the synaptic cleft, has been proven to be a clinically effective antidepressant (Belmaker and Agam, 2008).

The hyperactivity of hypothalamic pituitary adrenal (HPA) axis is also involved in the pathophysiology of MDD (Vreeburg *et al.*, 2009; Stetler and Miller, 2011). This hyperactivity is caused by malfunctioning of glucocorticoid receptors impairing the negative feedback circuit of the HPA-axis. Glucocorticoid receptor malfunction might also cause MDD via impaired neurogenesis and reduced hippocampus volumes (Manji *et al.*, 2003; Pariante and Lightman, 2008). Corticotropin releasing hormone (CRH) and vasopressin (AVP) are released from the hypothalamus in response to the perception of psychological stress by cortical brain regions. These hormones induce the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, which stimulates the adrenal gland to release glucocorticoid (cortisol in human) into plasma. Glucocorticoids then interact with their receptors in multiple target tissues including the HPA axis, where they are responsible for feedback inhibition on both CRH and AVP from the hypothalamus and directly on secretion of ACTH from pituitary (Vreeburg *et al.*, 2009). The activated HPA axis not only regulates body peripheral functions such as metabolism and immunity but also has profound effects on the brain. For example, glucocorticoids regulate neuronal survival, neurogenesis, the sizes of complex anatomical structures such as the hippocampus, the acquisition of new memories and emotional appraisal of events (Vreeburg *et al.*, 2009; Stetler and Miller, 2011).

Mounting data indicate that cytokine plays a major role in neuropsychiatric diseases, including MDD (Schiepers *et al.*, 2005; Miller *et al.*, 2009). Patients with MDD have been shown to exhibit increases in inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin (IL)-1 and interleukin (IL)-6 in the peripheral blood and cerebrospinal fluid (Miller *et al.*, 2009; Dowlati *et al.*, 2010). Studies have

demonstrated that the cytokines and pro-inflammatory cytokines can activate the HPA axis and impair the central serotonin system (Dantzer *et al.*, 2008; Miller *et al.*, 2013). These cytokine-induced changes in neurotransmitter and neuroendocrine function have, in turn, been correlated with the development of MDD (Dantzer *et al.*, 2008; Anisman, 2009; Raison *et al.*, 2009; Miller *et al.*, 2013). Moreover, administration of cytokines including interferon (IFN)-α and cytokine inducers such as lipopolysaccharide (LPS) have been shown to lead to host behavioural changes that overlap with those seen in MDD patients (Reichenberg *et al.*, 2001; Brydon *et al.*, 2008). A number of cytokines and their signalling pathways have been shown to activate the enzyme indoleamine 2,3 dioxygenase (IDO), which in turn breaks down tryptophan, the precursor of serotonin into kynurenine (Schwarcz and Pellicciari, 2002; Dantzer *et al.*, 2008). The breakdown of tryptophan is believed to contribute to reduced serotonin availability and cause depressive symptom (Neumeister *et al.*, 2004)

1.1.4 Diagnostic criteria of MDD

Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-V) (American Psychiatric Association, 2013) is the current reference used by mental health professionals and psychiatrist to diagnose mental disorders. According to DSM-V, for a diagnosis of MDD at least five or more of the following symptoms must be present during the same 2-weeks period and it represents a change from previous functioning. At least one of the symptoms is either depressed mood or loss of interest or pleasure.

i. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g. feels sad or empty) or observation made by others (e.g. appears tearful). Note: In children and adolescents, can be irritable mood.

ii. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation made by others).

iii. Significant weight loss when not dieting or weight gain (e.g. a change of more than 5 percent of body weight in a month), or decrease or increase in appetite nearly every day. Note: In children, consider failure to make expected weight gains.

iv. Insomnia or hypersomnia nearly every day.

v. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down).

vi. Fatigue or loss of energy nearly every day.

vii. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick).

viii. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others).

ix. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.

The symptoms must be accompanied by significant distress or impairment in social, occupational, or other important areas of functioning. The symptoms also are not due

to the direct physiological effects of a substance (e.g. drug abuse, medication) or medical condition (e.g. hypothyroidism)(American Psychiatric Association, 2013).

1.1.5 Assessment tools for MDD

Various assessment tools have been developed by medical practitioner to measure the depressive symptoms in patients. The use of rating scales is a well-established technique for assessing the severity of mental illness including MDD. In general, the rating scalecan be divided into two groups; patient-rated scale and clinician-rated scale. Total score obtained from the rating scales provides an indication of the severity of MDD for a time period (Cusin *et al.*, 2009; Uher *et al.*, 2012). According to Uher *et al.* (2012), a complete assessment of depression should include both patient-rated scale and clinician-rated scale (Uher *et al.*, 2012).

The commonly used patient-rated scales for MDD were Beck Depression Inventory (BDI), Patient Health Questionnaire (PHQ), Depression, Anxiety and Stress Scale (DASS), and Hospital Anxiety Depression Scale (HADS) (Firdaus Mukhtar and Tian P.S. Oei, 2011a). In Western studies, BDI has been established as the most commonly used outcome measure either in clinical setting or in research practice (Stahl, 2000). Previous studies also support the notion that BDI can be used as an instrument with confidence to measure the levels of depressive symptoms in Malaysian (Firdaus Mukhtar and Tian P.S. Oei, 2011a). BDI had been proven to have good psychometric properties with acceptable internal consistency and moderate concurrent validity (Schotte *et al.*, 1997; Enns *et al.*, 2000; Svanborg and Åsberg, 2001).

Beck Depression Inventory (BDI) was developed by J. Erbaugh based on the records of statements made by individuals with depressive disorders during psychotherapeutic sessions (Beck *et al.*, 1961). BDI has 21-items that assess all DSM-V diagnostic symptoms of depression and additional symptoms (e.g. irritability). A large proportion of BDI items focus on the cognitive symptoms of depression, such as guilt, self-esteem, feeling disappointed in oneself, feeling of being punished, and pessimism (Uher *et al.*, 2008). Each item is composed of four first-person statements graded by the degree of depression severity it typically represents and rated on a 4 point ordinal scale (0 to 3). BDI was originally designed to be read out to the patient by an interviewer, but has commonly been used as self-report questionnaire for literate patients. The total BDI score is calculated by summing the 21 items and the score can range from 0 to 63. Higher score reflects more severe depression. The advantages of BDI scale over clinician-rated scales are they may take less time, do not require trained personnel, and their administration and scoring process appear more standardized (Cusin *et al.*, 2009).

The commonly used clinician-rated scales in clinical studies were Hamilton Depression Rating Scale (HDRS), Montgomery-Asberg Depression Rating Scale (MADRS) and Quick Inventory of Depressive Symptomatology (QIDS) (Cusin *et al.*, 2009; Uher *et al.*, 2012). However, MADRS has been found to be internally consistent and discriminate levels of depression severity more accurately than other clinician-rating scales (Carmody *et al.*, 2006; Bernstein *et al.*, 2010). The inter-rater reliability of MADRS ranged from 0.89 to 0.97 (Montgomery and Asberg, 1979).

Montgomery–Åsberg Depression Rating Scale (MADRS) was develop in the late 1970. MADRS consist of 10 item assessing symptoms of depression that were found to be responsive to treatment (Montgomery and Asberg, 1979). It is commonly used in clinical practice and clinical studies for weekly administration. MADRS focused more toward psychological, as opposed to somatic aspects of depression (Cusin *et al.*, 2009). Sad mood is assessed by two item that capture the observer perspective, and reported subjective experience respectively. The other eight items assess tension, sleep, appetite, concentration, lassitude (activity), inability to feel (anhedonia), pessimism, and suicidal thoughts. Each item is rated on a 7 point (0 to 6) ordinal scale. A total score is computed as the sum of the 10 items and the score range from 0 to 60. A higher total scores will reflect more severe depression (Müller *et al.*, 2003). MADRS can be used by both psychiatrists and professionals without a specific or with minimal psychiatric training (Cusin *et al.*, 2009).

1.1.6 Treatment of MDD

Several treatment approaches to MDD are currently available. These approaches include pharmacotherapy, psychotherapy, electroconvulsive treatment (ECT) and transcranial magnetic stimulation (TMS). Antidepressants drugs were used to reduce the symptoms of depression and help depressed people feel the way they did before they became depressed. Antidepressant drugs commonly used for the treatment of MDD are selective serotonin reuptake inhibitors (SSRIs) (e.g. Citalopram, Escitalopram, Fluoxetine, Paroxetine, Sertraline), serotonin-norepinephrine reuptake inhibitors (SNRIs) (e.g. Venlafaxine, Duloxetine), tricyclic antidepressants (e.g. Amitriptyline, Imipramine, Doxepin) and monoamine oxidase inhibitor (MAOIs) (e.g. phenelzine and tranylcypromine) (Valdivia and Rossy, 2004;

Halverson, 2016). Although choices of antidepressant drugs appear to be many, all current antidepressant drugs have essentially similar mechanism of action through the monoaminergic pathways (S. W. Tang *et al.*, 2010).

Besides pharmacological treatment, MDD patients also have the alternative choice of psychotherapy treatments. Several studies stated that a combination of pharmacotherapy and psychotherapy provides the quickest and most sustained response in MDD patients (Trivedi Madhukar, 2008; Karyotaki *et al.*, 2016). Psychotherapies normally used for adult with MDD including interpersonal psychotherapy (IPT), cognitive-behavioural therapy (CBT), problem-solving therapy (PST), behavioural activation (BA) and contingency management therapy (Valdivia and Rossy, 2004; Halverson, 2016).

Electroconvulsive treatment (ECT) and transcranial magnetic stimulation (TMS) be an option for those who have not responded to antidepressants. The treatments are known to impact the function of neurotransmitters in our brain and typically offers immediate relief of even severe depression (Halverson, 2016). The use of ECT and TMS are usually limited to MDD patients that are highly resistant to antidepressant treatment or have high risk of medical morbidity and mortality (Valdivia and Rossy, 2004; National Institute of Mental Health, 2016a). However, the use of ECT can give several side effect to patients like headache, muscle pain, nausea, fatigue and temporary loss of memory. In serious cases, ECT may cause cardiovascular complication and hypertension (Datto, 2000).

1.2 Human Immune System and Lymphocyte Subsets

1.2.1 Cell of immune system

Human immune system consists of complex network of cells, tissues and organs that work together to protect the body against foreign substances (antigen) such as bacteria, viruses, fungi and other parasites. The immune cells are known as white blood cells or leukocytes. Leukocytes can be divided into five different types based on their physical and functional characteristics. They are neutrophils, eosinophils, basophils, lymphocytes, and monocytes (Elgert, 2009). Lymphocytes are the smallest member of the leukocytes family that capable of complex biological response and activities. Lymphocytes are round cells with 6 to 15 μm in diameter and have a large nucleus and a coarse, dense cytoplasm. They represent 20-40% of the body's leukocytes and are found circulating between blood and lymphoid tissues (Abbas *et al.*, 2014).

All lymphocyte develop from pleuripotent haematopoietic stem cells originate in the bone marrow through a process called haematopoiesis. Pleuripotent haematopoietic stem cells (HSCs) are capable of giving rise to multiple cell lineages including erythrocytes, platelets, myeloid progenitor cells and lymphoid progenitors cells. Myeloid progenitor cells give rise to neutrophils, basophils, and eosinophils. They also give rise to monocytes that can further differentiate to macrophages or dendritic cells (DCs), depending upon exposure to different cytokine millieus. While, lymphoid progenitors cells develop into lymphocytes in the microenvironment of the primary lymphoid organ (thymus or bone marrow). The newly formed lymphocytes further mature and proliferate in these organs and finally give rise to various

lymphocyte subsets known as T lymphocyte, B lymphocytes or natural killer (NK) cells (Abbas *et al.*, 2014). Although all lymphocytes are morphologically similar and rather remarkable in appearance, they are extremely heterogeneous in lineage, function, and phenotype (Elgert, 2009).

T lymphocytes and B lymphocytes express specific receptors for antigen and thus the key mediators of adaptive immunity. In contrast, NK cells do not express specific receptor for antigen and they play a crucial role in innate immunity (Elgert, 2009). These cells are often distinguished by surface protein that may be identified by panels of monoclonal antibodies. The standard nomenclature for these proteins is the "CD" (cluster of differentiation) with numerical designation (e.g. CD3, CD4, CD8, CD25, etc.), which is used to delineate surface protein that define a particular cell type or stage of cell differentiation (Figure 1.1) (Abbas and Lichtman, 2009).

Circulating lymphocytes play a key roles in maintaining immune homeostasis against invading pathogens and damaged host-derived cells that might otherwise destroy the immune balance. Many immune-related disorders such as autoimmune diseases, immunodeficiency syndromes, allergies, transplantation rejection, and leukemia are involved in alterations of lymphocyte subsets (Abul and Andrew, 2011). Recently, it has been reported that mental disorder like post-traumatic stress disorder, panic disorder and major depressive disorder were also associated with the alteration of various lymphocyte subsets (Li *et al.*, 2010; Marazziti *et al.*, 2010).

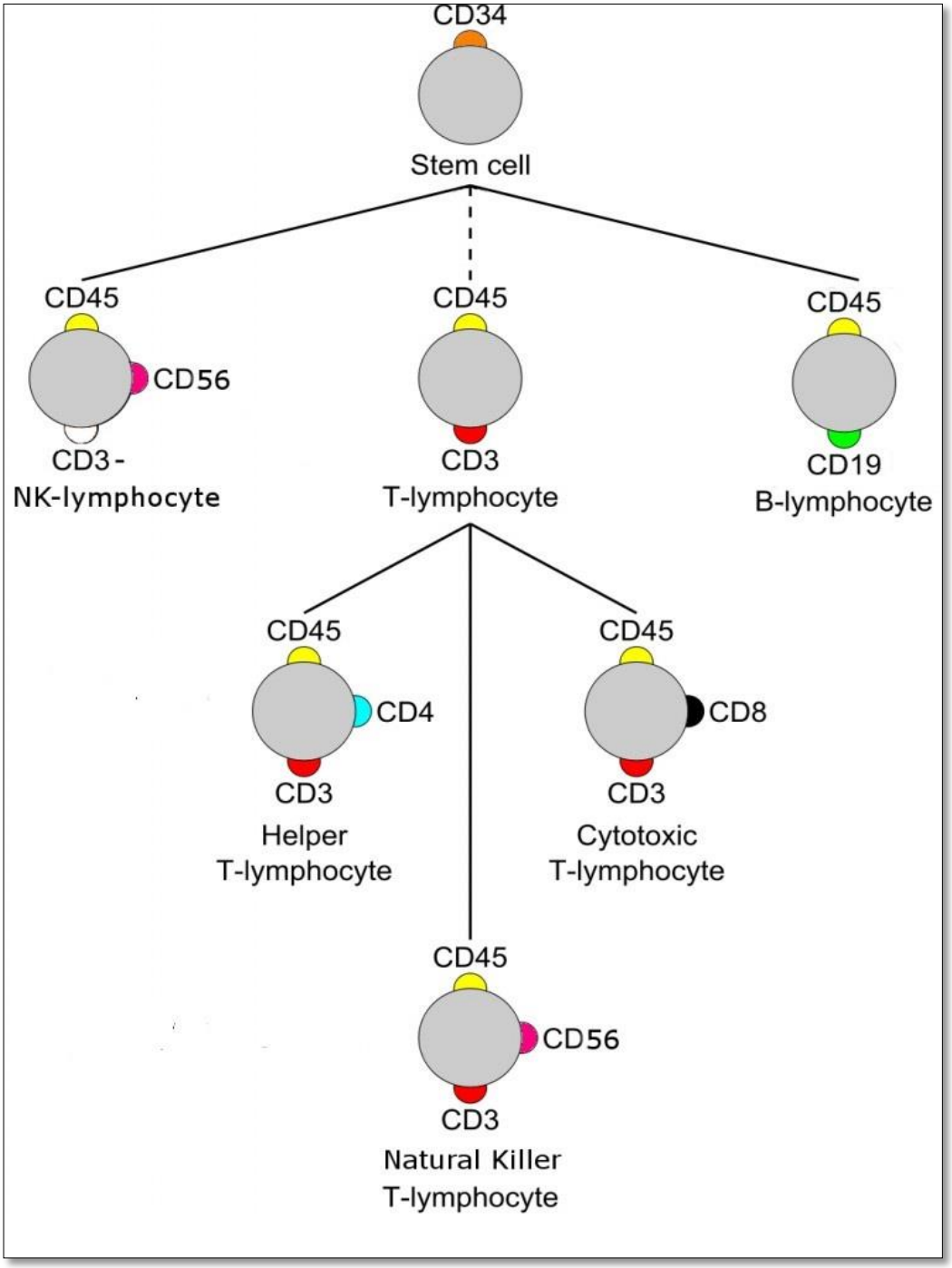

Figure 1.1. Lymphocyte subsets and cluster of differentiation

1.2.2 Total T cells ($CD3^+$ T cells)

Lymphocytes that mature in the thymus after having originated in the foetal liver and adult bone marrow are known as T lymphocytes or T cells. Majority of T cells are small lymphocytes, possessing a large nucleus with very few intracytoplasmic organelles. T cells play a central role in cell mediated immunity and are functionally and phenotypically heterogeneous. T cells express T cell receptors (TCR) on the cell surface which can recognize a specific antigen. T cells that express αβ TCRs are considered part of the adaptive immune response and those expressing γδ TCRs are considered part of the innate immune response. αβ T cells can be divided into several subsets depending upon the co-receptor expressed and each have a distinct function. The main subsets of αβ T cells are T helper cells ($CD4^+$ T cells) and T cytotoxic cells ($CD8^+$ T cells) (Abbas *et al.*, 2014).

In 1990s, a new subset of T cells were discovered known as regulatory T cells (Tregs). Tregs has been found to play a crucial role in immune regulation. T cell dysregulation is present in many diseases including autoimmune diseases, immunodeficiency diseases and also psychological stress-related diseases (Atanackovic *et al.*, 2006; Yamanouchi *et al.*, 2007; Kuhn *et al.*, 2009; Hisamatsu *et al.*, 2016). Through their neuroprotective and anti-inflammatory effects, T cells may play a pivotal role in both the development of MDD as well as its treatment (Miller, 2010).

1.2.3 T helper cells ($CD4^+$ T cells)

T helper (T_H) cells are a subset of T lymphocytes that express the surface protein CD4 and are also referred as $CD4^+$ T cells. The main function of $CD4^+$ T cells is to secrete cytokines for the activity of other immune cells (Zhu *et al.*, 2009). $CD4^+$ T cells generally provide positive signals to other subsets, for example, they cooperate with B cells in the production of antibodies and in the maturation of T cytotoxic cells (Crotty, 2015). In addition, these cells also release cytokines that help macrophages to kill micro-organisms (Zhu and Paul, 2008).

T helper cells can be divided into three broad subsets, T_H1, T_H2, and T_H17. The distinction between the subsets is essentially phenotypic and is based on the profile of the cytokines they expressed (Zhu *et al.*, 2009). T_H1 cells synthesize IL-2, IFN-γ, TNF-α and TNF-β (lymphotoxin). These cytokine activate $CD8^+$ T cells and NK cells. Once these cells have been activated, they kill host cells that have been infected with viruses or intracellular bacteria (Zhu *et al.*, 2009).

T_H2 cells synthesize IL-4, IL-5, and IL-13. IL-4 and IL-13 influence B cells class switch to IgE and IgG in humans, while IL-5 activates eosinophils. T_H17 cells synthesize and secrete the IL-17 family of cytokines (particularly IL-17A anf IL-17F) and IL-22. These cytokine promote the inflammatory response at the mucosal sites. It has been found that, T_H17 cells and cytokines are involved in many autoimmune diseases including rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease (Bettelli *et al.*, 2008; Hisamatsu *et al.*, 2016). Loss of functional $CD4^+$ T cells leads to the symptomatic stage of infection known as the acquired immunodeficiency syndrome (AIDS) (Janeway *et al.*, 1997).

1.2.4 T cytotoxic cells ($CD8^+$ T cells)

T cytotoxic (T_C) cells are a subset of T lymphocytes that express the surface protein CD8 and referred as $CD8^+$ T cellsor cytotoxic T lymphocyte (CTL). $CD8^+$ T cell express a dimeric co-receptor CD8, usually composed of one CD8α and one CD8β chain. $CD8^+$ T cells are very important for immune defence against intracellular pathogens, including viruses and bacteria, and for tumour surveillance (Abbas and Lichtman, 2009). When a $CD8^+$ T cell recognises its antigen and becomes activated, it has three major mechanisms to kill infected or malignant cells. The first is secretion of cytokines, primarily tumornecrosis factor (TNF)-α and interferon (IFN)-γ, which have anti-tumour and anti-viral microbial effects (Price *et al.*, 1999).

The second mechanism is the production and release of cytotoxic granules. These granules, also found in NK cells, contain two families of proteins; perforin and granzymes. Perforin forms a pore in the membrane of the target cell. This pore allows the granzymes to enter the infected or malignant cell. Granzymes are serine proteases which cleave the proteins inside the cell, shutting down the production of viral proteins and ultimately resulting in apoptosis of the target cell. $CD8^+$ T cells can contribute to protection against subsequent encounters with the same antigen (Stenger *et al.*, 1998).

The third mechanism is the destruction of infected cells via Fas/Fas ligand interactions. The cell-surface Fas receptor (Fas) is a member of the tumour necrosis factor (TNF) and nerve growth factor (NGF) family of receptors (Schulze-Osthoff *et al.*, 1998). Activated $CD8^+$ T cells express Fas ligand (FasL) on the cell surface,

which binds to its receptor, Fas, on the surface of the target cell. This binding causes Fas molecules on the surface of the target cell to trimerise, which pulls together the signalling molecules. These signalling molecules result in the activation of the caspase cascade, which cause the apoptosis of the target cell (Hanabuchi *et al.*, 1994; Waring and Müllbacher, 1999). Since $CD8^+$ T cells can express both molecules, Fas/FasL interactions are a mechanism by which $CD8^+$ T cells can kill each other, to eliminate immune effector cells during the contraction phase at the end of an immune response (Hanabuchi *et al.*, 1994).

However, in addition to their critical role in immune defense against viruses, and tumors, $CD8^+$ T cells can also contribute to an excessive immune response that leads to immunopathology, or immune-mediated damage. For example, self-antigens released from $CD8^+$ Tcell lysis during the process of killing virus-infected cells can be presented by antigen-presenting cells to $CD4^+$ or $CD8^+$ T cells, causing them autoreactive. Eventually, this might lead to damage at the site of inflammation or tissue containing self-antigen (Fujinami *et al.*, 2006)**.**

1.2.5 Natural killer cells ($CD16^+$ $CD56^+$ NK cells)

Natural killer (NK) cells are large granular lymphocytes comprising ≈15% of peripheral blood lymphocytes (Robertson and Ritz, 1990). NK cells develop from $CD34^+$ hematopoietic progenitor cells (HPCs) within the microenvironment of the bone marrow. They are characterized phenotypically by the expression of CD16 and CD56 (Trinchieri, 1989). NK cells are the crucial component of innate immune system which display natural cytotoxic activity (Robertson and Ritz, 1990). They lack both immune memory and major histocompatibility complex (MHC) restriction, and are activated by the secretion of interleukin-2 (IL-2) and interferon-γ (IFN-γ) (Trinchieri, 1989).

NK cells express a functional heterodimeric interleukin-2 receptor (IL-2Rβγ), with intermediate affinity for IL-2 (Caligiuri *et al.*, 1990). NK cells also express constitutively several receptors for monocyte-derived cytokines (monokines), including IL-1, IL-10, IL-12, IL-15 and IL-18 (Carson *et al.*, 1995; Kunikata *et al.*, 1998; Fehniger *et al.*, 1999). Besides that, several other studies have identified human NK cells also expressed other surface molecule like CD43 (Aguado *et al.*, 1999), CD55 and CD59 (Solomon *et al.*, 1995), CD57 and HLA-DR (Sedlmayr *et al.*, 1996).

NK cells play a major function in the elimination of compromised host cells, such as tumor or virus-infected cells. NK cell mediated killing involves exocytosis of cytoplasmic granules containing perforin and granzyme through a metabolically active process (Winkler *et al.*, 1996). They also have the ability to kill target cells via death receptors like tumor necrosis factor (TNF)-related apoptosis-inducing ligand

(TRAIL) and Fas ligand (FasL) (Screpanti *et al.*, 2001; Takeda *et al.*, 2005). In addition, NK cells have the capacity to produce an early source of immunoregulatory cytokines. During the innate immune response to infection, monokines stimulate NK cells to produce immunoregulatory cytokines like IFN-γ, TNF-β and granulocyte macrophage-colony stimulating factor (GM-CSF) that are important to the early host defense against a variety of viral, bacterial, and parasitic pathogens (Scharton-Kersten and Sher, 1997; Biron *et al.*, 1999).

NK cells also act as regulatory cells to influence various other cell types, such as dendritic cells (DCs), T cells, B cells and endothelial cells. For example, NK cells can kill immature DCs in humans and thereby influencing DC homeostasis. In addition, by means of IFN-γ and TNF, NK cells can promote the maturation of DCs which in turn activate the NK cells by means of IL-12 (Walzer *et al.*, 2005). IL-2 can promotes NK cell proliferation, cytotoxicity and cytokine secretion. In the inflamed lymph node, NK cells can promote the priming T_H1 cells by secreting IFN-γ (Martín-Fontecha *et al.*, 2004; Morandi *et al.*, 2006). Futhermore, NK cells can also kill activated T cells and suppress autoreactive B lymphocytesin vitro (Takeda and Dennert, 1993).

NK cells are unique as they have the ability to recognize stressed cells in the absence of antibodies and major histocompatibility complex (MHC), which allowing for a much faster immune reaction (Robertson and Ritz, 1990; Cooper *et al.*, 2001). This role is important because harmful cells that are missing MHC class I markers cannot be detected and destroyed by other immune cells. In addition to their major functions in innate immune response, NK cells also play a role in adaptive immune response.

Numerous studies have demonstrated their ability to readily adjust to the immediate environment and formulate antigen-specific immunological memory which are fundamental for responding to secondary infections with the same antigen (Pyzik and Vidal, 2009; Rölle *et al.*, 2013).

1.2.6 B cells ($CD19^+$ B cells)

B cells also known as B lymphocytes are the effectors of humoral immunity. B cells are the only immunoglobulin (Ig) producing cells. They constitute approximately 15% of peripheral blood leukocytes and are found in the bone marrow, blood, lymphoid organs and lymph. B cells mature in the bone marrow (in mammals) or Bursa of Fabricus (in birds) (Cooper and Alder, 2006). Unlike T cells and NK cells, B cells express B cell receptor (BCRs) on their membrane. BCRs allow the B cell to bind a specific antigen, against which it will initiate an antibody response. Mature B cells differentiate into either plasma B cells or memory B cells (Elgert, 2009)**.** During their development stages, B cells expressed several surface molecule such as CD19, CD20, CD21, CD22, CD23, CD24, CD40, CD72 and CD81. However, among all the surface molecule, only CD19 is expressed in all stages of B cell development including the mature B cell (but not the plasma cell) (LeBien and Tedder, 2008). So the expression of CD19 is a useful marker of all cells in the B-cell lineage up to the plasma cells. Besides that, B cells also express MHC class I and II molecules. The expression of MHC class II molecules allows them to present antigen to T helper cells (LeBien and Tedder, 2008).

The main function of B cells is antibody production. The antibodies are secreted by plasma B cell in large amount to assist in the destruction of microbes by binding to

them and making them easier targets for phagocytes and activation of the complement system. In addition to their essential role in humoral immunity, B cells also mediate or regulate many other functions essential for immune homeostasis. Of major importance, B cells are required for the initiation of T cell immune responses. The antigen-specific interactions between B and T cells may require the antigen to be first internalized by the BCR, processed, and then presented in an MHC-restricted manner toT cells (LeBien and Tedder, 2008). Previous studies also demonstrated that B cells are essential for optimal CD4 T cell activation during immune responses to low-dose foreign antigens and autoantigens (Bouaziz *et al.*, 2007).

While critical for normal immune system development, B cells are also important for its maintenance. B cells can release immunomodulatory cytokines that can influence a variety of T cell, APC, and dendritic cells functions, regulate lymphoid tissue organization and neogenesis, regulate wound healing and transplanted tissue rejection, and influence tumor development. B cells can also function as cytokine-producing effector cells that influenceT-cell differentiation (Harris *et al.*, 2000). One phenotypically distinct subset, designated as B10 cells has been shown to uniquely regulate T cell mediated inflammatory responses through the production of IL-10 (Mizoguchi and Bhan, 2006).

1.2.7 Regulatory T cells (Tregs)

1.2.7 (a) Discovery of Tregs

Tregs is a newly discovered lymphocyte subsets compared to T helper, T cytotoxic, NK cells and B cells. In the early 1970s, Gershon and Kondon proposed that a specialized populations of T cells supressed the immune responses of other lymphocytes (Gershon and Kondo, 1970). The suppressor T cells, which were characterized by expression of the CD8 cell surface marker, have been intensively studied over the following years in various fields of immunology. However, because of the poor characterization of the cells and the lack of specific markers, the concept of suppressor T cells was largely abandoned by the end of the 1980s (Sakaguchi *et al.*, 2007).

In mid-1990s, renewed interest in suppressor T cells emerged with the identification of $CD4^+$ T cells population which have the ability to downregulate T cell function. The suppressor T cells was named as regulatory T cells (Tregs). Tregs was observed to expressed CD25, the IL-2 receptors α chain, and are known as $CD4^+$ $CD25^+$ Tregs. However, further studies shows that CD25 is not unique to Tregs only, as it was also expressed on activated effector $CD4^+$ T cells. This has made it difficult to isolate a pure Tregs subsets for functional studies (Sakaguchi *et al.*, 2007).

Studies on the Scurfy disease in mice and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans led to the identification of a marker specific for Tregs, known as Foxp3 (forkhead box P3). Foxp3 is a transcription factor required for the development of Tregs in the thymus and has been

shown to induce non-regulatory T cells to acquire suppressive activity in the periphery (Hori *et al.*, 2003). Therefore, $Foxp3^+$ transcription factors is considered as the most reliable intracellular marker for functional Tregs to date and the Treg cells was known as $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells.

1.2.7 (b) Characteristics of Tregs

Natural Tregs represent approximately 5-10% of the total $CD4^+$ T cell population. In mice, $CD4^+$ Tregs are homogenous population in which all $CD4^+$ and $CD25^+$ cells are Tregs. In human, the Treg are a heterogeneous population, in which not all $CD25^+$ cells are Tregs. Studies by groups of scientists at Havard and the Royal Free and University College Medical School in London revealed that only those $CD4^+$ T cells that expressed very high level of $CD25^+$, representing approximately 2-3% of total $CD4^+$ T cells were Treg (Baecher-Allan *et al.*, 2005).

There are a number of Tregs subtypes with the best understood being those that express CD4, CD25, and Foxp3. Foxp3 is the transcription factor required for $CD4^+$ $CD25^+$ Treg cells development and function (Thompson and Powrie, 2004). $Foxp3^+$ Treg cells use the αβTCR for antigen recognition, and have a broad repertoire, comparable in size to, but largely distinct in composition from conventional $CD4^+$ T cells (Pacholczyk *et al.*, 2006; Wong *et al.*, 2007). It have been found that, when Tregs lose this transcription factor they become ‘ex-Tregs’ which are effector-like T cells that no longer serve to regulate immune function (Zhou *et al.*, 2009). Loss of function or mutations in the human Foxp3 gene lead to IPEX syndrome, a severe multi-organ autoimmune and inflammatory disorder (Rudensky, 2011).