UNIVERSITI SAINS MALAYSIA

IDENTIFYING THE EFFECT OF BIOSYNTHESIZED AgNP FROM *GARCINIA ATROVIRIDIS* AQUEOUS EXTRACT ON INDUCED T-REGULATORY CELLS FROM NON-OBESE RESISTANCE (NOR) MOUSE MODEL

BY

NUR AZIRAH BT MD YUSOF

DISSERTATION SUBMITTED IN PARTIAL FULLFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER IN SCIENCE (TRANSFUSION SCIENCE)

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August 2018

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2018

DECLARATION

I hereby declare that this research has been sent to Universiti Sains Malaysia for the degree of Master of Science in Transfusion Science. It is not to be sent to any other universities. With that, this research might be used for consultation and can be photocopied as reference.

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NUR AZIRAH BT MD YUSOF

Matric No. : P-IPM 0043/17

Date : 8 AUGUST 2018

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Bismillahirrahmanirrahim

In the name of Allah, the Most Gracious, the Most Merciful

Alhamdulillah, all praises be to Allah, with his guidance and blessing, this dissertation is finally completed in time. This is all because of the strength, patience and wisdom bestowed by Him.

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LIST OF SYMBOLS AND ABBREVIATION

AgNP	-	Silver nanoparticles
NOR	-	Non-Obese Resistance
Th	-	T helper
Tcon	-	Conventional T cell
Treg	-	Regulatory T cell
iTreg	-	Induce regulatory T cell
nTreg	-	Natural regulatory T cell
ctrl	-	Control
STAT	-	Signal Transducer and Activator of Transcription
RORC2	-	Retinoic acid-related Orphan Receptor C
RORɤt	-	Retinoic acid-related Orphan Receptor gamma
TCR	-	T cell receptor
APC	-	Antigen presenting cell
IL	-	Interleukin
TGF-β	-	Tumour growth factor β
TNF	-	Tumour Necrosis Factor
CCL	-	50% of concentration causing lethal
CCR	-	Standard deviation
TIGIT	-	T-cell immunoreceptor with Ig and ITAM
ITIM	-	Immunoreceptor tyrosine-based inhibition motif
ICOS	-	Inducible co-stimulator
MHC	-	Major Histocompatibility
ECM	-	Extracellular matrix

ER	-	Endoplasmic reticulum
TAP	-	Transporter associated with antigen processing
TCR	-	T cell receptor
NFAT	-	Nuclear factor of activated T-cells
P38 MAP	-	*P38* Mitogen-Activated Protein
ERK 1/2	-	Extracellular signal-regulated ½
TLR	-	Toll-like receptors
P13K	-	Phosphatidylinositol 3-kinase
PIP_3	-	Phosphatidylinositol 3,4,5-triphosphate
PPAR	-	Peroxisome proliferator-activated receptor
RXR	-	Retinoid X receptor
PBS	-	Phosphate-Buffered Saline
FBS	-	Fetal Bovine Serum
DMSO	-	Dimethyl Sulfoxide
nm	-	Nanometers

ABSTRAK

CD4 + CD25 + Foxp3 + T *regulatory* (Tregs) adalah penting untuk keseimbangan imunisasi. Oleh itu, memanfaatkan fungsi mereka sebagai modulator imunisasi boleh digabungkan dengan perkembangan pesat pembangunan nanoteknologi. Dalam kajian semasa, nanopartikel perak biosynthesis (AgNP) daripada ekstrak berair *Garcinia Atroviridis* (GA) digunakan untuk mengenal pasti kesan sinergistiknya terhadap sifat-sifat modulasi sel Treg (iTreg) yang disebabkan oleh Model Tikus Bukan Obesiti (NOR). Sel CD4 + CD25- T konvensional telah diasingkan menggunakan pemisahan magnetik dari limpa tikus NOR. Seterusnya, 1x105cells / mL dirangsang dengan anti CD3 / CD28, TGF-β dan IL-2 untuk mendorong CD4 + Foxp3 + sel (iTreg). Sel-sel telah dirawat dengan atau tanpa GA-AgNP dan dibiakkan selama 120 jam. Ekstraselular dan molekular dinilai dengan menggunakan pewarna fluorochrome untuk mengesan CD4 +, Foxp3, IL-17R, ICOS, TIGIT, P38, STAT5 dan ERK1 / 2. Sel-sel telah dianalisis menggunakan sistem analisis BD FACS DIVA Cell analysis. Kumpulan kawalan ditetapkan sebagai CD4 + IL17Rhigh dan dirangsang dengan anti-CD3 / CD28 dan IL-2 sahaja. Kumpulan yang dirawat telah ditetapkan seperti berikut: Kumpulan 1. anti-CD3 / CD28, IL-2, TGF- β Kumpulan 2. anti-CD3 / CD28, IL-2, TGF- β, GA-AgNP dan 3. anti-CD3 / CD28, IL-2, GA-AgNP. Keputusan semasa menunjukkan bahawa GA-AgNP memodulasi beberapa molekul pada populasi sel CD4 + IL17Rhigh T yang terisolasi. Tahap ekspresi CD4 + telah dikurangkan dengan ketara dalam Kumpulan dan Kumpulan 3 yang dirawat dengan GA-AgNPs berbanding kumpulan kawalan. Begitu juga, Kumpulan 1 menyatakan corak ekspresi molekul hampir sama dengan kumpulan kawalan. Sebaliknya, faktor transkripsi Foxp3 tidak banyak berlaku dalam semua kumpulan berbanding dengan kumpulan kawalan. Ungkapan penanda

ICOS dan TIGIT dalam sel CD4 + IL17Rhigh dalam semua kumpulan tetap tidak berubah berbanding dengan kumpulan kawalan. Sebaliknya, faktor transkripsi Foxp3 dalam semua kumpulan tidak nyata disebabkan oleh kumpulan kawalan. Fosforilasi protein p38 dan ERK1 / 2 laluan MAPK tidak dapat dikesan dengan ketara dalam semua kumpulan sel berbanding dengan kumpulan kawalan. Tiada kumpulan sel menunjukkan tahap fosforilasi STAT5 yang signifikan berbanding dengan kumpulan kawalan. Penemuan ini mungkin mencadangkan GA-AgNP dalam memodulasi populasi sel CD4 + IL-17Rhigh dan tidak menjejaskan pembentukan CD4 + Foxp3 + sel. Oleh itu, penambahan GA-AgNP dalam persekitaran TGF-β / IL2 boleh mencetuskan kepekaan CD4 + sel ke dalam populasi yang berlainan. Kajian lanjut perlu mengenal pasti mekanisme molekul yang terlibat. Penemuan semasa boleh berfungsi sebagai asas untuk kajian imunotoxicity bahan nanom yang digunakan dalam produk bio-terapeutik.

Kata kunci: *modulator imunisasi, peraturan imunisasi, NOR, GA-AgNP, iTreg*

ABSTRACT

CD4+CD25+ Foxp3+ T regulatory cells (Tregs) are important for maintenance of immune homeostasis. Thus, harnessing their function as immune modulator may be coupled with the rapid advancement of nanotechnology development. In the current study, biosynthesized silver nanoparticle (AgNP) from aqueous extract of Garcinia Atroviridis (GA) was used to identify its synergistic effect on modulatory properties of induced Treg (iTreg) cells from Non-Obese Resistance (NOR) Mice Model. Conventional CD4+ T cells were isolated using magnetic separation from NOR mice spleen and then cultured. Next, 1x105cells/mL were stimulated with anti CD3/CD28, TGF-β and IL-2 to induce CD4+Foxp3+ cells (iTreg). The cells were treated with or without GA-AgNP and were cultured for 120 hours. Expression of extracellular and intracellular markers were evaluated by using fluorochrome dye to detect CD4+, Foxp3, IL-17R, ICOS, TIGIT, P38, STAT5 and ERK1/2. Cells were analysed using BD FACS DIVA Cell analysis system. Control group was set as CD4+IL17Rhigh and stimulated with anti-CD3/CD28 and IL-2 only. Treated groups were designated as following: Group 1. anti-CD3/CD28, IL-2, TGF- β Group 2. anti-CD3/CD28, IL-2, TGF- β, GA-AgNP and 3. anti-CD3/CD28, IL-2, GA-AgNP . Current results showed that GA-AgNP modulated the expressions of several molecules on isolated CD4+IL17Rhigh T cell population. Levels of CD4+ expression were significantly reduced in Group and Group 3 which were treated with GA-AgNPs as compared to control group. Similarly, Group 1 expressed almost similar patterns of molecular expression with control group. In contrast, transcription factor Foxp3 was not significantly induced in all groups as compared to control group. The expression of ICOS and TIGIT markers in CD4+IL17Rhigh cells in all groups remained unchanged as compared to control group.

In contrast, transcription factor Foxp3 in all groups was not significantly induced compared to control group. The phosphorylation of p38 protein and ERK1/2 of MAPK pathways did not significantly detected in all groups of cells as compared to control group. None of the cell groups showed significant STAT5 phosphorylation level compared to control group. These findings may suggest GA-AgNP in modulating CD4+IL-17Rhigh cell population and did not significantly induce the formation of CD4+Foxp3+ cells. Thus, the addition of GA-AgNP in TGF-β/IL2 environment may trigger the plasticity of CD4+ cells into different population. Further studies are necessary to identify the molecular mechanisms involved. Current findings may serve as a basis for immunotoxicity study of nanomaterial used in therapeutic bio-products.

Keywords: *immune modulator, immune regulation, NOR, GA-AgNP, iTreg*

CHAPTER ONE

INTRODUCTION

1.1 Background

Our immune system consists of innate immunity and adaptive immunity. Innate immunity is the first line defence and involves macrophages as well as neutrophils. It is difference for the adaptive immune system which it serves for second line defence and involves lymphocytes. For the adaptive immune system it has two different classes which are humoral immune response and cell mediated immune response. These responses carried out by different type of lymphocytes known as B cell lymphocytes and T cell lymphocytes (T cytotoxic cell and T helper cell) respectively (Alberts et al., 2007). B lymphocyte is important in producing the antibody which can eliminate extracellular microbes. Meanwhile T cytotoxic cell have an ability to kill intracellular microbes and T helper cell can activate macrophages to kill the microbes (Abbas et al., 2012). T helper cell also known as $CD4^+$ effector T cells and it can be further classified into T helper 1 (T_H 1) and T helper 2 (T_H 2) (Betteli et al., 2007). Later, the researchers found that CD4+ effector T cells also can be differentiated into T_H 17 as well as T reg (Langrish et al., 2005; Sergio et al., 2009).

Th17 is the subset of T helper cell and its production of from naive $CD4^+$ T cell occurs when IL-21, IL-6 and TGF-B phosphorylate the Signal Transducer and Activator of Transcription 3 (STAT 3) (Wei et al., 2007; Yang et al., 2008). The phosphorylations of STAT 3 will upregulates of RORC2 transcription factor inside the naive $CD4^+$ T cell (Wei et al., 2007). Retinoic acid-related Orphan Receptor C (RORC2) is the analogue Retinoic acid-related Orphan Receptor gamma (RORγt) genes in mouse which usually being used by researchers to study on Th17 cell. This upregulation will then produce

Th17 cell population. Later, IL-23 will help Th17 to stabilize and proliferate (Bettelli et al., 2007; Onishi and Gaffen, 2009). The Th17 is highly pro-inflammatory. It functions to kill the bacteria as well as involve in autoimmune disease (Wei, 2007; Pecks & Mallins, 2009). When the bacteria invade our body, antigen presenting cell (APC) (eg: lymphocytes, B cells, dendritic cells) will engulf and lyse it into the peptide. This peptide is then presenting on the surface of the antigen presenting cell (APC). However, Th17 need high dose of peptide for them to be stimulated (Pecks & Mallins, 2010). Once, the peptide being presented, the APC produce interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour growth factor β (TGF-β) (Pecks& Mallins, 2010; Onishi & Gaffen, 2010). As a result, Th17 responds to the APC-peptide complex by binding of co-stimulatory (CCL20 on the APC and CCR6 on the Th17 cell) (Onishi & Gaaffen, 2010). Th17 starts to produce IL-17 cytokine and will further kill the bacteria. IL17 have six family ligands which are IL-17A, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F. This IL-17 will further kill the bacteria.

Similar to Treg, it also is a subset of T helper cells. Treg can be natural regulatory T cell (nTreg) or induce Treg (iTreg). In this study, focus is more on iTreg which its induction need various signalling pathways. It involves stimulation of T cell receptor (TCR) on the surface of $CD4^+$ naïve T cells by TGF-β and IL-2 (Chen et al., 2003). This stimulation activates STAT5 and prostaglandin to transcribe Foxp3 gene and eventually produce $CD4^+$ $Foxp3^+$ T cell (iTreg) (Fantini et al., 2004; Zhu & Paul, 2008; Chen et al., 2011; Villarino et al., 2016). In contrast with Th17, iTreg acts as an anti-inflammatory (English et al., 2009). It also expressed T-cell immunoreceptor with Ig and ITAM (TIGIT) and inducible co-stimulator (ICOS) on its surface which has their own roles in immunology system (Kornete et al., 2012; Lozano et al., 2013).

The application of nanoparticles in boosting immune system regulation is now an emerging field. Nanoparticles may act differently towards innate and adaptive immunity (Luo et al., 2015). Previous study has shown that silver nanoparticles may help in immune regulation in the body (Catillo et al., 2008; Murphy et al., 2016). It induces IL-8 pro-inflammatory cytokines which activates phagocytic activity. Many studies have been conducted on the effect of silver nanoparticle to pro-inflammatory immune activity (Murphy et al., 2016). However, silver nanoparticle effects on anti-inflammatory immune activity still remain as hypothesis since none of the studies provide a clear evidence and yet to be discovered.

1.2 Aim

To understand the synergistic effect of silver nanoparticles (AgNP) synthesized from Garcinia Atriviridis in inducing iTreg from CD4+ IL17R^{high} cells.

1.2 Objectives

1. To identify potential use of Garcinia Atriviridis Silver nanoparticles (GA-AgNP) on induced Treg cells from CD4+ IL17Rhigh cells in Non Obese Resistance (NOR) mice.
2. To identify potential use of Garcinia Atriviridis Silver nanoparticles (GA-AgNP) on induced Treg cells from CD4+ IL17Rhigh cells in Non Obese Resistance (NOR) mice.

1.3 Hypothesis

Garcinia Atriviridis Silver nanoparticles (GA-AgNP) may have synergistic effect on iTreg induction from CD4+ IL17Rhigh cells in Non Obese Resistance (NOR) mice

CHAPTER TWO

LITERATURE REVIEW

2.1 Immune System

Immune system is a host defence system which protects against disease within the organism. It is a system that involves multiple components of cells, tissue and organ. It consists of innate immunity and adaptive immunity. The innate immunity is also called as natural immunity. It is essential for first line defence as it can invade the microbes in the first few hours or days. Macrophage is one of importance cells involve in this system. Macrophage are largely abundant in tissue especially in area easily get infected including lungs and guts. They also can be found in connective tissues, liver and spleen. Neutrophils also involves in innate immune system as a phagocytic. However, neutrophils only can be found in the blood but not in tissues. It only presents in infected tissues after being activated by macrophage or the molecules release by the microbes itself. These lead to microbe degradations. In contrast, for the adaptive immune system which it serves for second line defence and involves lymphocytes. Adaptive immune system is stronger and specialized which it capable to eradicate the pathogen that can resist the innate immune system (Abbas et al., 2012). It also can provide long lasting protection by production of memory antibody. For the adaptive immune system it has two different classes which are humoral immune response and cell mediated immune response.

The humoral mediated immunity, B cell will recognize antigens or pathogens which circulating in the blood or lymph. The production of B cells is originated from haematopoietic stem cell with the growth factors (cytokines). The haematopoietic stem cell in the bone marrow will transforms into common lymphoid progenitor and later to committed lymphoid progenitor cells which are pre-B cell. The pre-B cell is the undergo maturation process in the bone marrow. Once the B cell matured, it can be activated. In most cases, the activation of B cells needs interleukins and helper T cells as a co-stimulator. Once B cells activated, it will proliferate and produce plasma cell. Plasma cell later produce antibody which circulate in blood and activate the humoral immune response to eliminate extracellular microbes (Janeway et al., 2001; Alberts et al., 2007).

Meanwhile, the cell mediated immune response is mediated by T lymphocytes (T cytotoxic cell and T helper cell) which can eliminate intracellular and extracellular microbes (Betteli et al., 2007; Koretzky, 2010; Heberman, 2012). The T lymphocyte arises from bone marrow like B cell. However its maturation occurs at the thymus. The maturation of T cell started to differentiate into effector T lymphocytes ($CD4^+$ or $CD8^+$). The differentiation occurs with the help of cytokines (IL, TNF, and TGF-β). IL2 will help in producing $CD8^+$ (T cytotoxic cell) whereas IL-4 will produce $CD4^+$ (T helper cell). Matured T cell will also express T cell receptor (TCR) and CD4 or CD8 molecules. The TCR have ability to recognize and bind to major histocompatibility complex (MHC) derived complex on the antigen presenting cell (APC) through ligands binding (*Figure 2.1*) (Andersen et al., 2006; Eisenbarth & Hooman, 2011). T cytotoxic or $CD8^+$ effector T cell can recognize antigen peptide presented by MHC I (intrinscic pathway) while T helper cell which also known as $CD4^+$ effector T cells bind to MHC II (extrinsic pathway) (*Figure 2.2*). T helper cell can be further classified into T helper 1

(Th 1) and T helper 2 (Th 2) (Betteli et al., 2007). Later, the researchers found that T helper cell also can be differentiated into T reg as well as Th 17.

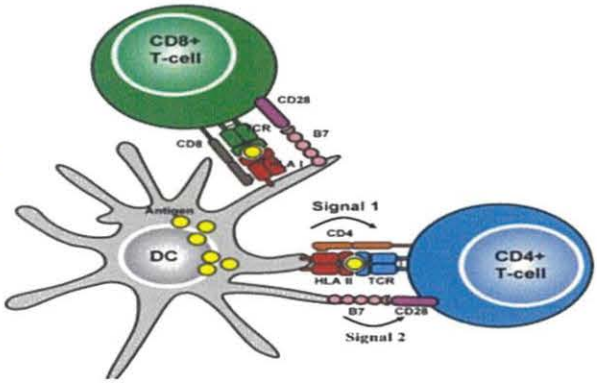

Figure 2.1 The binding and activation of CD8+ and CD4+ T cell to the APC are depend on the ligands binding (Source: http://knowledge-forlife.com/cd4-t-cell-activate-cd8-t-cell/).

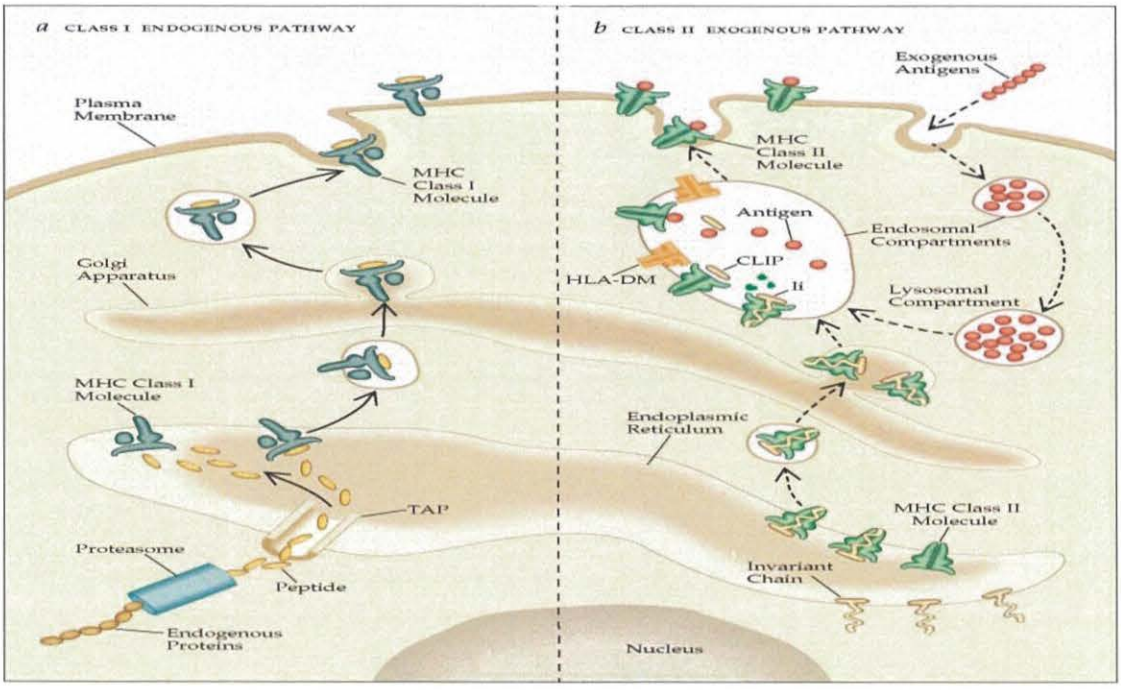

Figure 2.2 (a) Intrinsic pathway. The pathogens degraded by proteasome and enter endoplasmic reticulum (ER) through Transporter associated with antigen processing (TAP) to assembles with MHC I. The MHC I – peptide complex is then presented on the APC. (b) Extrinsic pathway. The APC engulf the pathogens and lysosome will surround the pathogens. The lysosome will secrete lysozyme to degrade pathogen into peptide. In the ER, the MHC II is form and release from ER. The MHC II is then enters the vesicles and release TAP. Once TAP released, the peptide bind to MHC II. The MHC II – peptide complex is present on the surface of APC (Betteli et al., 2007)

2.2 Immune Regulations of $CD4^+$ Th17

Production of Th17 cell from naive $CD4^+$ T cell occurs when IL-21, IL-6 and TGF-β phosphorylate the STAT 3 (Wei et al., 2007; Yang et al., 2008). Phosphorylation of STAT 3 will upregulate the Retinoic acid-related Orphan Receptor C (RORC2) transcription factor inside naive $CD4^+$ T cell (Wei, 2007). RORC2 is the analogue Retinoic acid-related Orphan Receptor gamma (RORγt) genes in mouse which upregulate Th17 cell production. In addition, IL-23 will help Th17 to stabilize and proliferate (Bettelli et al., 2007; Onishi and Gaffen, 2010). This T cell subset is highly pro-inflammatory. Its function is to kill the bacteria as well as involve in autoimmune disease (Wei, 2007; Pecks & Mallins, 2010). When bacteria invade our body, antigen presenting cell (APC) (eg: lymphocytes, B cells, dendritic cells) will engulf and lyse it into the peptide. This peptide is then presented on the surface of APC and recognized by Th17 cell by binding to co-stimulatory (CCL20 on the APC and CCR6 on the Th17 cell) (Pecks& Mallins, 2009; Onishi & Gaaffen, 2009). This cell starts to produce IL-17 where it will further kill the bacteria. However excessive response of Th17 can lead to autoimmune reaction. Thus, there is counter-check mechanism to overcome this. One of them is via production of T regulatory cell (Treg).

2.3 Immune Regulations of T Regulatory Cell (Treg)

Regulatory T cell or T reg is one of the $CD4^+$ subset. T regulatory ($CD4^+$ $CD25^+Foxp3^+$) can be divided into two which are natural T regulatory (nTreg) and induce T regulatory cell (iTreg). The nTreg is develops from T cell lienage inside the thymus while iTreg arise from conventional T cell ($CD4^+$ $CD25^-$ $Foxp3^-$) at the peripheral site (Lafaille & Lafaille, 2009; Schmitt & Williams, 2013). Treg cells play a role in regulating and suppressing other immune cells response.

In *in vitro*, the iTreg can arise when there is decrease or defective nTreg (Schmitt & Williams, 2013). Most of the immune depletion cases such as diabetes, arthritis and encephalitis there is absence of optimal condition to encountered foreign antigen (Korn et al., 2007). Thus, the body try to compensate it by inducing the production of iTreg from $CD4^+$ $CD25^-$ Foxp3- in the periphery which later become $CD4^+CD25^+$ $Foxp3^+$ (Apostolou & Boehmer, 2004; Schmitt & Williams, 2013). This induction is influence by T cell receptor (TCR) stimulation and cytokine involvement. The most essentials TCR stimulation and cytokines involve are anti-CD3, anti-CD28, IL-2 and TGF-β.

The stimulation of TCR on CD4+ naïve T cell can induce transcription factors Signal Transducer and Activator of Transcription 5 (STAT 5) and Nuclear factor of activated T-cells (NFAT) at Foxp3 gene with the help of TGF-β and IL-2 (Zhu & Paul, 2008; Chen et al., 2010). This event leads to the transcription of Foxp3 and form $CD4^+$ $Foxp3^+$ T cell (*Figure 2.3*) (Fantini et al., 2004, Villarino et al., 2016).

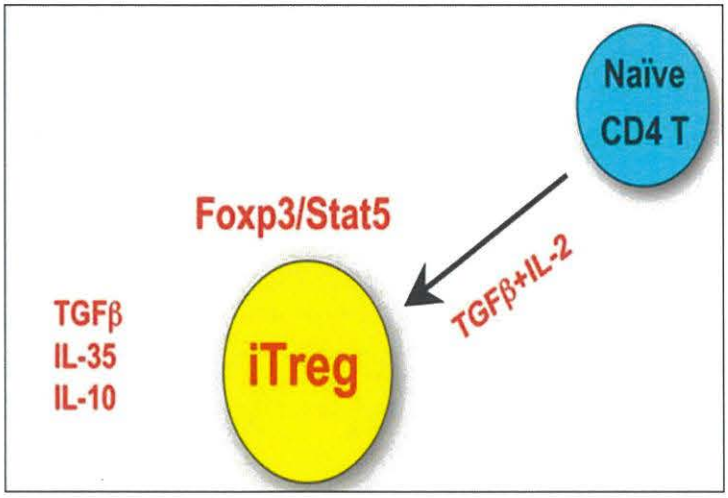

Figure 2.3: TGF-β and IL-2 cytokines involve in phosphorylation of STAT5 and Foxp3 leads to formation of iTreg. The iTreg later produce TGF-β, IL-35 and IL-10 (Zhu & Paul, 2008).

In addition, the CD3/CD28 TCR stimulation and TGF-β will activate P38 Mitogen-Activated Protein (P38 MAPK) kinases and extracellular signal-regulated 1/2 (Erk 1/2). These pathway activation help in iTreg cell growth, proliferation and survival (Pacini et al., 1998; Huber et al., 2008; Zeiser & Negrin, 2008).

2.4 Effect of Silver Nanoparticles (AgNP) Extracted From *Garcinia Atroviridis* to Immune Regulation.

Garcinia Atroviridis is natively come from Malaysia, India, Myanmar and Thailand where it live in humid forest. It is widely used as herbal medicine for antioxidant, antimicrobial and weight loss. This *Garcinia Atroviridis* has higher content of phenol which makes the silver ions inside it become more stable.

According to Bar et al. (2009), nanoparticles are particulate materials that have one dimension of less than 100 nanometers (nm). Metal nanoparticles have become the focus of intense work due to their various application as well as benefits (Rassaei et al., 2008). Nanoparticles have high surface area to volume ratio with decrease in the size of the particles which make them more effective to their activity (Bae et al., 2010; Liu et al., 2010; Munkuthan 2011). Silver, gold and platinum are most commonly used in medicine serving as a carrier for drug delivery (Munkuthan, 2011).

Silver nanoparticles (AgNP) is non-hazardous element and is use in many health studies especially as an antibacterial agent, wound dressing and burn injuries (Haase et al., 2014; Rai et al. 2009; Liu et al., 2010). It can activate Toll-like receptors (TLRs) which play an important role in innate immunity (Klippstein et al., 2014; Luo et al., 2015). TLR will recognize certain components on the surface of microbes and induces subsequent activation of adaptive immunity (Akira et al., 2006). Another study

conducted by Haase et al., 2014 and Murphy et al., 2016 show that AgNP responsible in formation of neutrophils and macrophages by activate pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α. These cytokines are essentials in production of Th17 which later acts as a pro-inflammatory cell.

In contrast, some of the study found out that, AgNP may also act as anti-inflammatory by inhibition of IL-6 secretion mediated by TLR (Castillo et al., 2008). Excessive of pro-inflammatory cytokines will lead to its inhibition and enhancement of anti-inflammatory by AgNP (Wong et al., 2009).

Thus, it is believe that AgNP extracted from *Garcinia Atroviridis* may give effect on the activity of TCR stimulations and formation iTreg.

2.5 TIGIT and ICOS ligands on the iTreg surface.

T-cell immunoreceptor with Ig and ITAM (TIGIT) was expressed on $CD4^+$ T Regulatory cell (Treg), natural killer cell and memory cell (Lozano et al., 2013). In Treg, TIGIT was expressed in natural Treg (nTreg) and induce Treg (iTreg). However, not all Treg express TIGIT but the study conducted by Joller et al. (2014) indicated that most of the Treg expressed TIGIT. TIGIT have immunoglobulin like structure and contains immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic (*Figure 2.4*)

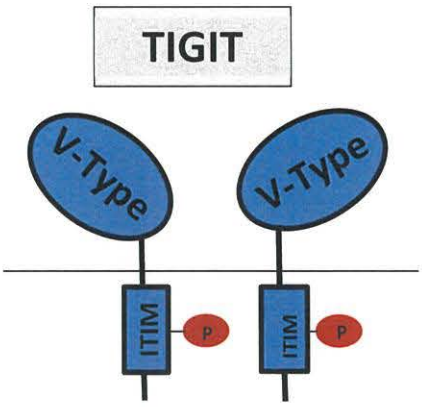

Figure 2.4: Structure of TIGIT (Source:https://www.omicsonline.org/articles-images/2155-9899-S12-004-g001.html).

Similar to CD28/CTLA4, this TIGIT interacts with two ligands which are Nectin (CD112) and PVR (CD155) (Stanietsky et al., 2013). Many studies proved that CD112 and CD155 exist on the surface of dendritic cell, natural killer cell, T cell, fibroblast, endothelial cell and some tumor cell (Pauken & Wherry, 2014; Yu et al., 2009; Lozano et al., 2012). According to Yu et al. (2009), TIGIT inhibit the activation of T cell through dendritic cell. It will compete with CD226 to bind to the CD112 and CD155 although TIGIT have higher affinity towards the ligands (Lozano et al., 2012;

Yu et al., 2009). CD226 is expressed on the activated T cells but not on Treg (Lozano et al., 2009). The binding of CD226 on the ligands will increase activation of natural killer cell and T cell in contrast with TIGIT (Hanaoka et al., 2004; Yu et al., 2009). Thus, the inflammatory response also increases. During the competition, TIGIT will prevent CD226 homodimerization and it's binding to the ligands. Instead of CD226, TIGIT binds to the ligands and inactivate the dendritic cell and T cell (Th1, Th2, and Th17 except Treg). Thus, the production of inflammatory response is decreases (Pauken and Wherry, 2014). For example in Th17, the binding of TIGIT to the ligands, will inhibit the production of IL6. Thus, the STAT3 cannot be phosphorylated and transcription factor RORγt/ RORCt will not be expressed (Qin et al., 2009). Due to this, the pro-inflammatory cytokines and inflammatory response will not produce.

Most of the studies conclude that binding of TIGIT to the ligands may decrease the proliferations of T cells (eg: Th 17, Th1 and Th2), secretions of IL2, (Lozano et al., 2012; Joller et al., 2014). In contrast, it promotes the Treg differentiation and increase the secretion of anti-inflammatory cytokines such as IL10, IL4 (Yu et al., 2009; Lozano et al., 2012; Joller et al., 2014). For example, TIGIT binding will upregulate the transcription factor Foxp3 genes and cause expression of Foxp3 to promote Treg cells (Kurtulus et al., 2015). Expressions of Foxp3 then cause Treg differentiation and proliferation (Villarino and Laurence, 2015).

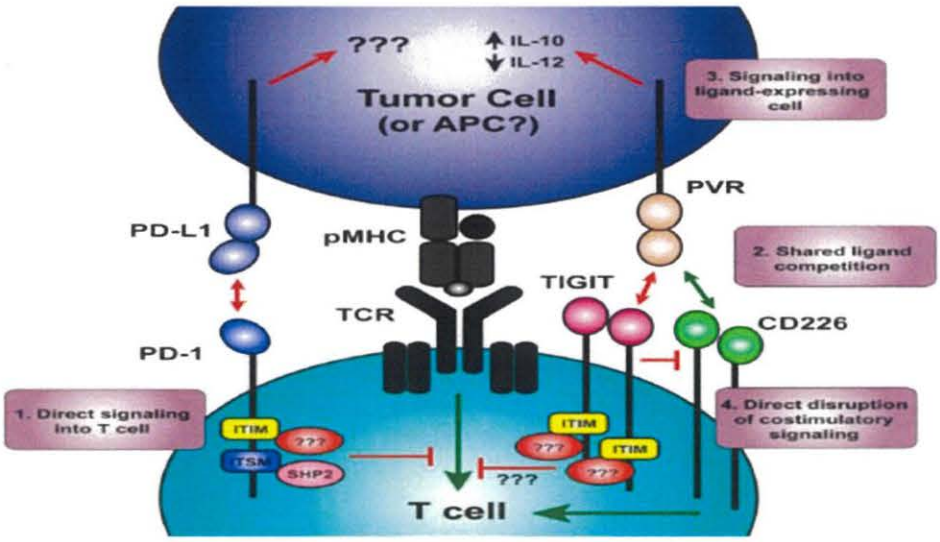

Figure 2.5: (2) Costimulatory (CD226) and coinhibitory (TIGIT) receptors that share ligands can compete for ligand binding (e.g., TIGIT and CD226 competing for PVR). (3) Inhibitory receptor binding can deliver an inhibitory signal to the ligand-expressing cells (e.g., TIGIT binding PVR induce functional changes in dendritic cells). (4) Novel mechanism of inhibitory receptor function identified by Johnson et al. (2014) where inhibitory receptors can directly block homodimerization of costimulatory receptors, mediating the inhibitory effect by preventing the costimulatory signal from acting (e.g., TIGIT physically preventing CD226 from signalling). Red lines indicate inhibitory signals; green lines indicate stimulatory signals (Pauken and Wherry, 2014).

In addition to that, Inducible co-stimulator (ICOS) also expressed on the iTreg surface. ICOS is one of the family members of CD28 which is homodimeric protein (Hutloff et al., 1999; Kornete at al., 2012). It contains YMFM motif in its cytoplasmic tail (Wikenheiser & Stumhofer, 2016) and is highly expressed on dendritic cell, macrophages, B cell, T cells (eg: Treg) and non-lymphoid cells (Herman et al., 2004; Burmeister et al., 2008). ICOS has significant homology to the co-stimulatory molecule CD28 and the immune-attenuator CTLA-4 (Wikenheiser & Stumhofer, 2016). It will bind to its ligands, ICOS-L (B7 homologous protein) and produce signal that induce the recruitment of phosphatidylinositol 3-kinase (P13K) which eventually will help in

production of membrane-bound phosphatidylinositol 3,4,5-triphosphate (PIP_3). This will lead to the activation of Akt-kinase which is important in promoting cellular proliferation and survival (Wikenheiser & Stumhofer, 2016).

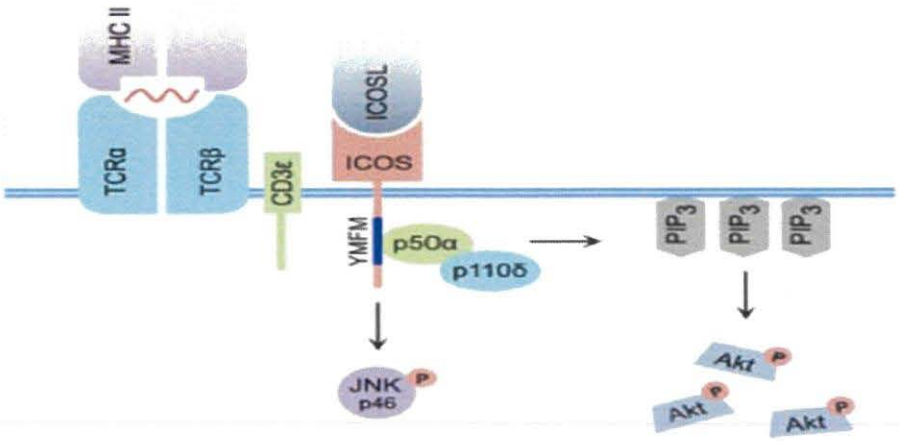

Figure 2.6: ICOS:ICOSL interaction induces P13K regulatory subunit recruitment via its YMFM signalling motif, resulting in strong PIP3 production and enhanced Akt phosphorylation (Wikenheiser & Stumhofer, 2016)

Many studies showed that ICOS can upregulate the inflammatory response by promoting Th1 and Th2 proliferation. Thus, the production of pro-inflammatory cytokines such as IL-4, IL-3 and IL-13 was increased (Burmeister et al., 2008). However, its role on Th17 remains controversial. The study conducted by Park et al. (2006) showed that ICOS is essential in the proliferation of Th17. It also reported that blockage of ICOS will increase pro-inflammatory cytokine such as IL-6. Other studies conducted by Kornete et al, (2012) and Wikenheiser & Stumhofer (2016) revealed that $ICOS^{+}Foxp3^{+}$Treg cells have higher production of IL-10 compared to $ICOS^{-}$

Foxp3^{+}Treg. Thus, it indicates that, ICOS expression may help in the activation of Treg and production of anti-inflammatory cytokines. In conclusion, ICOS may help in balancing hemostasis as it can produce pro-inflammatory and anti-inflammatory cytokines. A study done by Herman et al. (2004), found that the blockage of ICOS leads to the rapid onset of diabetes as it disrupt the balance of T effector and T reg.

2.6 Peroxisome-Proliferator Activated Receptor (PPAR)

Peroxisome proliferator-activated receptor (PPAR) is a three dimensional structure which form heterodimer with retinoid X receptor (RXR) and bind to specific DNA element called PPAR response element (Straus & Glass, 2007; Ehrenborg & Krook, 2009). This process leads to transcription of various genes involved in physiological processos (Gorniak, 2014). PPAR can inhibit the activation of Th17 by inhibit the pro-inflammatory cytokines (eg: IL17). It can be found in many tissues such as adipocytes, endothelial cells, hepatocytes and muscles (Straus & Glass, 2007; Gorniack, 2014). It also presents on dendritic cells, macrophages and T cells (Choi & Bothwell, 2012). Besides, PPAR may repress the transcription of certain genes which indicates the dual functions of PPAR. PPAR ligands comprises of three isoform which are PPAR α, PPAR γ and PPAR β/δ. These three isoform have different roles in tissue distributions, activation and proliferation (Gornick, 2014). Activation of PPAR α is capable in lowering the lipid levels while PPAR ɤ can regulate energy balance and adipogenesis. Whereas PPAR β/δ contributes in oxidation of fatty acid, regulates cholesterol level and blood glucose. Many studies have been done regarding the role of PPAR γ in inhibiting pro-inflammatory cytokines (Choi & Bothwell, 2012). PPAR γ negatively regulates T cell Receptor (TCR) stimulations and lead to inactivation of T cells. It also showed that PPAR γ reduces the production of IL-2 which is important in T cell differentiation and proliferation (Choi & Bothwell, 2012). A study by Klotz et al. in 2009 reported that

PPAR γ will reduce the TGF-β and IL-6 and inhibit transcription of RORγt. Inhibition of RORγt leads to inactivation of Th17 and its pro-inflammatory cytokines in autoimmune disease.

In autoimmune disease, the inhibitory blocking could limit the activation of Th17 to regulate the physiological balance in the body. For example, the person suffer from encaphilitis indicates higher pro-inflammatory cytokines such as IL-6, TGF-β and IL-17 (Klotz et al., 2009; Biswas et al., 2010). However, the presences of PPAR can ameliorate the encephalitis. Research was done on the mice expressed PPAR γ shown the suppression of Th17 differentiation by inhibit transcription of RORγt (Kanakasabai et al., 2009; Klotz et al., 2009). Besides, TIGIT which present on Treg also have the ability to inhibit activation of Th17 in encephalitis. Mice with TIGIT -/- developed more severe encephalitis as there is no mechanism to regulate balance between pro-inflammatory and anti-inflammatory cytokines (Joller et al., 2011).

Similar condition was observed in rheumatoid arthritis. The Th1, Th2 and Th17 were highly activated and produced excess level of pro-inflammatory cytokines such as IFN-ɤ, IL-2, IL4 and IL-17 (Dolhain et al., 1996; McInnes et al., 2016, Zhao et al., 2016). These cytokines induce the expression of matrix metalloproteinases (MMP) and cause degradation of extracellular matrix (ECM) at the joint. The degradation of ECM eventually leads to the erosion of cartilage. Besides, IL17 also increased the resorption of bone which usually occurs in the patients with rheumatoid arthritis (Onishi & Gaffen, 2010). However, the presence of TIGIT on $CD4^+$ T cell can knockdown IFN-ɤ, IL-2, IL4 and IL-17 and increased secretion of anti-inflammatory cytokines, IL-10. The higher levels of pro-inflammatory cytokines are then regulate the ICOS-ICOSL signalling and cause activation of Treg. These condition leads to production of anti-inflammatory cytokines like IL-10. Some of the research indicated that activation of

Th17 was regulated by ICOS in normal conditions. However, in encephalitis, it was found the ICOS-/- mice expressed higher activation of Th17 and IL-17, IL-6 and TGF-β cytokines production. This proved that ICOS may inhibit the activation of Th17 and secretion of pro-inflammatory cytokines (Dong et al., 2001; Galicia et al., 2009).

Another autoimmune disease usually associated with imbalance of cytokines is multiple sclerosis. Multiple sclerosis occurs when there is imbalance in Th17 (pro-inflammatory cytokines) /Treg cells (anti-inflammatory cytokines). Basically, Treg cell expressed CD-39.This CD-39 plays a role in inhibition of Th17 cells. However, in multiple sclerosis patients, Treg cell is defective which cause disability for CD-39 to inhibit over production of Th17. This cause high concentration of pro-inflammatory cytokine IL-17 which leads to brain inflammation (Noack & Miossec, 2014).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 MATERIALS AND EQUIPMENTS

Table 3.1 presented the apparatus and equipments used during this experiment.

Table 3.1 List of Apparatus and Equipment

Apparatus and Equipment	Brand and Manufacturer
Centrifuge 5810R	Eppendorf, Germany
Water Bath	Memmert
Vortex Lab Dancer V	Yellowline, Germany.
Electronic weighing balance	Mettler Toledo, US
Cryovial	Favorit, Malaysia
Beaker	Duran, Germany
Falcon tube (15 ml and 50 ml)	Becton Dickinson, USA
12 and 24 -well tissue culture testplate	Fisher Scientific, US
Disposable syringe (30 cc/mL)	Terumo, Philippines
Micropipette	Eppendorf, Germany
Parafilm	Bemis, USA
Flow cytometer tube	BD Bioscience
Biosafety Cabinet	ESCO

MACS Stand Magentic Cell Separator	Miltenyi Biotech
Incubator	Thermo Scientific
Light Microscope	Carl Zeiss, USA
Alcohol Swab	Terumo Corporation, Philippines
Neubauer Improved Hemacytometer	Hirschmann EM Technicolor, Germany

Table 3.2 showed all the solvents and chemicals used during this experiment.

Table 3.2 List of Solvents and Chemicals

Solvents and Chemicals	Brand and Manufacturer
Sterile Phosphate Buffer Saline (PBS)	Sigma-Aldrich Co., USA
RPMI 1640	Gibco BRL, UK
Ethanol (absolute)	BDH Chemical, UK
Tryphan Blue 0.4%	Gibco BRL, UK
Transcription Factor Buffer Set	BD Pharmanigen
i. TF Fix/Perm Buffer (4x)	
ii. TF Diluent Buffer	
iii. TF Perm/Wash Buffer (5x)	
Antibodies	Miltenyi Biotec
i. Anti-CD4+ (APC)	
ii. Anti-FOXP3 (FITC)	
iii. Anti- ICOS (VIOBLUE)	
iv. Anti- TIGIT (PE)	
v. Anti-IL17 (VIOBLUE)	
vi. Anti- P38 (ALEXA FLOUR)	
vii. Anti-STAT5 (ALEXA FLOUR)	
viii. Anti-ERK1/2 (ALEXA FLOUR)	
Total Complete Media (TCM)	
Fetal Bovine Serum (FBS)	Gibco Thermo Fischer Scientific
Dimethyl Sulfoxide (DMSO)	Fisher Bioreagents, US

3.2 METHODOLOGY

3.2.1 Ethics approval.

Approval was obtained from Institutional Animal Ethics Committee, Universiti Sains Malaysia (USM) [USM/animal ethics approval/2015/97/704]. All protocols including the euthanasia and samples collection were carried out according to the recommendations of the Animal Ethics Committee.

3.2.2 Animal care.

Five female Non-Obese Resistance (NOR) mice were purchased from the Jackson Laboratory, Miane USA. The permission from Veterinary Department, Ministry of Agriculture, Malaysia at Seberang Jaya District, Penang was obtained to import the animals. These animals were acclimatised at the Animal Research Centre, USM, prior to commencement of the experiment. The animals were housed in the cage with dimension of 29.5 cm × 12.5 cm × 15.5 cm and bedded with sterile dry sawdust. The bedding was replaced twice a week and the floor of the room was sterilized with disinfectant weekly to decrease risk of infection. The animals were given standard contamination-free solid mouse chow and water *ad libitum*. Temperature of the room was kept at 18-26°C with humidity of 40-70% for 24 hours. Each NOR mouse was weighed every week to monitor health conditions.

3.2.3 Samples Collection.

The NOR mice was sacrificed by cervical dislocation and signs of death such as absence of respiratory movement and heart rate were observed prior to sample collection. The spleen of NOR mice was harvested.

3.2.4 Negative and Positive Isolation of $CD4^{+}CD25^{-}$

Once the spleen sample collected, the spleen was placed in felcon tube contained Total Complete Media (TCM). Then, spleen was excised into small pieces. The cell fragments of spleen were placed onto a cell strainer attached to felcon tube. These cell fragments were then pressed through the cell strainer using the plunger end of a syringe. The cells was washed through the cell strainer with excess isolation buffer and centrifuged at 300 x g for 20 minutes. The supernatant was aspirated out and added with 950 µL Fetal Bovine Serum (FBS) and 50 µLDimethyl sulfoxide (DMSO). The cell was then separated manually by magnetic separator, MACS multistand. Separated cells $CD4^{+}CD25^{-}$ were then cryopreserved at -80°C.

3.2.5 Thawed and Resting Cells

$CD4^{+}CD25^{-}$ cells in cryovial were thawed in the water bath at 37°C. The thawed cells was added with 10 mL of TCM and centrifuged at 300xg for 10 minutes at room temperature. The supernatant was aspirated and cell pallet was added with 1 mL TCM. The $CD4^{+}CD25^{-}$ cell was rest by incubated in 12 wells plate at 37°C overnight.

3.2.6 Pre-Cultured Cellular Profiling.

The rested $CD4^{+}CD25^{-}$cells were counted by using haemocytometer (*see appendix 1*). The 1×10^{5} cells were stained extracellularly and intracellularly to determine population of cell contained in the sample prior to the activation with cytokines and addition of GA-AgNP.

In extracellular staining, cell was stained with CD4+ (FITC), TIGIT (PE) and ICOS (Vioblue). The cell was placed in three flow cytometer tubes. Every tube was stained with different fluorochromes dyes. The cell was centrifuged at 300xg for 10

minutes. The supernatant was aspirated out and the cell was added with 90µL, 45 µL and 90 µL to $CD4^+$ (FITC), TIGIT (PE) and ICOS (Vioblue) staining respectively. The 5µL of antibody anti-TIGIT (PE) and 10 µL anti-ICOS (Vioblue) was mixed well with the cell. The tubes were then incubated in 2-8°C dark refrigerators for 10 minutes. After 10 minutes, the cells in the tubes was washed with 1 mL isolation buffer by centrifuged it to remove unbound antibody. The 500 µL isolation buffer was added to the cell and the tubes were then covered with aluminium foil to avoid light from bleach the staining.

For intracellular staining Foxp3 (APC) and IL-17 (Alexa Fluor 466), the cell were placed on two different tubes and centrifuged to get cell pallet. Then, 1 mL of Fixation/Perm Working buffer was added to make a hole on the cells. The mixture of cell and Fixation/Perm Working Buffer was incubated in 2-8 °C dark refrigerators for 30 minutes. After 30 minutes, the cell was washed with 1 mL Permeabilization Buffer Wash and centrifuged at 4 °C, 300xg for 5 minutes. The supernatant was aspirated out. The left cell pallet was then added with 80 µL of Fixation/Permeabilization Working Buffer. For Foxp3 (APC), 10 µL anti-Foxp3 (APC) was added to the cell while for IL-17 (Alexa Fluor 647), 1 µL Golgi Stop was added first. Both cell tubes were incubated in 2-8 °C dark refrigerators for 30 minutes. The cell were then washed with 1mL Permeabilization Buffer Wash and centrifuged at 4 °C, 300xg for 5 minutes to remove unbound staining. The supernatant was aspirated out. For Foxp3 (APC) staining tube, 500 µL of Fixation/Permeabilization Working buffer was added and the tube was covered with aluminium foil. For IL-17 (Alexa Fluor 466), it need further process by added 80 µL Fixation/Permeabilization Working buffer and 20 microlitre antibody anti-IL-17. The cell was incubated again in 2-8 °C dark refrigerators for 30 minutes. After that, the cell was washed by using 1 mL Permeabilization Buffer wash and centrifuged

at 4 °C, 300xg for 5 minutes. The supernatant was aspirated out and 500 µL of isolation buffer was added to the cell pallet. The tube was then covered with aluminium foil.

Besides single staining as mentioned above, mix fluorochromes dyes were also prepared. There are 3 tubes which were $CD4^+$(FITC) Foxp3(APC) TIGIT(PE), $CD4^+$(FITC) Foxp3(APC) ICOS(Vioblue) and $CD4^+$(FITC) IL-17(Alexa Fluor).

Table 3.3: Summary of Tubes Prepared

TUBES	STAINING
1	**Unstained**
2	**$CD4^+$ (FITC)**
3	**TIGIT (PE)**
4	**ICOS (Vioblue)**
5	**Foxp3 (APC)**
6	**IL-17 (Alexa Fluor 647)**
7	**$CD4^+$(FITC) Foxp3(APC) TIGIT(PE)**
8	**$CD4^+$(FITC) Foxp3(APC) ICOS(Vioblue)**
9	**$CD4^+$(FITC) IL-17(Alexa Fluor)**

All the flow cytometer tubes were read by flow cytometer BD FACSCanto II