CYTOKINES EXPRESSION IN PRIMARY AND NON-PRIMARY MATERNAL CYTOMEGALOVIRUS (CMV) INFECTION

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
γ	Gamma
%	Percentage

LIST OF ABBREVIATIONS

AI	Avidity index
APC	Antigen presenting cell
CCD	Charge coupled device
cCMV	Congenital CMV
CD	Cluster of differentiation
CNS	Central nervous system
CMV	Cytomegalovirus
DAGS	Double antigen sandwich assay
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
FDA	Food and drug administration
g	Gram
gB	Glycoprotein B
HIG	Hyperimmune globulin
HIV	Human immunodeficiency virus
HUSM	Hospital Universiti Sains Malaysia
IE	Immediate early
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Inducible protein
IUGR	Intrauterine growth retardation
IQR	Interquartile range
kbp	Kilo-base pair
LED	Light emitting diode
MCP	Monocyte chemoattractant protein
MFI	Median fluorescence intensity
MHC	Major histocompability complex
MIP	Macrophage inflammatory protein
miRNA	Micro ribonucleic acid
ml	Milliliter

NK	Natural killer
nm	Nanometer
PCR	Polymerase chain reaction
PE	Preeclampsia
Pg/ml	Pictograms per milliliter
PL	Parameter logistic
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPL	Recurrent pregnancy loss
SNHL	Sensory hearing loss
TCL	T-cell receptor
TH1	T helper 1
TH 2	T helper 2
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TORCHES	Toxoplasma, rubella, cytomegalovirus, herpes simplex virus
U/ml	Unit per millilitre

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EKSPRESI SITOKIN DALAM JANGKITAN MATERNAL PRIMER DAN BUKAN PRIMER CYTOMEGALOVIRUS (CMV)

ABSTRAK

Jangkitan Cytomegalovirus (CMV) ketika kehamilan adalah penyebab jangkitan kongenital yang paling biasa berlaku di seluruh dunia. Jangkitan CMV primer pada kehamilan mempunyai risiko penularan pada janin yang lebih tinggi berbanding dengan jangkitan bukan primer. Jangkitan CMV primer pada awal trimester kehamilan boleh menyebabkan komplikasi serius seperti keguguran dan malformasi kongenital yang teruk. Jangkitan CMV mengubah keseimbangan Th1 dan Th2 semasa kehamilan melalui sitokin (IL-8, IL-6) yang diubah dalam sel plasenta. Maka, kajian ini bertujuan untuk menentukan ekspresi sitokin dalam jangkitan CMV primer dan bukan primer pada ibu hamil dan membandingkan ekspresi sitokin pada kedua-dua jenis jangkitan. Kajian keratan rentas ini dilakukan di Makmal Mikrobiologi, Hospital Universiti Sains Malaysia (HUSM) dari Jun 2019 sehingga Julai 2020. Keputusan kajian menunjukkan tujuh puluh empat wanita hamil dengan hasil kehamilan yang tidak normal dengan IgG CMV positif dengan atau tanpa IgM telah menjalani ujian aviditi IgG dengan kaedah pemeriksaan elektrokemiluminesensi (ECLIA) untuk membezakan jangkitan CMV primer dan bukan primer. Kajian juga menunjukkan sera pesakit dengan jangkitan CMV primer dan bukan primer tersebut menjalani pemeriksaan multiplex sitokin untuk analisis sitokin yang melibatkan tujuh sitokin dan dua kemokin untuk menentukan kepekatannya dalam jangkitan CMV primer dan bukan primer. Sitokin dan kemokin yang diuji adalah IL-12, IL-2, IFN- γ, TNF-α, IL-1β, IL-6, IL-10, IFN- γ, TNF-α, MCP-1 (CCL-2), dan IFN- γ IP-10 (CXCL-10). Tahap konsentrasi IL-1β, IL-6, dan

MCP-1 (CCL-2) meningkat dengan ketara pada wanita hamil dengan jangkitan CMV primer. Intensiti IFN-γ, IL-12, dan IL-2 lebih tinggi dalam jangkitan CMV primer berbanding dengan jangkitan CMV bukan primer. Penghasilan sitokin pro-inflammatori bersama-sama dengan kemokin MCP-1 (CCL-2) oleh wanita hamil dengan jangkitan CMV primer, adalah signifikan, di mana ia menunjukkan dominasi tindak balas Th1. Tahap sitokin yang rendah dalam jangkitan CMV bukan primer mungkin disebabkan oleh keadaan CMV jangkitan laten. Oleh itu, ia tidak mencetuskan pengaktifan semula sel-sel imuniti.

CYTOKINES EXPRESSION IN PRIMARY AND NON-PRIMARY MATERNAL CYTOMEGALOVIRUS (CMV) INFECTIONS

ABSTRACT

Cytomegalovirus (CMV) infection in pregnancy is the commonest cause of congenital infection worldwide. Primary CMV infection in pregnancy carries a higher risk of fetal transmission compared to non-primary infection. Primary infection in the early trimester of pregnancy can cause serious complications such as miscarriages and severe congenital malformations. CMV infection changes Th1 and Th2 balance during pregnancy by altered cytokine (IL-8, IL-6) in placental cells. Thus, this study aims to determine the cytokines expression in primary and non-primary maternal CMV infections and to compare the expression of cytokines in both types of infection. This cross-sectional study was conducted at Microbiology Laboratory, Hospital Universiti Sains Malaysia (HUSM) from June 2019 until July 2020. The result of this study showed that seventy-four pregnant women with abnormal pregnancy outcomes with positive CMV IgG with or without IgM were subjected to IgG avidity assay by electrochemiluminiscence immunoassay (ECLIA) method to discriminate primary and non-primary CMV infection. Then, study also showed sera of the patients in primary and non-primary CMV infection were subjected to multiplex cytokine assay for cytokine analysis that involved seven cytokines and two chemokines to determine their concentrations in both primary and non-primary CMV infection. Cytokines and chemokines tested were IL-12, IL-2, IFN- γ, TNF-α, IL-1β, IL-6, IL-10, IFN- γ, TNF-α, MCP-1 (CCL-2), and IFN- γ IP-10 (CXCL-10). Concentrations of IL-1β, IL-6, and MCP-1 (CCL-2) were significantly elevated in pregnant women with primary CMV infection. The intensity

of IFN-γ, IL-12, and IL-2 was higher in primary CMV infection compared to non-primary CMV infection. The productions of pro-inflammatory cytokine together with MCP-1 (CCL-2) in pregnant women with primary CMV infection were significant, which showed the predominance of Th1 response. The low level of cytokines in non-primary CMV infection might be due to the latent state of CMV in a host. Thus, it does not trigger the immune host cells' reactivation.

CHAPTER 1

INTRODUCTION

1.1 Background

Cytomegalovirus (CMV) is ubiquitous β-herpesvirus that infects immunocompetent hosts and causes lifelong persistent infection (Patro *et al.,* 2019). Ljungman *et al*. (2002) stated that CMV infection is diagnosed by isolation of CMV virus or the existence of viral proteins or nucleic acid from body fluid or tissue. CMV can cause severe diseases in the offspring of pregnant women with CMV infection (Dollard *et al.,* 2007; Kenneson and Cannon, 2007). CMV infects a high percentage of individuals throughout their life, and after disease recovery, it hides in leukocytes and become latent (Elbushra *et al*., 2019).

CMV infection is endemic, especially in developing countries, and the virus seroprevalence differed among populations. CMV infects most of the human population, 50 to over 90% of the people, depending on the region (Nikolich-Žugich *et al*., 2020). The prevalence of CMV is also higher in lower socioeconomic groups (Mussi-Pinhata and Yamamoto, 2020). CMV can become a severe health problem and cause a burden to the economy and society. In Malaysia, states of Selangor and Wilayah Perseketuan, the seroprevalence of CMV was 92% (Camalxaman *et al*., 2012). CMV is the common cause of congenital infection, and the prevalence rate is 0.2% - 2.4% in newborns (Revello and Gerna, 2002; Uchida et al., 2019). In infants, CMV can cause jaundice, microcephaly, developmental delay, and hepatosplenomegaly (Naing *et al.,* 2016).

There are two types of CMV infection, which are primary CMV infection, and non-primary CMV infection (Prince and Lapé-Nixon, 2014). Primary CMV

infection is the infection in a previously seronegative person and non-primary CMV infection term as sporadic excretion of the virus in the presence of host immunity (Bonalumi *et al*., 2011). Primary CMV infection can also be documented by seroconversion or through the presence of IgM with low-avidity IgG antibodies (Griffiths *et al*., 2015). Non-primary CMV infection can be either due to reactivation of endogenous virus (latent) or reinfection from an exogenous virus (Boppana *et al.,* 2001; Ross *et al.,* 2010; Yamamoto *et al*., 2010). Reinfection means the infection with a new or different CMV strain (Ross *et al.*, 2010).

Reactivation is the presence of viral DNA in tissues without transcription or translations of lytic genes (Reeves and Sinclair, 2008). Although primary infection in pregnant women raises a significant risk of transmitting the virus to the fetus and diseases, the virus transmission to the fetus in women with non-primary infection is frequent (Mussi-Pinhata and Yamamoto, 2020). The maternal CMV seroprevalence increases with maternal non-primary infections and causing prevalence of cCMV infection increases. In addition, maternal preconceptional CMV immunity does not protect the fetus and maternal reinfection by a new strain is a major source of cCMV. When CMV is in a life-long latency state, active genome replication and viral progeny production are not detectable. However, CMV can sporadically reactivate in response to stress or differentiation changes in cellular signalling and viral progeny can shed from the asymptomatic host (Collins-McMillen *et al.*, 2018; Gupta and Mahmoud, 2020; Nikolich-Žugich *et al.,* 2020).

Cytokines are known as small-secreted proteins released by cells to regulate and influence immune responses (Takeuchi and Akira, 2010). According to Zhang and An (2009), other names of cytokines are lymphokine (cytokine made by

lymphocytes), monokine (cytokines produced by monocytes), chemokines, and interleukin. Interleukins are secreted proteins that will bind to their specific receptors and play a vital role in the communications among leukocytes (Akdis *et al.*, 2011). Interleukins belong to cytokines superfamily and help induce and differentiate immune cells (Vaillant and Qurie, 2020).

Cytokines can either be pro-inflammatory or anti-inflammatory and classified according to their cells of origin or their mechanisms of action (Kassab *et al*., 2019). Proinflammatory cytokines are important for upregulation of inflammation and are produced mainly by activated macrophages reactions (Zhang and An, 2009). Over-production of pro-inflammatory cytokines can cause a "cytokine storm" and affect the host immune system (Srinivasan *et al.*, 2017; Tisoncik *et al.,* 2012). The term "cytokine storms" refers to inflammation due to the sudden release of cytokines to upregulate an inflammatory process (Tisoncik *et al*., 2012). High levels of circulating cytokines and immune cell hyperactivation can cause severe CMV infection (Fajgenbaum and June, 2020). Unregulated expression of MCP-1 (CCL-2) and TNF-α cause detrimental effects in pregnant women with CMV infection that lead to fetal injury and death (Hamilton *et al.,* 2012)

Anti-inflammatory cytokines are immunoregulatory molecules that regulate pro-inflammatory cytokines response (Zhang and An, 2009). Major anti-inflammatory cytokines include IL-10, IL-4, IL-11, and IL-13. In this research, only IL-10 was investigated. A study reported that among all anti-inflammatory cytokines, IL-10 is a cytokine with potent anti-inflammatory properties that help control and inhibit pro-inflammatory cytokines expression during the recovery phases of infection (Ouyang *et al*., 2011; Rojas *et al*., 2017). The interactions between pro-

inflammatory cytokines and anti-inflammatory cytokines help control the inflammatory response (Opal and DePalo, 2000).

Chemokines are a family of small chemoattractant cytokines that promote the migration and activation of leukocytes to the site of inflammation (Ferreira *et al.,* 2018). Examples of chemokines are monocyte chemoattractant protein [MCP-1 (CCL-2)], macrophage inflammatory proteins-1α (MIP-1α), and interferon-γ induced protein-10 [IFN-γ IP-10 (CXCL-10)]. This study focuses on two chemokines, which are MCP-1 (CCL2) and IFN-γ IP-10 (CXCL-10). These chemokines were selected from literature based on several studies (Hamilton *et al.*, 2012; Scott *et al.,* 2012; Weisblum *et al*., 2015).

Cytokines will activate the innate immune response and the development of adaptive antiviral immunity in reactions to the presence of the virus (Clement and Humphreys, 2019). If the cytokine production is uncontrolled, these processes can lead to an immune-driven pathological process. A study stated that CMV has a unique ability that able to evade host immune defenses while simultaneously changed the host immune systems (Nikolich-Žugich *et al*., 2020)

Various commercial assays were developed based on Immunoglobulin G (IgG) detection to determine CMV types in the infected person. This study used IgG Avidity assay to diagnose primary or non-primary CMV infection during pregnancy. Even though evidence of seroconversion is one of the confirmatory method to diagnose primary CMV infection, however it is not easily achieved as currently there is no health authority recommend it as a routine antenatal screening (Lazzaroto *et al.,* 2011). IgG avidity test is a useful method to differentiate primary CMV infection

from reactivation. Avidity refers to strength with which antibody bind to the antigen (Hazell, 2007). Low IgG avidity reflected weakly bound antibody to the antigen as it is produced during the first few months whereas high avidity prove that the antibodies are produced up to 6 months post-infection (bind tightly to antigen) (Hazell, 2007; Lazzarotto *et al*., 2008; Prince and Lapé-Nixon, 2014; Revello and Gerna, 2002). Low avidity is highly sensitive and specific to diagnose primary CMV infection (Manicklal *et al.,* 2013; Revello and Gerna 2002). CMV IgM is a poor detection marker to diagnose primary CMV infection since CMV IgM persist 6-9 months after primary infection and is detected during CMV reactivation (Lazzarotto *et al.,* 2011; Prince and Lapé-Nixon, 2014; Revello *et al.,* 2011).

Later, this study used the Multiplex cytokine assay to determine cytokines expression in both types of infection. Nine cytokines and chemokines were measured which were IL-2, IL-6, IL-12, IFN-γ, TNF-α, IL-1β, IL-10, MCP-1 (CCL2) and IFN- γ IP-10 (CXCL10).

1.2 Rationale of the study

CMV is a virus under the family of Herpesviridae that can encode many immune-evasion genes and triggers the presence of inflammatory cytokines during the infection (Clement & Humphreys, 2019). Cytokines play an essential role in CMV-induced pathologies and their regulation helps to fight against the disease.

Examining public health measures and encouraging the testing for CMV infection in pregnant women helps to predict risk of fetal transmission especially in pregnant women with primary CMV infection and to plan for further treatment of the neonates or infants. The antiviral treatment for prenatal and postnatal, was not

proven to prevent congenital and postnatal CMV infection effectively (Chiopris *et al.*, 2020).

There is no publishable research in Malaysia regarding cytokine expression in different types of CMV infection. We postulated that CMV infection, either primary or non-primary, liberated different cytokine profiles that may give new idea on the disease pathogenesis. Understanding the upregulation of the immune systems and expression of cytokines in various types of infection may help us understand the pathogenesis of viral infection.

1.3 Hypothesis

Primary CMV infection expresses higher level of pro-inflammatory cytokines and chemokines compared with non-primary CMV infection.

1.4 Study objective

1.4.1 General Objective

The general aim of this study is to evaluate the expression of cytokines in primary and non-primary maternal CMV infections.

1.4.2 Specific Objectives

1. To determine demographic data of study participants.
2. To determine the expression of the most dominant cytokines in maternal primary and non-primary CMV infection [IL-12, IL-2, IFN- γ, TNF-α, IL-1β, IL-6, IL-10, IFN- γ, TNF-α, MCP-1 (CCL-2), and IFN- γ IP-10 (CXCL-10)].

3. To compare cytokine expression in maternal primary and non-primary CMV infection [IL-12, IL-2, IFN- γ, TNF-α, IL-1β, IL-6, IL-10, IFN- γ, TNF-α, MCP-1 (CCL-2), and IFN- γ IP-10 (CXCL-10)].

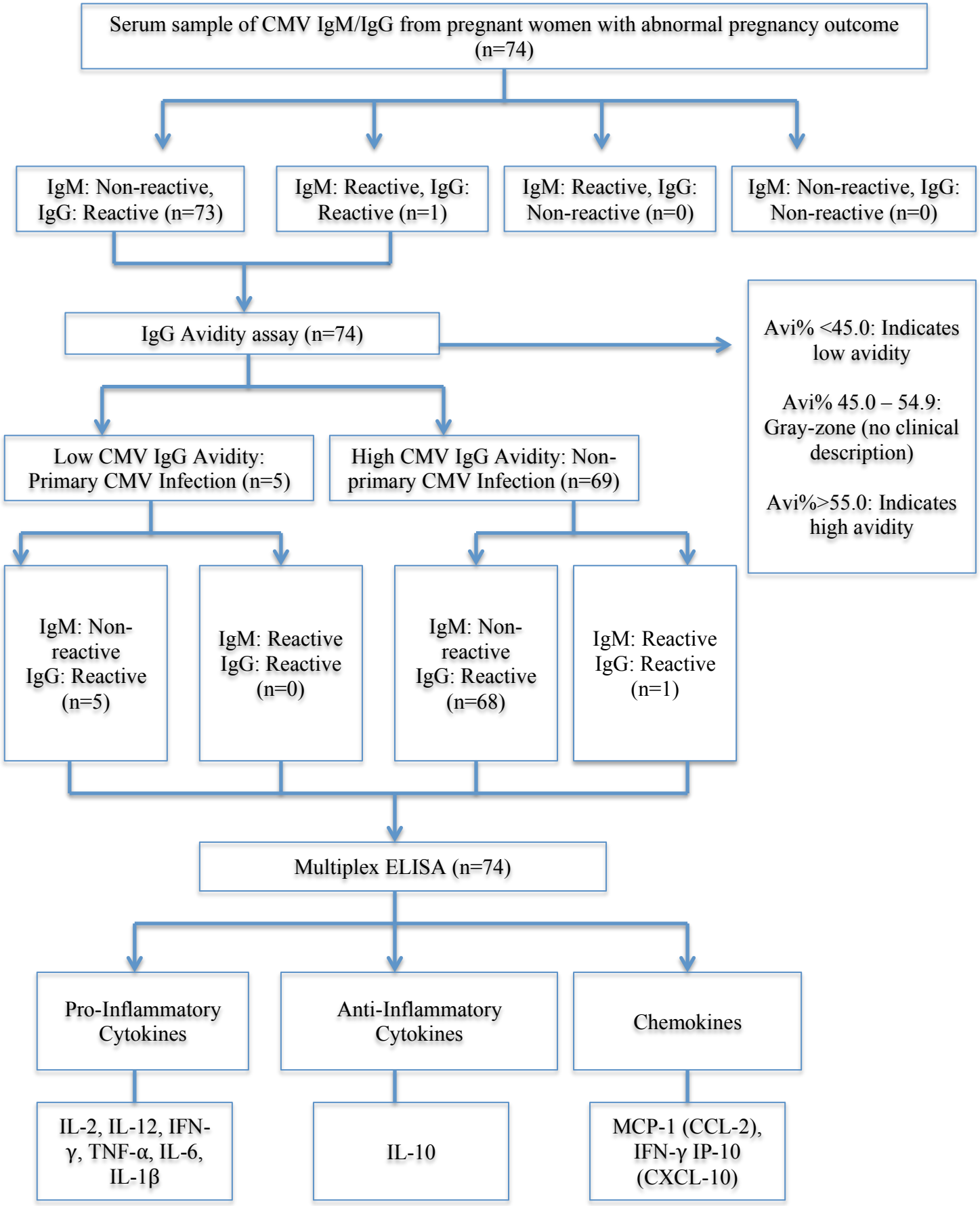

Figure 1.1 The flowchart of serological study

CHAPTER 2

LITERATURE REVIEW

2.1 Cytomegalovirus

Cytomegalovirus (CMV) belongs to the Herpesviridae family and possesses double-stranded DNA that is linear in shape (Brooks *et al.,* 2010). CMV was isolated for the first time in 1956, and the name derived from the fact that it causes the infected cell to expand (cytomegaly) (Schottstedt *et al*., 2010). This virus has the largest genome at 236 kilo-base pair (kbp) in size compare with other known human viruses with over 200 open reading frames (Dolan *et al.,* 2004; Gorzer *et al.,* 2015; Sijmons *et al*., 2015). An icosahedral capsid surrounds the genome with 162 capsomers (100-110 nm diameter), and a protein layer known as tegument is present between the capsid and the virus envelope (Seitz, 2010). CMV genome encodes 165 genes, four non-coding ribonucleic acid (RNAs), and 14 microRNA (miRNAs) (Forte *et al*., 2020). Five immediate early genes (0-4 hours after infection) are involved in transcription regulation, followed by early genes (4-48 hours after infection) that are involved in the replication of viral DNA and further transcriptional regulation (Torres and Tang., 2014).

CMV has two phases of the life cycle: lytic and latent (Poole *et al.*, 2011). The lytic stage is where the transcription of viral immediate-early (IE) gene, early gene and late genes resulting in the production of infectious virions (Poole *et al.*, 2011). CMV used a strategy known as molecular mimicry to regulate antiviral immunity and develop various functions that mimic host-encoded immunomodulatory proteins to latently infect the healthy human host (McSharry *et al*., 2012). CMV had the largest number of genes used to evade the host's innate and adaptive immune systems (Gupta

and Mahmoud, 2020). One of the critical characteristics of CMV infection is the potential to establish a long-life latent infection in the host (Dupont and Reeves, 2016).

2.2 Epidemiology

2.2.1 Prevalence

CMV had infected 50-90% of the world population depends on age and socioeconomic status (Cannon *et al.,* 2010). The prevalence of CMV is about 100% in Africa and Asia and 80% in Europe and North America (Al Mana *et al.*, 2019; Cannon *et al*., 2010). In Malaysia, the seroprevalence of CMV is about 92% in states of Selangor and Wilayah Persekutuan (Camalxaman *et al.*, 2012). Other studies showed that the seroprevalence of CMV in other countries varies widely such as Germany is 56.7% (Lachman *et al*., 2018), 22 to 66% in Ireland (Zuhair *et al*., 2019), 90% in Iran (Sharghi *et al*., 2019), 96.2% in China (Wang *et al*., 2017) and 100% in Thailand, Egypt, Iran and Turkey (Zuhair *et al.,* 2019) respectively.

About 59% of the population over six years old had been exposed to CMV, and infection may occur as a primary infection, reinfection, and reactivation (Gupta and Mahmoud, 2020). The adult female seropositivity ranges from 40% to 90% and at the highest level were found in individuals of lower socioeconomic background (Badami *et al.,* 2009; Bate *et al.,* 2010; Seale *et al*., 2006; Staras *et al*., 2008). Around 40% to 80% of pregnant women are susceptible to CMV at the early trimester of pregnancy (Petrov *et al.*, 2019). The seroconversion rate for seronegative females during pregnancy varies from 1% to 7% (Revello and Gerna, 2002).

2.2.2 Transmission and risk factors

There are two modes of CMV transmission, which are vertical transmission and horizontal transmission. Vertical transmission is defined as an infection transmitted directly from infected mother to the fetus or children (Jackson and Sparer, 2018). The vertical transmission rate is approximately 30-40% in early pregnancy of primary infection (Bhide and Papageorghiou 2008). Vertical transmission rate in women with primary infection is higher than in women with reactivated or chronic CMV infection (Jin *et al.*, 2017). Meanwhile, horizontal transmission is the transmission of CMV from one human to another that can happen through interaction with contaminated body secretions such as saliva, urine, and breast milk (Andronaco, 2020).

The transmission of CMV can occur through urine, saliva, or other bodily fluids of an infected person (Stowell *et al.*, 2014). It is reported that children who attend day-care centers have higher rate of CMV infection due to child-to-child transmission of CMV infection (Pass and Anderson, 2014). People who are in frequent contact with young children less than three years old such as women with child at home and day-care workers have higher risk of getting CMV infection (Adler, 2011; CDC, 2020). Young children are the common source of CMV and transmission occurs mainly through urine or saliva of the infected children (CDC, 2020; Navti *et al*. 2016). Children who are less than 2 years shed CMV for more than six months, and they have a higher viral load of CMV in saliva and urine than adults (Adler, 2011; Davis *et al*., 2017). The transmission from person to a person needs a very close contact (Petrov *et al*., 2019).

Primary CMV infection in pregnant women has approximately a 30% transmission rate of CMV to the fetus, and it depends on the gestational age (Boppana *et al.,* 2013; Enders *et al.,* 2011; Picone *et al.,* 2013). During pregnancy, the frequency of viral transmission increased from 30% during the first trimester to 70% in the third trimester (Bodéus *et al.,* 2010; Revello *et al.*, 2011). Primary CMV infection during the third trimester of pregnancy presents a greater chance of congenital transmission than in the first trimester of pregnancy (Munro *et al.,* 2005). About 0.15% to 2.0% of CMV primary infections occur in pregnancy, and the transmission rate to a fetus is 40% (Saraswathy *et al*., 2011). The transmission of CMV from pregnant women with primary CMV infection to fetus is about 30-40% and 1.1-1.7% for pregnant women with recurrent infection (Benoist *et al*., 2013; Hollier and Grissom, 2005; Kenneson and Cannon, 2007; Manicklal *et al.,* 2013; Rycel *et al.,* 2013).

CMV-infected pregnant women with Human immunodeficiency virus (HIV) have a higher risk of transmitting both viruses to the fetus and the risk of transmission was higher, especially in women who did not receive any antiretroviral therapy during pregnancy (Adachi *et al*., 2017). In-utero transmission can occur during primary infection and secondary infection, including in mothers who have prior immunity to CMV (Boppana *et al.,* 2001). Since the prevalence of CMV infection in the population was higher, most congenitally-infected infants are born to mothers with pre-existing immunity to CMV (Forte *et al*., 2020; Wang *et al.*, 2011).

2.2.2(a) CMV transmission to the fetus and infants

The maternal CMV transmission to the fetus occurs via placenta. CMV will attack the placenta and transmitted it to the fetus (Navti *et al*., 2016). There are two

routes of transplacental transmission of CMV to the fetus. The first one is across syncytiotrophoblasts and the second one is through invasive cytotrophoblasts within the uterine wall (Fisher, 2000). Syncytiotrophoblasts are specialized layers of epithelial cells covering the entire surface of the villous and in direct contact with maternal blood. Meanwhile, cytotrophoblasts are beneath the syncytiotrophoblasts and will continue to differentiate into syncytiotrophoblasts during villous formation and development (Wang *et al*., 2010).

Newborns get infected as CMV crosses via human placenta, birth canal passage during delivery or ingestion of infected milk (Kenneson and Cannon, 2007; Mocarski *et al.,* 2013; Roizman *et al.,* 2013). Intrapartum transmission occurs during birth as the virus sheds in the cervix, and 50% of neonates acquired the virus (Pass and Anderson, 2014). The neonates will begin to shed the virus at 3-6 weeks of age. Most of the infants infected during the first year of life through breast milk transmission, and will shed the virus in saliva or urine. Thus, they will spread the virus to the caregivers and other children that can cause great impact on CMV seroprevalence (Bardanzellu *et al.*, 2019; Pass and Anderson, 2014). Breast milk transmission might cause symptomatic CMV infection in preterm infants with low birth weight of less than 1500g and gestational age of less than 32 weeks (Rabe *et al.*, 2020). CMV excretion through breast milk was more than 30% from CMV seropositive mothers (Davis *et al.,* 2017; Dworsky *et al.,* 1983). CMV usually sheds in human milk from two to twelve weeks of postpartum, and more than 90% of CMV seropositive women have milk that tested positive by polymerase chain reaction (PCR) (Boppana *et al.,* 2001).

2.3 Types of CMV infection in pregnant women

There are two types of CMV infection in pregnant women, which are primary and non-primary CMV infection. Primary CMV infection in pregnant women carriers a greater risk for congenital infection than secondary infection; with the risk of vertical transmission is 30-40% and 1-2% for secondary infection (Bhide and Papageorghiou, 2008). Non-primary CMV infection is due to the reactivation of CMV that remains latent in the host cell or reinfection with a new viral strain (Ornoy and Diav-Citrin *et al.,* 2006). The reactivation of CMV from latency occurs when the virus is actively sheds the virus in various body fluids, including saliva, urine, tears, semen, cervicovaginal fluid, and breast milk (Steininger, 2007).

Latency is characterized by the absence or low level of replication of viruses and viral genomes within the nuclei of CD33+ and CD34+ bone-marrow cells and peripheral blood mononuclear cells (Martí-Carreras and Maes, 2019). The virus remains latent in lymphocytes, polymorphonuclear leukocytes, renal epithelium cells, and salivary glands (Yagmur *et al*., 2016). CMV is known for its ability to establish latent infection in bone marrow, stem cells, and myeloid cells (Jackson *et al*., 2017). The viral genome is retained in the host cell in the latent state without active replication or new viral progeny development. However, CMV can be reactivated from latency in response to stress or differentiation changes in cellular signalling (Collins-McMillen *et al.*, 2018; Gupta and Mahmoud, 2020).

2.4 CMV infection and its sequelae

The incubation period for CMV approximately one month from exposure to symptoms onset (Johnson *et al.,* 2012). CMV infection is usually asymptomatic in healthy individual. However, it can cause morbidity and mortality in immunocompromised patients such as pregnant women, HIV infected person, and transplant recipient (Dollard *et al*., 2007; Kedhar and Jabs, 2007; Torres-Madriz and Boucher, 2008; Tuthill *et al.,* 2009). CMV infection during pregnancy can cause birth defects and other health problems to the developing baby (CDC, 2020).

2.4.1 CMV in fetus

The fetal abnormalities are related to intrauterine CMV infection and can be detected using ultrasound, as it is a valuable tool for determining fetal growth (Naing *et al*., 2016). The presumed onset of maternal infection was around 14-15 weeks of gestation (Revello *et al.,* 2015). CMV cervical shedding was more common among pregnant women as pregnancy gestation advances; less than 5% in the first trimester, 6 to 10% in the second trimester and 11 to 28% in the third trimester (Cannon *et al.,* 2011; Davis *et al.,* 2017). In pregnant women, CMV infection causes severe diseases to the fetus, such as reduced growth, jaundice, enlargement of liver and spleen, and neurological damage (Ford *et al.,* 2013; Springer and Weinberg, 2004). CMV can also cause a higher risk of pregnancy loss (Petrov *et al.*, 2019). Pregnancies with primary CMV infection have a higher rate of miscarriage compared with secondary infection (Usta *et al.*, 2016). There is no serological immunity to CMV prior to conception in maternal primary CMV infection while in non-primary CMV infection the serological immunity is presence prior to conception (Britt *et al.,* 2018). Pregnant women with recurrent pregnancy loss (RPL) had a higher IgG titer level, which showed that they

had frequent exposure to CMV (Sherkat *et al.*, 2014). Therefore, recurrent exposure to CMV is a risk factor for RPL and it can occur in primary and non-primary CMV infection. CMV usually attacks the fetus's nervous systems, which cause miscarriages at early pregnancy and a high number of defects in newborns that lead to 30% mortality (Raynor, 1993). Asymptomatic newborns might have low birth weight and earlier gestational age than non-infected neonates, but this finding will rarely lead to CMV diagnosis (Davis *et al.*, 2017).

2.4.2 CMV in neonates and infants

Neonate is defined as a child under 28 days of age, whereas infants is defined as a child from 0 to 1 year of age (CDC, 2021; WHO, 2021). Congenital CMV (cCMV) infection is one of the common cause of sensorineural hearing loss (SNHL) and neurodevelopmental disabilities (Dobbins *et al.*, 2019). An estimated 13% of infected infants will develop neurological disorders at birth, and 10 to 15% will exhibit SNHL within the first two years of life (Itell *et al.*, 2017). Primary infection during the first and early second trimester has a higher risk of neurological sequelae that varies between 14.2 to 52.4% (Kenneson and Cannon, 2007). The cCMV infection symptoms include microcephaly, ventriculomegaly, increased periventricular echogenicity, periventricular pseudocysts, calcifications, and cerebral hemorrhage (Benoist *et al*., 2013; Malinger *et al.,* 2011).

cCMV infection may present with intrauterine growth retardation (IUGR), jaundice, petechiae, retinitis, hepatosplenomegaly, thrombocytopenia, and fetal death in cCMV infected infants (Angélica *et al.,* 2004; Boppana *et al*., 2001; Chakravarty *et al.*, 2005; De Vries *et al.,* 2011; Friedman and Ford, 1999; Fowler *et al*., 1997).

cCMV is symptomatic in about 10 – 15% cases, with 10% morality rate and 90% who survive will develop SNHL defects (Mocarski and Shenk, 2007). Although most of the newborns with cCMV are normal at birth and asymptomatic, 15% may develop hearing loss, vision damage, cognition and motor function (Dollard *et al.,* 2007). In symptomatic newborns, 35% develop hearing loss, 43% experience cognitive deficits, and 6% develop vision impairment (Bilavsky *et al*., 2016; Cannon *et al.,* 2014; Dollard *et al*., 2007; Goderis *et al.,* 2014). CMV can also cause severe diseases in premature infants, such as worsening respiratory status, neutropenia, bradycardia, and bowel distention (Buxman *et al.,* 2013; Kurath *et al.,* 2010).

If the symptoms of CMV such as influenza-like illness with the presence of abnormal sonographic findings is present during pregnancy, serological testing may be performed (Bonalumi *et al.*, 2011). The enzyme-linked immunosorbent assay is the common method that had been used to detect CMV antibodies (IgM and IgG) (CDC, 2020). Infected mothers may choose to do amniotic fluid testing to diagnose intrauterine infection by PCR but not routinely done due to its invasive nature of amniotic fluid sampling. It can be carried out after 21 weeks of gestation and at least six weeks after the maternal infection's presumed time (Cannon *et al.,* 2011; Griffiths *et al*., 2015).

Though CMV is not highly contagious, it has spread across households and children in day-care facilities (Petrov *et al.,* 2019). Practicing exemplary hygiene practices for newly pregnant mothers at risk have been seen to decreases the rate of maternal infection during pregnancy (Wizman *et al*., 2016).

2.5 Immune responses to CMV infection

CMV can also be transmitted from mother to fetus if the mother had a history of previous CMV infection and was immune at the time of conception (Pass and Arav-Boger, 2018). It had been suggested that these are because of the reinfection with new CMV strain also known as non-primary CMV infection (Yamamoto *et al.,* 2010). Premature newborns were not protected by maternal CMV antibodies as the passive transfer of antibodies to the fetus occur after 28 weeks (Bryant *et al.,* 2002).

Immune cells and cytokine signalling pathway act as mediators to promote healthy pregnancy (Yockey and Iwasaki, 2018). Pregnancy itself is associated with altered cytokine profiles. There is a complex interaction between innate and adaptive immune responses occur if the pregnant women infected with CMV.

During pregnancy, CMV infection will induce innate immune response in the placenta that will significantly changes the decidual cytokine and chemokine environment (Emery and Lazzarotto, 2017).

2.6 Innate and adaptive immune responses to CMV infection

Immunity is divided by two parts, which are innate and adaptive response. Innate immunity is the first line of defence against pathogen. It is non-specific defence mechanisms since it has no immunological memory and the host uses it immediately when the host encounter with antigen (Marshall *et al.,* 2018). The activation of Pattern recognition receptors (PRRs) is important in the initiation of innate immunity as it can recognize the specific molecular structure on the surface of pathogens (Jang *et al*., 2015; Li and Wu, 2021). Example of PRR families is Toll-like receptors (TLRs). As CMV enters the cell, the TLR 2 recognize the virions and activate several mechanisms

of innate immune response (La Rosa and Diamond, 2012). The activation of innate immune system includes recruitment of antigen presenting cells (APC), phagocytes (neutrophils and macrophage) and Natural killer (NK) cells to the site of infection (Marshall *et al.,* 2018; Isaacson *et al.,* 2008). When a virus is detected, APC will processed it to form many different fragment of antigen and stimulate the antigen specific immune response (La Rosa and Diamond, 2010). NK cells provide rapid cytotoxic function in host against viral infection (La Rosa and Diamond, 2012). NK cells and type I interferon (IFN) are key regulatory factors of early CMV control (Verma and Benedict, 2011). A study showed that CMV strongly induces NK receptor on T cells to dampen T cell activation when the viral load reducing and preventing harmful activation to the host in latent viral infections, which indicate the importance of NK cell in innate immune response (van Stijn *et al.*, 2008).

Adaptive immune responses are specific to the particular pathogen and are carried out by white blood cells knows as lymphocytes which are called B cells and T cells (Marshall *et al*., 2018). The APC surfaces express cell-surface proteins known as Major histocompatibility complex (MHC), which display fragments of antigen (Rock *et al.,* 2016; Warrington *et al.,* 2011). MHC class will identify the antigen and presented the antigen peptides on cell surface. $CD8^+$ T cells (cytotoxic T lymphocytes) will recognize the antigen presented with MHC class I meanwhile $CD4^+$ T cells recognize the antigen presented with MHC class II (Wieczorek *et al.,* 2017). T helper (Th) cells play vital role in maximizing immune response. It is activated when T-cell receptor (TCR) recognize the antigen bound to MHC class II molecules and as activated it release cytokines that influence the activity of many cells type such as macrophages and B cell (Marshall *et al*., 2018; Warrington *et al.,* 2011). T-cells can be divided into Th1 and Th2. CMV can interfere with the MHC class I and MHC class

II antigen presentation to avoid T cell recognition (Miller-Kittrell and Sparer, 2009). A study showed that CMV able to hijack host IL-10 by expressing a viral homolog of the immunomodulatory cytokine IL-10 during latency (cmvLA IL-10), which reduce the expression of MHC class 1 and class II molecules. CMV also inhibit the proliferation of peripheral blood mononuclear cells and the production of inflammatory cytokines (Jenkins *et al.,* 2008). CMV able to counteract host's immune response by encoding multiple proteins that homologous to cytokines, chemokines and their receptors (McSharry *et al.*, 2012). This showed that CMV able to manipulate and escape the host the host immune systems to allow the survival of the CMV within the host.

2.7 Secretion of cytokines in CMV

Cytokines are glycoproteins or regulatory peptides made by every cell in the body and have regulatory effects in many different cell types (McEwan *et al.,* 2009). Cytokine is secreted molecules that play a role on immune cells to coordinate and propagate immune responses within the body (Lee and Margolin*,* 2011). Other names for cytokine include lymphokine (cytokine made by lymphocytes), monokine (cytokines made by monocytes), chemokines, and interleukin (Zhang and An, 2009). Interleukins can either be pro-inflammatory or anti-inflammatory. It plays a role in the intercellular communication in the immune systems, such as cell maturation, cell proliferation, cell migration, and cell adhesion. It also plays a vital role in inflammatory responses (Vaillant and Qurie, 2020). The functions of IL include facilitate the communication among immune system cells, control of inflammation and transcription factors, cell and differentiation, and antibody secretion (Salazar *et al.*, 2007).

T lymphocytes are a significant source of cytokines. T lymphocytes are divided into two main subsets determined by the cell surface markers known as CD4 and CD8. Naïve $CD4^+$ T differentiation into helper T cells will be divided into Th1 and Th2, and the cytokines they produce known as Th-1 type cytokines and Th-2 types cytokines (Berger, 2000). Th1-mediated immune responses are essential for antiviral effects on the host and related to immunity or infection resistance, whereas Th2 cells influence with B-cells development and involved with persistence of infection by suppressing Th1 response (Vu *et al.*, 2014).

CMV has immune-modulatory properties that can change the host cell's innate and adaptive immune responses (Powers *et al.*, 2008). During pregnancy, HCMV infection can affect the cytokine profile within a placenta and shift the cytokine expression to a pro-inflammatory state with adverse consequences to the pregnancy outcomes (Hamilton *et al*., 2012; Scott *et al*., 2012). Cytokine plays a role in the reactivation and pathogenesis of CMV infection (Sakharkar and Deb, 2017). Clement and Humphreys (2019) mentioned that cytokine plays a role in the immune response by activating innate immunity and orchestrate adaptive immunity, but uncontrolled production of cytokine triggers off-target effects.

The virus-host immune system interaction is regulated by cytokines through the balance between Th1 that consists of pro-inflammatory cytokines, IFN- γ, TNF-α, IL-2, and Th2 anti-inflammatory cytokine such as IL-10 (Romagnani, 1994; Sakharkar and Deb, 2017). IL-6 and TNF-α are the essential pro-inflammatory cytokines liberated at the early stage of infection (Halwachs-Baumann *et al*., 2006).

2.7.1 Secretion of cytokines in primary CMV infection

Primary CMV infection can be defined as infection in a previously seronegative person or the presence of CMV IgM pregnancy with low IgG avidity (Prince and Lapé-Nixon, 2014). The diagnosis of maternal primary CMV infection relies on virus-specific Immunoglobulin (IgG) antibodies in pregnant women who previously seronegative or presence of IgM antibody with low IgG avidity (Sonoyama *et al.,* 2012). Type 1 cytokine and chemokines levels such as INF γ IP-10 (CXCL-10) and MCP-1 (CCL-2) were increase during primary CMV infection after renal transplantation (Van de Berg *et al.*, 2010). A study by Scott *et al*. (2012) showed an increase in IFN-γ IP-10 (CXCL-10) only from sera of pregnant women with primary CMV infection compared to control.

2.7.2 Secretion of cytokines in non-primary CMV infection

Non-primary or secondary CMV infection means sporadic excretion of the virus in the presence of the host immunity. It may be due to either reactivation of an endogenous virus or exposure to a new virus strain from an exogenous source. (Bonalumi *et al.*, 2011; Britt, 2020). CMV reinfection is the condition where the host acquires a new or different CMV strain (Ross *et al*., 2010). CMV reactivation defined as if the two viral strains (prior and current) are found to be indistinguishable by sequencing specific regions of the viral genome using molecular techniques (Ljungman *et al.,* 2017). The expression level of IL-10 is higher during the CMV reactivation in immunocompetent critically-ill patients (Frantzeskaki *et al.*, 2015).

2.7.3 Secretion of pro-inflammatory cytokines in CMV

Pro-inflammatory cytokines are essential mediators against infectious pathogens in immune responses (Gram *et al.*, 2012). Pro-inflammatory cytokines activated by macrophages and play a role in the up-regulation of inflammation (Zhang and An, 2009). These cytokines serves to localize and resolve the inflammatory foci by activating a systemic inflammatory response (Srinivasan *et al.,* 2017). Examples of pro-inflammatory cytokines are IL-2, IL-6, IL-12, IFN-γ, TNF-α and IL-1β.

During pregnancy, CMV infection cause dysregulation of Th1 and Th2 cytokine by altering the cytokine expression in placental cells and increases the pro-inflammatory cytokines in amniotic fluid of congenital CMV infection (Scott *et al*., 2012). Thus, this will cause placental dysfunction and fetal injuries (Hamilton *et al*., 2012).

2.7.3(a) IL-2

IL-2 is produced mainly by activated CD4+ T cells, CD8+ T cells, and also can be expressed by dendritic cells (Ferreira *et al.*, 2018). IL-12 is a pro-inflammatory cytokine that can affect the Th1 cells involved in the host immune response against infection (Pijanowska *et al.*, 2020). IL-2 contributes to the development of regulatory T cells that regulate the expansion and apoptosis of activated T cells (Capobianco *et al.,* 2016; D'Souza and Lefrancois, 2003). IL-2 secreted by adaptive CD4+ T cell enhanced the activity of NK cells after re-exposure to CMV (Wu *et al*., 2015). It plays a role in NK development, proliferation, survival, and cytotoxic activity, helping pathogen clearance (Becknell and Caligiuri, 2005). A study reported that the

production of IL-2 by T cell is crucial to stimulate CD4+ and CD8+ T cell proliferation (Salkowitz *et al*., 2004).

2.7.3(b) IL-6

IL-6 is produces by different cells such as monocytes and T-lymphocytes, which play a role in the interface of adaptive and innate immunity (Naila and Siyavash, 2014). IL-6 is responsible for first-line defence against the virus's spread and prevents the transmission from mother to child (Calquist *et al.,* 1999; Halwachs-Baumann *et al*., 2006). IL-6 triggers the recruitment, adhesion, activation and survival of neutrophil and innate lymphoid cell populations including NK cells (Rose-John *et al*., 2017; Vilaar-Fincheira *et al.,* 2021). In innate immunity, IL-6 regulates leukocyte transportation and activation (Hurst *et al.,* 2001). IL-6 together with TNF-α and IL-1 are primary mediators of pro-inflammatory response during CMV infection (Chinta *et al.,* 2020). In vitro study founds that CMV infection increased IL-6 in CMV-infected placenta cultures (Kovács *et al.*, 2007). During pregnancy, the high level of IL-6 together with TNF-α due to CMV infection lead to immune disturbance and might have effect in CMV intrauterine infection and fetal impairments (Zhang *et al*., 2008)

2.7.3(c) IL-12

IL-12 consists of p35 and p40 subunit which when combined together it will become bioactive IL-12p70 (Gee *et al.*, 2009). Popescu *et al*. (2016) stated that IL-12 regulates CMV-specific CD4+ T cell proliferation during acute primary infection. IL-12 is also known as a T cell stimulating factors, which together with IFN- γ, it enhances Th1 cell differentiation by activating T-bet and activate macrophages (transcription factor T-bet) (Muñoz-Carillo *et al.,* 2019; Placek *et al.,* 2009). IL-12 can