THE EFFECT OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) ON THE EXPRESSION OF FOXP3, TIGIT, ICOS AND HISTONE PROTEINS IN NATURAL T-REGULATORY CELLS

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LIST OF SYMBOLS

-	negative/minus/to
#	Number
%	percentage
&	And
/	Slash
+	Positive
<	less than
>	more than
±	plus-minus
Δ	Delta
®/TM	registered trademark
°C	degree Celsius
cm	Centimeter
CO_2	carbon dioxide
G	Gauge
g	Gram
h	Hour
M	Molar
mg	Miligrams
ml	Milliliters
mM	Milimolar
mm	Millimeter
ng	nano gram
nm	Nanometer
O_2	Oxygen

U	Units
X	Times
xg	times gravity
α	Alpha
β	Beta
γ	gamma
δ	delta
ε	Epsilon
κ	kappa
μg	micrograms
μl	microliters
μM	Micromolar

LIST OF ABBREVIATIONS

15d-PGJ2	15-Deoxy-Δ(12,14)-prostaglandin J2
a.k.a	Also known as
AB	Assay buffer
ADP	Adenosine diphosphate
AF	activation function
AIRE	Autoimmune Regulator
AITD	Autoimmune Thyroid Diseases
Akt/PKB	Protein Kinase B
AMDI	Advanced Medical and Dental Institute
AMP	Adenosine monophosphate
APC	Antigen Presenting Cell
APC’	Allophycocyanin
ARC	Animal research centre
ATP	Adenosine triphosphate
BAFF	B cell activating factor of the tumour necrosis factor family
BBDP	Diabetes-Prone BioBreeding
Bcl-2	B-cell lymphoma 2
BD	Becton Dickinson
BRCA	Breast cancer
BRCT	BRCA1 C Terminus
BrD	bromodomain
BSA	Bovine Serum Albumin
BUZ	binding of ubiquitin zinc finger
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
CA	Capture antibody
CBP	CREB binding protein
CD	Cluster of Differentiation
Chomo	Chromatin Organization Modifier
CLA	Conjugated linoleic acid
CLIP	Class II associated Ii peptide

COX-2	Cyclooxygenase-2
CPG	Clinical Practice Guidelines
CPS	cataract-prone strain
CREB	cAMP Responsive Element Binding
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated molecule-4
CXCR3	Chemokine receptor 3
DA	Detection antibody
DAPI	4’,6-diamidino-2-phenylindole
DBC1	Deleted in breast cancer 1
DBD	Deoxyribonucleic acid binding domain
DC	Dendritic cell
DiCARE	Diabetes in Children and Adolescents Registry
DKA	Diabetic Ketoacidosis
DKK-1	Treg-derived membrane-bound Dickkopf-1
DMSO	Dimethyl sulphoxide
DN	Double Negative
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DS	Developing solution
DP	Double Positive
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EKLF	Erythroid Krüppel-like factor
ELISA	Enzyme-linked immunosorbent assay
ENaC	epithelial sodium channel
EOMES	omesodermin
ER	Edoplasmic reticulum
ERK	Extracellular signal-Regulated Kinase
ES	Enhancer solution
FACS	Fluorescence-activated cell sorting
Fas	first apoptosis signal
FasL	Fas Ligand
FBS	Fetal Bovine Serum

FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
GAD	Glutamic acid decarboxylase
GALT	gut-associated lymphoid tissue
GARP	Glycoprotein A repetitions predominant
GITR	glucocorticoid-induced TNFR family related gene
GM	ganglioside
GNAT	Gcn5-related N-acetyltransferases
GVHD	graft-versus-host disease
GW9662	2-Chloro-5-nitro-N-phenylbenzamide
GWAS	Genome-wide association study
HAT/KAT	Histone acetyltransferase
HCL	Hydrochloride acid
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α	hypoxiainducible factor 1α
HLA	Human Leukocyte Antigen
HSP	Heat Shock Protein
i.e.	That is
IAA	anti-insulinoma associated antigen 2
IACUC	Institutional animal care and use committee
IBD	Inflammatory Bowel Disease
ICAs	Islet cell antibodies
ICOS	Inducible T cell costimulator
IDF	International Diabetes Federation
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
ILC	Innate Lymphoid Cell
iNOS	Inducible nitric oxide synthase
IPEX	Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked

ITAM	immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
Itk	interleukin-2-inducible T-cell kinase
iTreg	inducible T Regulatory
KDM	K-demethylase
KMT	K-methyltransferase
LAG-3	Lymphocyte-activation gene 3
LAP	latency associated peptide
LBD	ligand-binding domain
LBP	Ligand binding pocket
LPS	Lipopolysaccharides
LRRC32	leucine rich repeats containing 32
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MBD	methyl-CpG-binding domain
MBT	Malignant Brain Tumor
MCF7	Michigan Cancer Foundation-7
MHC	Major Histocompatibility Complex
miRNA	microRNA
MOH	Ministry of Health
MOZ	monocytic leukemia zinc finger
mTEC	Medullary thymic epithelial cell
mTOR	mammalian target of rapamycin
MYST	MOZ, Ybf2/Sas3, Sas2 and TIP60
NAD	Nicotinamide adenine dinucleotide
NCoR	Nuclear receptor corepressors
ncRNA	non-coding Ribonucleic acid
NES	Nuclear Export Signal
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa B
NK	Natural Killer
NKT	natural killer T
NLS	Nuclear localization signal
NOD	Non Obese Diabetic (NOD/ShiLtJ)

NOR	Non Obese Diabetic Resistant (NOR/LtJ)
nTreg	natural T Regulatory
OGTT	Oral Glucose Tolerance Test
PBC	Primary Biliary Cirrhosis
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PCAF	P300/CBP-associated factor
PD-1	Programmed cell death-1
PE	R-phycoerithrin
PGE2	Prostaglandin E2
PGJ	prostaglandin
PHD	plant homeodomain
PI3K	phosphoinositide 3-kinase
PIC	protease inhibitor cocktail
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	PPAR-responsive elements
PSS	Progressive Systemic Sclerosis
PTM	post translational modification
PTPN22	Protein tyrosine phosphatase non-receptor type 22
PTX3	Pentraxin 3
PVR	Poliovirus receptor
QTLs	Quantitative Trait Locus
RA	rheumatoid arthritis
RAR	Retinoic Acid Receptor
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROR	RAR-related orphan receptor
RPMI	Roswell Park Memorial Institute
Rtt	regulator of Ty1 transposition gene product
RUNX1	runt-related transcription factor 1
RXR	retinoid X receptor
SAM	S-adenosyl-methionine
SDS	sodium dodecyl sulfate
SEM	Standard error mean

SHMT2	serine hydroxymethyltransferase 2
SIRT	sirtuin
SJL	Swiss Jim Lambert
SjS	Sjögren's Syndrome
SLE	Systemic Lupus Erythematous
SMRT	silencing mediator of retinoid and thyroid hormone receptor
SS	Stop solution
SP	Single Positive
SPF	Specific-pathogen free
SRC	steroid receptor coactivator
ssRNA	single stranded Ribonucleic acid
SUMO	small ubiquitin-like modifiers
T1D	Type 1 Diabetes
T2D	Type 2 diabetes
TAFII250	Transcription initiation factor TFIID subunit 1
TAP	Transporter associated with antigen processing
TBX/Tbet	T-box transcription factor
TCM	Total complete media
Tconv	T conventional cell
TCR	T cell receptor
Teff	T effector
TET	ten-eleven translocation
Tfh	T follicular helper cells
TFIIE	Transcription factor II E
TG2	Transglutaminase 2
TGF-β	Transforming growth factor-beta
Th	T helper
TIF-2	transcriptional mediators/intermediary factor 2
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TIL	Tumour infiltrating lymphocytes
TIP	Tat Interacting Protein
TNF	Tumor necrosis factor
TNFRSF18	Tumor necrosis factor receptor superfamily member18
Tr1	Type 1 regulatory T cells

TRAs	Tissue-Restricted Antigens
Treg	T Regulatory
TRPC5	Transient receptor potential canonical 5
TSDR	Treg-specific demethylated region
tTreg	Thymus Regulatory T cells
TZD	Thiazolidinedione
UC	ulcerative colitis
UHRF	ubiquitin-like, containing PHD and RING finger domain
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia
VAT	visceral adipose tissue
WB	Wash buffer
WHO	World Health Organization
α-TAT	Anti-α-Tubulin

KESAN RESEPTOR TERAKTIF PEMPROLIFERATOR PEROKSISOM GAMMA (PPARγ) KE ATAS EKSPRESI FOXP3, TIGIT, ICOS DAN PROTEIN HISTONE DALAM SEL T-REGULATORI

ABSTRAK

Sel Regulatori T semula jadi (nTreg) mewakili kira-kira 8-10% daripada jumlah populasi CD4+ T sel. Sel-sel ini penting untuk homeostasis imun dan pencegahan autoimun. Kajian terdahulu menunjukkan bahawa ligan reseptor teraktif pemproliferator peroksisom gamma (PPARγ) dapat menindas ekspresi FoxP3 dalam sel-sel nTreg setelah dikultur *in vitro* selama 72 jam. Kajian ini dilakukan untuk menjelaskan kesan-kesan ligan PPARγ pada ekspresi TIGIT dan ICOS dalam sel-sel nTreg dari mencit Balb/c. Kami juga menumpu kepada pengubahsuaian histon pada ekspresi gen FoxP3 dalam sel-sel nTreg berikutan rawatan ligan PPARγ dan perencatnya dalam mencit T1D. Limpa mencit Balb/c, Non Obese Diabetic (NOD) dan Non Obese Resistant (NOR) diekstrak melalui dislokasi serviks. Sel T CD4+CD25+ dipencil menggunakan MoFlow pengisih automasi ataupun MACS magnetik dan ketulenan dianalisis oleh sitometri aliran. Sel T CD4+CD25+ dikultur selama 72 jam dalam media RPMI dengan kehadiran antibodi anti-CD3/CD28 dan sitokin IL-2. Sel nTreg dari mencit Balb/c telah dirawat dengan atau tanpa 15d-PGJ2 atau ciglitazone untuk kajian mengenai profil penanda permukaan TIGIT dan ICOS. Sel nTreg dari mencit NOD/NOR dirawat dengan15d-PGJ2 atau ciglitazone dengan atau tanpa perencatnya, GW9662. Ekspresi protein FoxP3 diperhatikan oleh analisis imunofluoresensi dan aktiviti enzim histon diukur oleh ELISA. Dalam kajian model mencit T1D, kami mendapati berat badan NOD dan NOR meningkat selari dengan

peningkatan umur. Hiperglikemia NOD bermula pada umur 12 minggu. Pengisihan automatik adalah sebanding dengan cara MACS magnetik di mana efisiensi pengasingan sel-sel nTreg adalah lebih daripada 90% dan 40-70% daripada populasi ini adalah sel FoxP3+. Ligan PPARγ dan perencatnya tidak mempengaruhi rangsangan sel dan proliferasi dalam kultur *in vitro*. Kedua-dua 15d-PGJ2 dan ciglitazone tidak memberi kesan pada ekspresi molekul TIGIT dan ICOS dalam mencit Balb/c yang sihat. Analisis imunofluoresensi menunjukkan protein intraselular FoxP3 tidak berubah oleh kesan 15d-PGJ2 dalam kedua-dua kumpulan mencit NOD/NOR. Walau bagaimanapun, kombinasi 15d-PGJ2 dengan GW9662 telah memberi kesan balikan pada protein FoxP3 di NOR. Di samping itu, kami mendapati aktiviti HAT asetilasi histon tidak dikawal oleh ligan PPARγ dan GW9662 dalam mencit NOR. Tetapi aktivitinya menurun sedikit dalam mencit NOD. Sebaliknya, penemuan kami mengenai deasetilasi histon menunjukkan pengurangan aktiviti HDAC6 di kedua-dua mencit apabila sel-sel nTreg dirawat dengan 15d-PGJ2 dan ditindas oleh GW9662 berbanding dengan kumpulan yang tidak dirawat. Penemuan serupa dicatatkan pada aktiviti HDAC11 dengan rawatan yang sama. Kesimpulannya, ekspresi TIGIT dan ICOS tiada perantaraan dengan laluan PPARγ semasa keadaan tidak keradangan. Pengawalaturan ekspresi gen FoxP3 dikaitkan dengan aktiviti asetilasi HAT dan deasetilasi HDAC oleh ligan PPARγ, 15d-PGJ2 dan perencatnya GW9662 mempamerkan potensi tutur silang dengan enzim-enzim ini melalui laluan isyarat bebas PPARγ. Analisis lanjutan diperlukan untuk mengenalpasti peranan sebenar 15d-PGJ2 sebagai perencat HDAC, terutamanya dalam mengaktifkan kedua-dua pengaktif dan repressor untuk aktiviti enzim HDAC6/11 untuk pembangunan terapi berasaskan histon dalam model autoimun.

THE EFFECT OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) ON THE EXPRESSION OF FOXP3, TIGIT, ICOS AND HISTONE PROTEINS IN NATURAL T-REGULATORY CELLS

ABSTRACT

Natural T Regulatory (nTreg) cells represent approximately 8-10% of the total CD4+ T cell population. These cells are crucial for immune homeostasis and autoimmunity prevention. Previous study showed that peroxisome proliferator-activated receptor gamma (PPARγ) ligands suppress Forkhead box P3 (FoxP3) expression in nTreg cells following 72 hours *in vitro* culture. Current study was performed to elucidate the effects of PPARγ ligands on T cell immunoreceptor with Ig and ITIM domains (TIGIT) and Inducible T cell costimulator (ICOS) expressions in activated nTreg cells isolated from Balb/c mice. We also focused on histone modifications on FoxP3 gene expression in activated nTreg cells following PPARγ ligand, 15-Deoxy-△(12,14)-prostaglandin J2 (15d-PGJ2) and its inhibitor, GW9662 treatment in type 1 diabetes (T1D) mouse model. Spleens of Balb/c, NOD and NOR mice were harvested through cervical dislocation. CD4+CD25+ cells were isolated using MoFlow automated sorter or Magnetic-activated cell sorting (MACS) magnetic separation and purity was analyzed by flow cytometry method. Isolated CD4+CD25+ cells were cultured for 72 hours in supplemented RPMI in the presence of anti-CD3/CD28 antibodies and IL-2 cytokine. In Balb/c mice, sorted cells treated with or without 15d-PGJ2 or ciglitazone for TIGIT and ICOS surface marker profiling. In NOD/NOR mice, isolated cells were treated with 15d-PGJ2 or ciglitazone with or without GW9662 inhibitor. FoxP3 protein expression was

observed by immunofluorescence analysis and histone enzyme activities were measured by Enzyme-linked immunosorbent assay (ELISA) method. In T1D mouse model study, we found the body weight of NOD and NOR increased parellel by age. Hyperglycemia of NOD was started at age of 12 week old. Automated sorting was comparable with magnetic selection where the efficiency of nTreg cell isolation was more than 90% and 40-70% of the population was FoxP3+ cells. PPARγ ligands and its inhibitor did not affect cell stimulation and proliferation *in vitro* culture. Both 15d-PGJ2 and ciglitazone had no effect on TIGIT and ICOS molecules expression in healthy Balb/c mice. Immunofluorescence analysis showed that intracellular FoxP3 protein was not altered by 15d-PGJ2 in both NOD/NOR mice. However, combination of 15d-PGJ2 with GW9662 had reversed effect on FoxP3 protein in NOR mice. In addition, we found histone acetylation HAT activities were not regulated by PPARγ and GW9662 in NOR mice. However it was slightly downregulated in NOD mice. In contrast, histone deacetylation shown reduction of HDAC6 activities in both mice groups when nTreg cells treated with 15d-PGJ2 and further suppressed by GW9662 compared to untreated groups. Similar findings were recorded on HDAC11 activities with the same treatment. As a conclusion, TIGIT and ICOS expressions are not mediated by PPARγ pathway during non inflammation condition. Regulation of FoxP3 gene expression attributed to HAT acetylation and HDAC deacetylation activities by PPARγ ligand and its inhibitor exhibit potential crosstalk with these enzymes through PPARγ-independant signaling pathways in T1D mouse model. Further analysis is required to corroborate the putative role of 15d-PGJ2 as HDAC inhibitor, particularly in profilling both activators and repressors for HDAC6/11 enzyme activities regarding histone-based therapy development in autoimmune models.

CHAPTER 1

INTRODUCTION

1.1 Study background

Immune system is a collective function mediated together by the molecules, cells, tissues and organs to provide immunity protection for the host from internal and external assaults. The response is regulated by stringent mechanisms in order to maintain balance between protection and self-destruction. One of the immune regulatory mechanism is through the suppressive effect of Treg cells on autoreactive T effector (Teff) cells. Treg cells naturally express surface markers CD4+CD25+ and intracellular transcription factor FoxP3 (Sakaguchi, 2005). These cells are originally developed in the thymus and mainly engaged in peripheral self-tolerance, homeostasis and prevent autoimmune disease through their five putative suppressive mechanisms (Sakaguchi *et al.*, 1995). In cancer, high number of nTreg cells are correlated with tumorigenesis which indicate poor prognosis (Adeegbe and Nishikawa, 2013). While in autoimmune diseases, depletion of CD4+CD25+FoxP3+ cell population lead to the development of autoreactivity.

FoxP3 expression in nTreg cells is transforming growth factor-beta (TGF-β) independant whereas for peripheral inducible T Regulatory (iTreg) cells required TGF-β and IL-10 for FoxP3 expression (Fahlén *et al.*, 2005). Treg cells secrete IL-10 and TGF-β which are involved in immunosuppressive towards dendritic cells (DCs) (Onishi *et al.*, 2008), stimulate T lymphocytes such as T helper 1 (Th1), Th2 dan Th17 cell proliferation (Corthay, 2009). They also suppress allergy reactions from mast cells, basophils and eosinophils (Palomares *et al.*, 2010). Naturally occurring mutations in the FoxP3 gene cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) in human and scurfy in mouse

model which accelerate the development of autoimmune diabetes (Bennett *et al.*, 2001; Chen *et al.*, 2005; Bacchetta, Barzaghi and Roncarolo, 2018). As the master regulator for transcription factor gene of forkhead box family, stable expression of FoxP3 is important to maintain its suppressive effect (Horwitz *et al.*, 2008; Chen *et al.*, 2011). The development of nTreg cells start in developing thymocytes and mature nTreg cells where Treg-specific demethylated region (TSDR) of the FoxP3 locus exhibit hypomethylation or demethylated at CpG motif (Floess *et al.*, 2007), thus expression of FoxP3 is regulated by both genetic and epigenetic factors. Both nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB) are the target protein suppressed by FoxP3 resulting in downregulation of other effector T cells cytokines including IL-2 (Bettelli, Dastrange and Oukka, 2005; Lopes *et al.*, 2006). As a transcriptional repressor, FoxP3 requires direct deoxyribonucleic acid binding domain (DBD) in regulating T cell activation (Schubert *et al.*, 2001) to form oligomeric complexes with other proteins dynamically.

Recently, FoxP3 expression was found to be regulated negatively by PPARγ ligands in activated nTreg cells through PPARγ-independant mechanism (s) (Nor effa, Yaacob and Norazmi, 2018). The role of PPARγ as an immune suppressor has been widely studied. Activation of PPARγ by its ligands lead to binding of PPAR/retinoid X receptor (RXR) heterodimer, subsequently conformational change of the ligand-binding domain (LBD) causes corepressor releasing bind on the coactivators resulting modulation of PPARγ activity (Peters and Heuvel, 2002; Laudet and Gronemeyer, 2002). Therefore, PPARγ becomes an important nuclear receptor in diabetes as it acts as insulin sensitizer (Kletzien, Clarke and Ulrich, 1992) besides playing roles in adipogenic differentiation, energy storage and fatty acid metabolism (Stump *et al.*, 2015). 15d-PGJ2 which is a natural ligand and prostanoids

to activate PPARγ translocation into nucleus (Forman *et al.*, 1995; Kliewer *et al.*, 1995). However, *in vivo* production of the 15d-PGJ2 is insufficient to be a significant agonist (Erzin and Cakir, 2017). Thus natural ligands could potentially provide beneficial effect as adjuvant factor for understanding the role of PPARγ and its ligands on FoxP3 expression in Treg cells which may benefit as a potential molecular target in treating immune-related diseases.

TIGIT molecules are expressed on Treg cells, NK and CD8+ T cells but not in B cells and monocytes (Yu *et al.,* 2009). Ligation of CD155 and TIGIT has been found to inhibit CD8+ T cell metabolism resulting in anergy antitumor immunity (He *et al.,* 2017). TIGIT molecules that are expressed on activated T cells interact with high affinity polio receptors on DC to suppress IL-10 production (Yu *et al.,* 2009). In cancer, enriched TIGIT signalling have been studied in tumor-infiltrating lymphocytes (TIL), this signalling is exclusively found in Treg cells but not in effector CD8+ T cells due to its malfunction (Kurtulus *et al.,* 2015). Recently, Liu *et al.* (2019) revealed administration of TIGIT-Ig effective in lupus treatment and prevention.

ICOS, homologue to CD28, is an inducible co-stimulator receptor expressed on activated T cells but not in naïve T cells (Hutloff *et al.*, 1999; Tafuri *et al.*, 2001). T cell activation cascade initiates the ligation of T cell receptor (TCR)-Major Histocompatibility Complex (MHC) class I/II complex following secondary co-stimulation signals for proper T cell responses, whereas Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) was found to attenuated this stimulation (Krummel and Allison, 1995; Greenwald, Latchman and Sharpe, 2002; Rudd and Schneider, 2003). Notably, ICOS was found to be crucial in maintaining Treg cells stability in T1D animal model (Kornete, Sgouroudis and Piccirillo, 2012). During pre-diabetes,

islet-derived ICOS+ Treg cells adopt a Th1-like Treg phenotype in immunoregulation (Kornete *et al.,* 2015) and Teff cells coexist with Treg cells where this depends on ICOS regulation (Herman *et al.,* 2004) in delaying the onset of T1D.

T1D is defined by World Health Organization (WHO) as deficiency of insulin production and requires administration of insulin by daily. Previously T1D known as insulin-dependant, juvenile or childhood-onset and is not preventable like Type 2 diabetes (IDF, 2017). The lacking of insulin production leads to hyperglycaemia, which cause various complications such as cardiovascular disease, neuropathy, nephropathy and retinopathy (IDF, 2017). According to International Diabetes Federation (IDF) Diabetes Atlas 8th edition (2017), T1D has been diagnosed in 169,900 children and adolescent in United States of America (USA), < 20 years old age group made up the highest in the world (IDF, 2017). Globally, it is estimated 96,100 children and adolescents aged < 15 years, and 132,600 of them with age range < 20 years to be diagnosed with T1D annually (IDF, 2017).

In 2015, Ministry of Health (MOH) Malaysia has published Clinical Practice Guidelines (CPG) regarding Management of T1D in Children and Adolescent to guide the clinical practitioners (MOH, 2015). According to Malaysian Diabetes in Children and Adolescents Registry (DiCARE) annual report (2008) published in the same CPG, 69.2% of children had T1D and 57.1% has presented with Diabetic Ketoacidosis (DKA); the ratio male to female was 1:1.2 indicating female (54.2%) higher than male (45.8%). Whereas, Malaysia National Diabetes Registry (volume 1, 2009-2012) has reported that 0.6% of 657,839 diabetes patients have been diagnosed as T1D. Hence, the early management of T1D is critical as development of DKA that has been associated with high morbidity and mortality (MOH, 2015; IDF, 2017).

Similar to diagnosing Type 2 diabetes (T2D), which is based on blood glucose test, however diagnosis of T1D requires detection of autoantibodies against 4 different markers: cytoplasmic protein in the pancreatic β-cell, glutamic acid decarboxylase (GAD), anti-insulinoma associated antigen 2 (IAA) and anti-insulin G (Taplin and Barker, 2008; Chiang *et al.*, 2018). Innate immune cells including DCs, macrophages and neutrophils infiltrate into the pancreas which attract the infiltration of T lymphocytes into islet cells eventually develop insulitis that contribute to the development of diabetes (Miyazaki *et al.*, 1985). T1D is a T-cell mediated disease, NOD mice that develop spontaneous autoimmune diabetes has favoured animal model to study T1D as compared to lymphopenic bio-breeding rat (Jackson *et al.*, 1981). In addition, NOD mice have similarities of immunopathology in human T1D (Pearson, Wong and Wen, 2016).

Epigenetics study involves covalent modifications of deoxyribonucleic acid (DNA) strands such as methylation, acetylation, ubiquitination, phosphorylation and SUMOlylation in gene expression without affecting the DNA sequence. Of note, four epigenetic mechanisms have been identified including DNA CpG methylation, histone post-translational modifications (PTMs), non-covalent mechanisms e.g. incorporation of histone variants, and non-coding Ribonucleic acids (ncRNAs) including microRNAs (miRNAs). It is well- known that this field has becoming the focus of worldwide researchers to investigate mesmerizing topics such as neuropsychiatric disorders, cancer, metabolic diseases, nutritional development and novel therapeutics. For example, T1D susceptibility correlates with loci-dependant methylation where data showed significant CpG count across Human Leukocyte Antigen (HLA)-DR-DQ locus by using bioinformatics. It was found that HLA-DR3-DQ2 and DR4-DQ8 cis-met Quantitative Trait Locus (QTLs) at CpG sites are

present in both children and adults. However, methylation pattern was found to be significantly different due to the difference in CpG site abundance between these groups which may contribute to development of diabetes (Kindt *et al.*, 2018). Hence, epigenetic factors may as well complement the genetic and environment factors contributing to disease development.

PTMs have been recognized strongly affects gene expression regulation in cancer (Chrun, Modolo and Daniel, 2017) and inflammatory diseases (Barnes, Adcock and Ito, 2005; Villagra, Sotomayor and Seto, 2010; Shakespear *et al.*, 2011). Histone acetylation is regulated by the opposite actions of histone deacetylases (HDACs) and histone acetyltransferases (HATs) enzymes (Brownell *et al.*, 1996; De Ruijter *et al.*, 2003). Therefore, development of histone deacetylase inhibitor (HDACi) as small-molecular to regulate these enzyme activities has become major interest among therapeutics and pharmaceutical fields. The synergistic effect of HDACi has enhance the efficacy of standard chemotherapy when use in combination (Suraweera, O'Byrne and Richard, 2018).

HATs covalently transfer acetyl group from acetyl-coenzyme A to lysine residues on proteins whereas HDACs remove acetyl group enabling the negatively charged DNA bind to nucleosome. In addition, HATs enabling transcriptional activation as a result of more relaxed chromatin but HDACs act as transcriptional repressors in multi-subunit complexes and gene silencing under closed chromatin condition (Cress and Seto, 2000). Based on phylogenetic classification, mammals have four classes of HDACs based on homology to the yeast original enzymes and domain organization (Dokmanovic, Clarke and Marks, 2007) while HATs are group into five families according their catalytic domain and substrate specificity (Allis *et al.*, 2007).

Primarily, cytoplasmic Class IIb HDAC6 modulates histone and non-histone protein with its unique of two functional catalytic domains (Zou *et al.,* 2006). Furthermore, inhibition of HDAC6 increases the immunosuppressive function of FoxP3+ Treg, thus promotes Treg-dependant suppression of autoimmunity and transplant rejection when bind to Heat Shock Protein (HSP) 90 (de Zoeten *et al.*, 2011). Thus, they are often deregulated in diseases and inhibition of their enzymatic activities by the lacking of DNA binding remains of therapeutic interest. On the other hand, HDAC11 initially was identified as negative regulator of anti-inflammatory cytokine IL-10 (Villagra *et al.*, 2009) until HDAC6 found physically interact with HDAC11 in nuclear compartments to modulate the expression of IL-10 (Cheng *et al.*, 2014). As the sole member of Class IV which is zinc-dependant HDAC, HDAC11 is an immunomodulator in DNA replication resides in nuclear (Gao *et al.*, 2002). HDAC11 was found to bind to FoxP3 and promote deacetylation to enhance FoxP3 expression that increases Treg cells suppressive function and gene expression when HDAC11 deletion (Huang *et al.*, 2017). In transplantation, deletion of HDAC11 in Treg cells can prolong allograft survival (Huang *et al.*, 2017). Thus, this inhibition of HDAC11 is important for the maintenance and development of Treg lineage (Mantel *et al.*, 2006; Huang *et al.*, 2017).

1.2 Study Objectives

Current study was performed to determine the influence of PPARγ activation in nTreg cells on TIGIT and ICOS expression as well as to elucidate its role in histone proteins regulation on the expression of FoxP3 in nTreg cells from autoimmune diabetic condition and its control strain, NOR. Understanding the relation between these transcription factors will help in underlining the potential

synergistic effect for the establishment of molecular therapeutic target in treating autoimmune diseases. In this study, we measured histone activities in these cells following treatment with PPARγ ligands and its inhibitor. Schematic workflow of the experiments is stated as in Figure 1.1.

The objectives of this study are:

1. To identify the effects of PPARγ ligands on TIGIT and ICOS expression in nTreg cells isolated from Balb/c.

2. To determine histones modifications on FoxP3 gene expression following PPARγ activation by its ligands in nTreg cells from NOD and NOR mice.

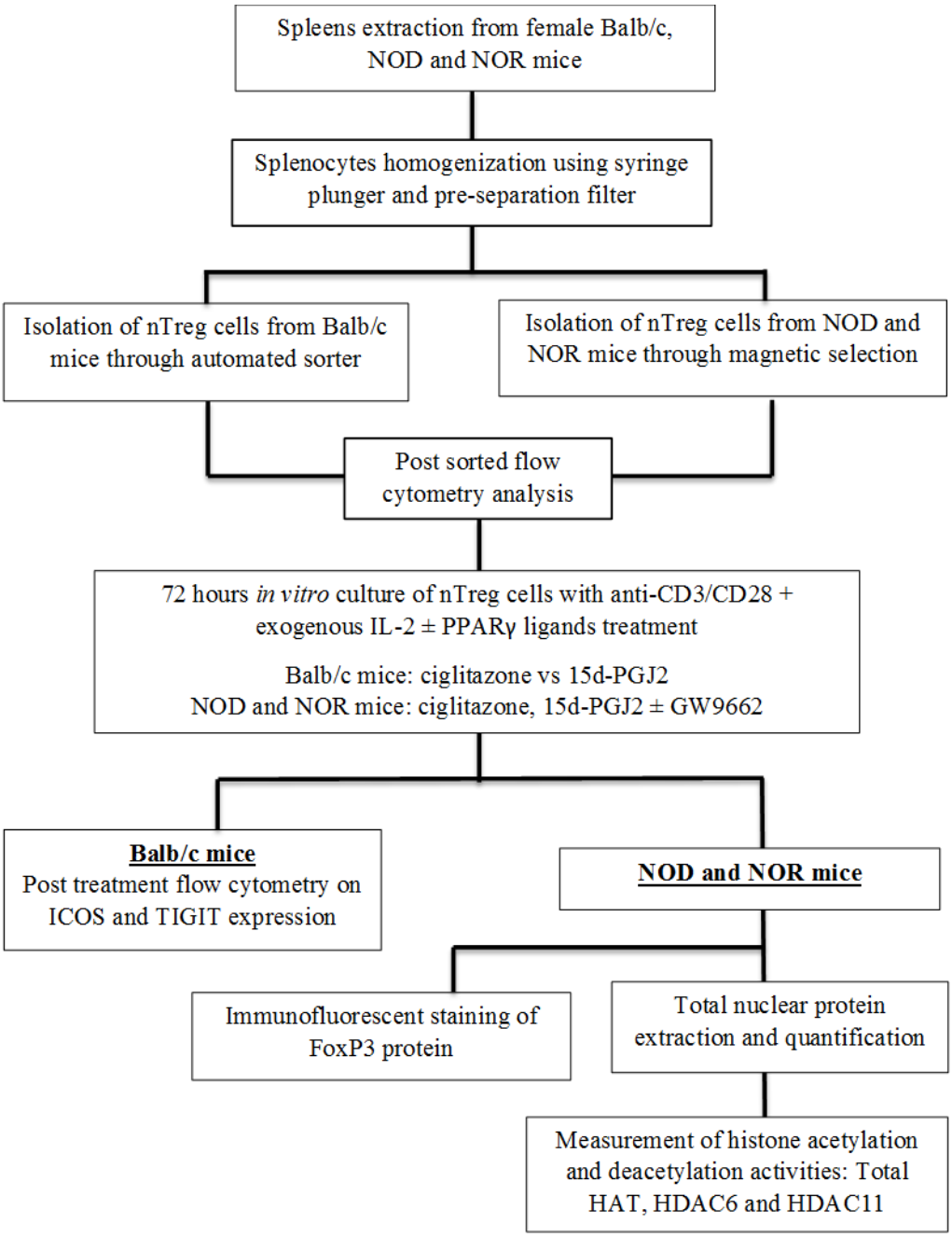

Figure 1.1 Schematic workflow of *in vitro* study in Balb/c, NOD and NOR mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of the immune system

Immune system is a host defence network which cells (lymphocytes, antigen-presenting cells, and effector cells), tissues, and organs that work together to protect the body from infections. It can generally be divided into two: innate also known as natural or native immunity and adaptive immunity also known as specific or acquired immunity (Figure 2.1).

Innate immunity is the first line host defence, capable of a rapid response to microbes and stimulate adaptive immune responses. Innate immunity components include epithelial barriers (skin or mucosa tissues) and cells (phagocytes, natural killer cells and the complement system) that protect the host agaist repeated infections and tissue injury (Abbas, Lichtman and Pillai, 2014). Thus, inflammation and anti-viral defenses are the two protective reactions in innate immune system.

Adaptive immunity is the second line of defence and comprises of two types of responses: humoral and cell-mediated immune response. This immunity requires clonal expansion and differentiation of the cells before it becomes effective (Alberts *et al.*, 2002; Abbas, Lichtman and Pillai, 2014). Memory cells will be developed from proliferated clones and provide rapid and specific response against similar pathogen in the future (Burnet and Fenner, 1953).

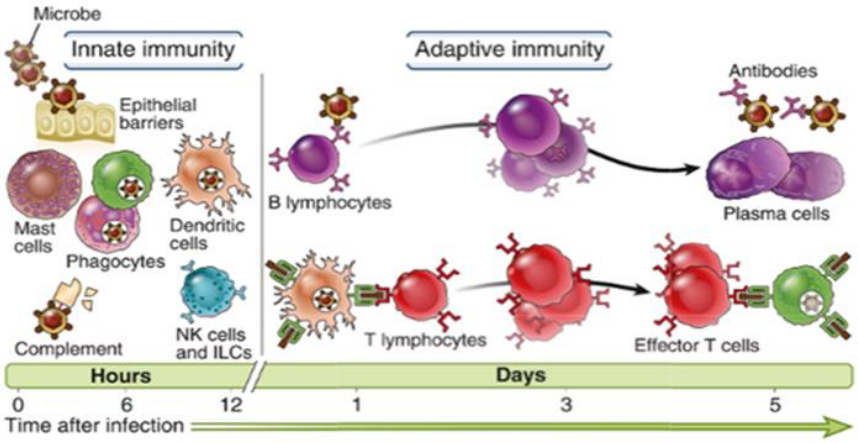

Figure 2.1 Principal mechanisms of innate and adaptive immunity. NK, natural killer; ILCS, Innate Lymphoid Cell. [Adapted from Abbas, Lichtman and Pillai, (2014)].

2.2 Antibody-mediated immunity

2.2.1 Antibody production by B cells

All lymphocytes arise from stem cells in the bone marrow (Abbas, Lichtman and Pillai, 2014). B cells undergo maturation in the bone marrow (generative lymphoid organ) and circulate to peripheral lymphoid organs (lymph nodes, spleen, mucosal and cutaneous lymphoid tissues).

Burnet and Fenner (1953) coined the clonal selection theory which explained that an individual B cell that produces all antibody molecules supposedly has the same antigen-binding site (Figure 2.2). The newly formed B cell is not secrete out its first antibody but this antibody will be inserted into the plasma membrane to be served as receptor for antigen (Alberts *et al.*, 2002).

When a naïve or memory B cell is activated by antigens, it proliferates and differentiates into an antibody-secreting effector cell (Alberts *et al*., 2002). Effector B cells can begin secreting antibody while they are still small cells, but the end stage of their maturation pathway is large plasma cells (Alberts *et al*., 2002; Abbas, Lichtman and Pillai, 2014). The continuously secretion of antibodies are short-lived plasma cells die after several days, some long-lived plasma cells survive in the bone marrow for months or years and continue to secrete antibodies into the blood (Abbas, Lichtman and Pillai, 2014; Churlaud *et al*., 2015).

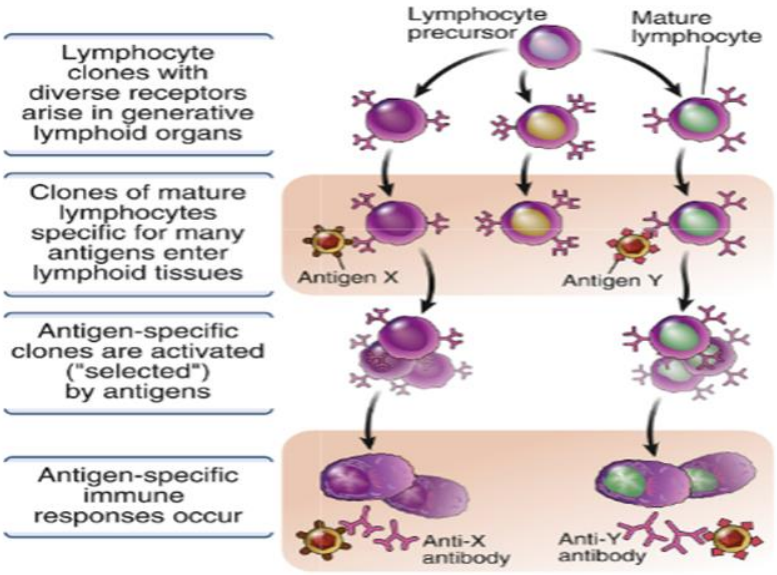

Figure 2.2 Clonal selection.
Mature cells with receptors for many antigens develop before encountering these antigens. A clone refers to a population of cells with identical antigen receptors and therefore specific to all of these cells are presumably derived from one precursor cell. Each antigen (e.g., X and Y) selects a pre-existing clone of specific cells and stimulates the proliferation and differentiation of that clone. The diagram shows only B cell giving rise to antibody-secreting cells, but the same principle applies to T cell. The antigens shown are surface molecules of microbes, but clonal selection is also true for extracellular soluble and intracellular antigens [Adapted from Abbas, Lichtman and Pillai (2014)].

2.3 T cell mediated-immunity

2.3.1 T cell development

T cell is derived from haematopoietic precursor cells which are found in the bone marrow. Progenitors of these cells undergo series of complex maturation stage in the generative lymphoid organs mainly thymus. Thymus is made up by an outer cortex, inner cortex and medulla region (Figure 2.3).

The earliest developing thymocytes are lack of the expression of TCR, CD4 and CD8, therefore termed as double negative (DN) cells at cortex region (Germain, 2002). DN population can be further sub-divided into 4 stages of differentiation: (DN1) CD44+CD25−; (DN2) CD44+CD25+; (DN3) CD44− CD25+; and (DN4) CD44− CD25− (Germain, 2002; Abbas, Lichtman and Pillai, 2014).

When thymocytes passage from DN2 to DN4, pre-TCR is expressed then undergo transition and replacement of α- and β-TCR chains which eventually progress to essential cell proliferation from DN4 to be αβ-TCR+CD4+CD8+ double positive (DP) (Germain, 2002; Abbas, Lichtman and Pillai, 2014). The interaction of DP cells with cortical epithelial cells associated to high expression of MHC class I and class II molecules with self-peptides.

The fate of DP thymocytes depends on the signal between TCR with these self-peptide-MHC ligands (Robey and Fowlkes, 1994). Low signalling lead to delayed apoptosis (death by neglect), while too high signalling can result acute apoptosis (negative selection). The appropriate intermediate levels of TCR signalling initiate effective maturation i.e. positive selection.

Thymocyte that express TCRs bind to self-peptide-MHC-class I complexes become CD8+ T cell, whereas those that express TCRs that bind to self-peptide-

MHC-class-II ligands become CD4+ T cell. Both cells are ready to migrate from medulla to peripheral lymphoid sites (lymph nodes, spleen, mucosal and cutaneous lymphoid tissues) as single positive (SP) (Germain, 2002; Abbas, Lichtman and Pillai, 2014).

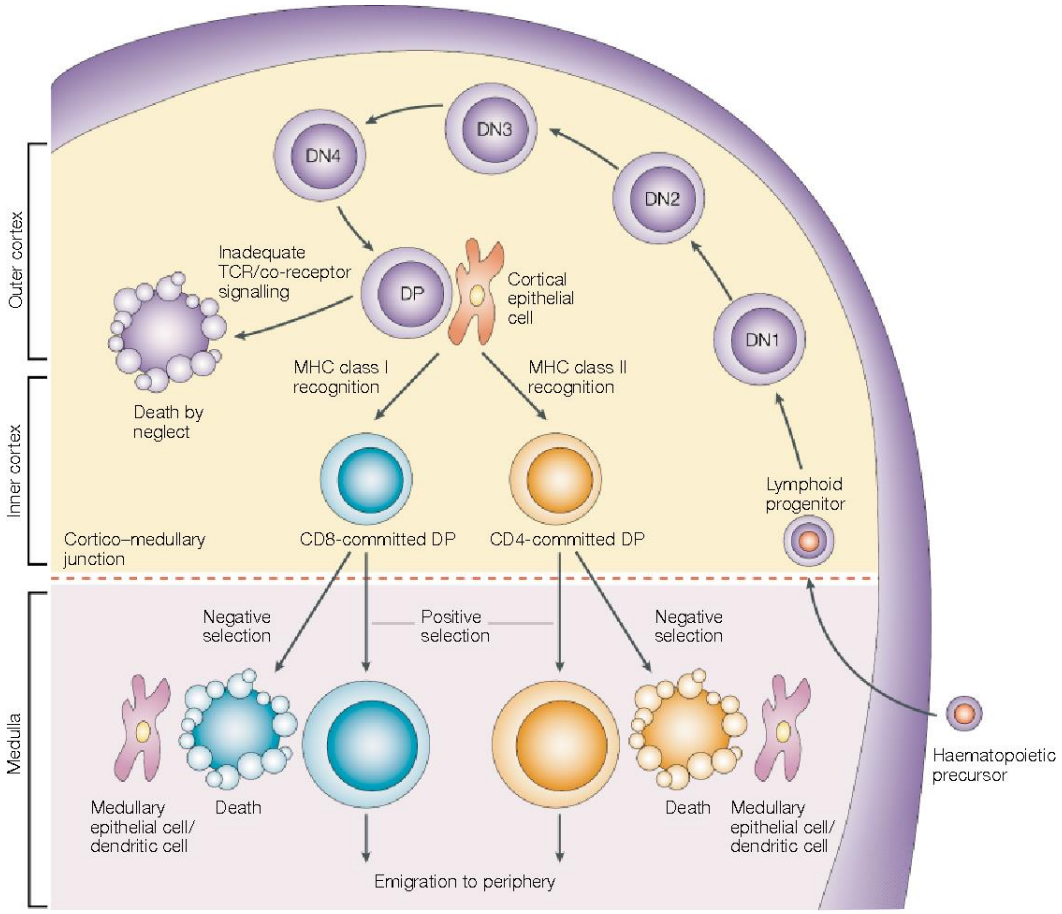

Figure 2.3 T cell development in the thymus.
Lymphoid progenitor undergo series of selection and maturation stages to be mature T cell possess co-receptor molecule (CD4 or CD8) expression that commited to MHC-binding property that match with its TCR [Adapted from Germain (2002)].

2.3.2 Subsets of T cells

CD4+ and CD8+ T cells are two major arms of T cell-mediated immunity. CD4+ Th cells enhance killing o extracellular and intracellular microbes by eliminating phagocytosed microbes through cytokines production and recognition of class II MHC (Abbas, Lichtman and Pillai, 2014). On the other hand, CD8+ T cells are called cytotoxic T lymphocytes (CTLs) complete the adaptive immune system by killing tissue cells that harboring viruses through recognition of class I MHC. CTLs. also play important role in tumors eradication besides critical in acute rejection of organ allografts (Kindt *et al*., 2007; Abbas, Lichtman and Pillai, 2014).

CD4+ Th cells consist of four major effector subsets called type 1, 2, 17 and T follicular helper (Tfh) cells that produce distinct sets of cytokines, immune reactions, different host defense as well as immunologic diseases (Figure 2.4). The differentiation of Th1, Th2 and Th17 are developed from naïve CD4+ T cells in response to the cytokines produced by antigen presenting cells (APCs) mainly DCs and macrophages, NK and mast cells in the lymphoid organs (Abbas, Lichtman and Pillai, 2014).

When intracellular microbes present during innate immune response, IL-12 is produced by DCs and macrophages while interferon gamma (IFN-γ) produced by NK cells. These cytokines activate the transcription factors such as T-bet, STAT1 and STAT4 in naïve CD4+ T cells that promote Th1 development (Abbas, Lichtman and Pillai, 2014). After Th1 cells have developed, their secretion of IFN-γ is to amplify the differetiation, thus maintain Th1 population by inhibiting the development of Th2 and Th17 subsets. The signature role of Th1 cells is to activate macrophages so that the increase of phagolysosomes can destroy phagocytosed microbes. Besides this,

Th1 cells produce tumor necrosis factor (TNF) in recruitment of neutrophils that promotes inflammation.

Th2 subset is phagocyte-independent defense mainly in eradicate helminthic infections and allergens (Abbas, Lichtman and Pillai, 2014). The initiate development of Th2 is incompletedly defined, but the further proliferation is depends on its signature cytokine, IL-4 to start up transcription factor STAT6 simultaneously with TCR signals that induce GATA3 expression in naïve CD4+ T cells. As a result, the expression of GATA3, IL-4, IL-5 and IL-13 production are stimulated. IL-4 acts as growth factor to maintain the differentiation and development of Th2 effector cells from naïve T cells. When IL-4 works together with IL-13, they are involved in mucosal barriers immunity, activate macrophage through alternative pathway in tissue repair but suppress classical Th1-mediated macrophage activation (Abbas, Lichtman and Pillai, 2014). Lastly, IL-5 as an activator of eosinophils is mainly produced by Th2 cells in eliminating helminthic by releasing its granule contents and important in allergic diseases (Abbas, Lichtman and Pillai, 2014).

When DCs recognised extracellular bacteria and fungi, they produces proinflammatory cytokines including IL-1, IL-6 and IL-23. Cytokines such as IL-1, IL-6 and TGF-β collectively promote differentiation of naïve CD4+ T cells into Th17 subset by activating transcription factor RORγt and STAT3. Interestingly, IL-23 is crucial in maintainance and proliferation of Th17 cell population. In mucosal tissues, Th17 cells stimulate local tissue IL-17 production by recruiting neutrophils to combat intestinal infections. Furthermore, IL-17 also increase the production of antimicrobial peptides together with IL-22. In particular, IL-22 produced by Th17 in tissue cells increase barrier function and associated with tissue injury in inflammatory diseases.

In germinal center, naïve CD4+ T cells are exposured to antigen require sequential activation initially by DCs and then by activated B cells to differentiate into Tfh cells. The unite phenotype of Tfh cells is they express ICOS, PD-1, IL-21, chemokine receptor CXCR5 in high level and transcription factor Bcl-6. IL-21 as the signature cytokine of Tfh cells play role to facilitate B cell selection events and differentiated activated B cells into plasma cells. In addition, Tfh cells also secrete IFN-γ, IL-4 and low level of IL-17. Activated Tfh cells produce IL-4 that stimulates B cell isotype switching and production of IgE bind to mast cells leading degranulation and inflammation (Abbas, Lichtman and Pillai, 2014).

Similar to CD4+ T cells, naïve CD8+ T cells also undergo differentiation to be effector CTLs. The activation of naïve CD8+ T cells begins while the antigen is presented by DCs through effective cross-presentation involved CD4+ Th cells and cytokines. Cells infected by viruses or tumor cells are ingested by DCs and transported into cytosol to be processed in proteasomes, therefore the antigen is able to be presented by MHC class I pathway.

In addition, other T cell subsets are small populations but serve specialized host defense functions mainly located in epithelial tissues. These populations of the cells are including Treg cells, γδ T cells, natural killer T (NKT) cells and mucosa-associated invariant T (MAIT) cells. Some of these cells are not MHC restricted in antigen presentation and work in between innate and adaptive immunity. Unlikely other CD4+ Th cells, Treg cells is immportant immunosuppression cells to maintain self tolerance.

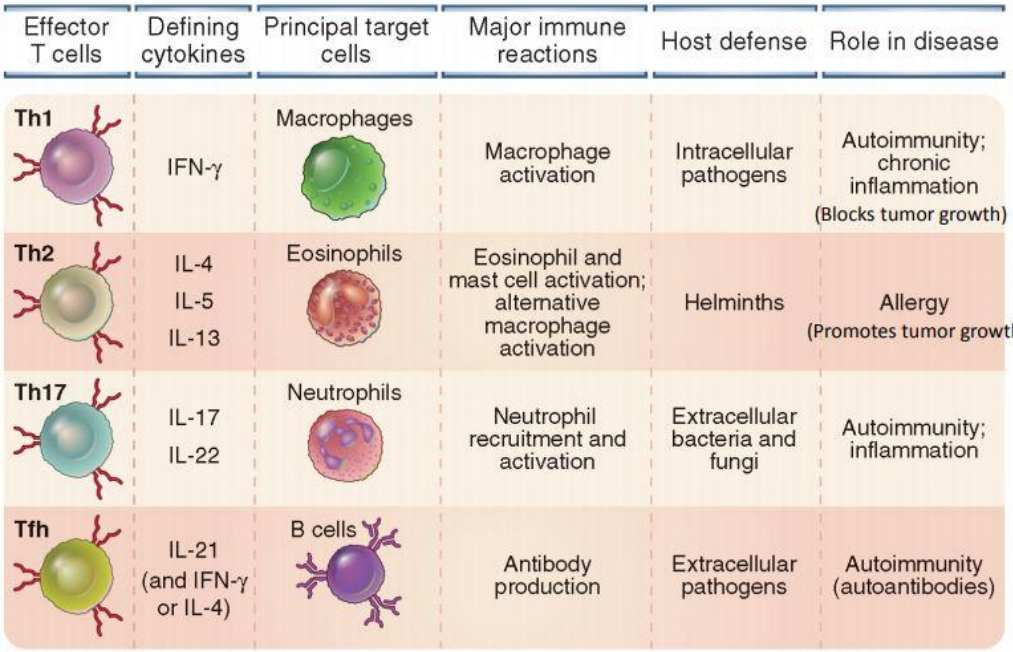

Figure 2.4 Properties of the major subsets of CD4+ helper T cells.
The cytokines produced by these T cell subsets determine their effector functions and role in diseases [Adapted from Abbas, Lichtman and Pillai (2014)].

2.3.3 Antigen processing and presentation by MHC

There are two pathways in antigen processing and presentation by MHC: endogeneous (intracellular antigens) by class I MHC and exogeneous (extracellular antigens) by class II MHC as stated in Figure 2.5.

In cytoplasm, class I MHC pathway begins when cytosolic protein of viral or infected cells undergo proteasomal degradation. Peptides are transported to the endosplamic reticulum (ER) to be bound with newly synthesized class I MHC molecules through transporter associated with antigen processing (TAP). Class I MHC molecules become stable after bound with peptides are moved through Golgi apparatus to be expressed on the cell surface. The class I MHC complex therefore is recognised by CD8+ T cells (Kindt *et al*., 2007; Abbas, Lichtman and Pillai, 2014).

On the hand, class II MHC pathway involved extracellular proteins captured by APCs such as macrophage, DCs and B cells to form endocytic vesicle. The internalized protein antigens are then processed with lysosome and endosome vesicles in peptide forms. Meanwhile, the sysnthesis of class II MHC molecules with class II associated I_i peptide (CLIP) happen in ER are transported to endosomes. The binding of processed peptides to class II MHC occur after proteolytic degradation of invariant chain (I_i). The class II MHC complex are stablized after bound with peptides then displayed to surface of APCs for recognition by CD4+ T cells (Kindt *et al*., 2007; Abbas, Lichtman and Pillai, 2014).

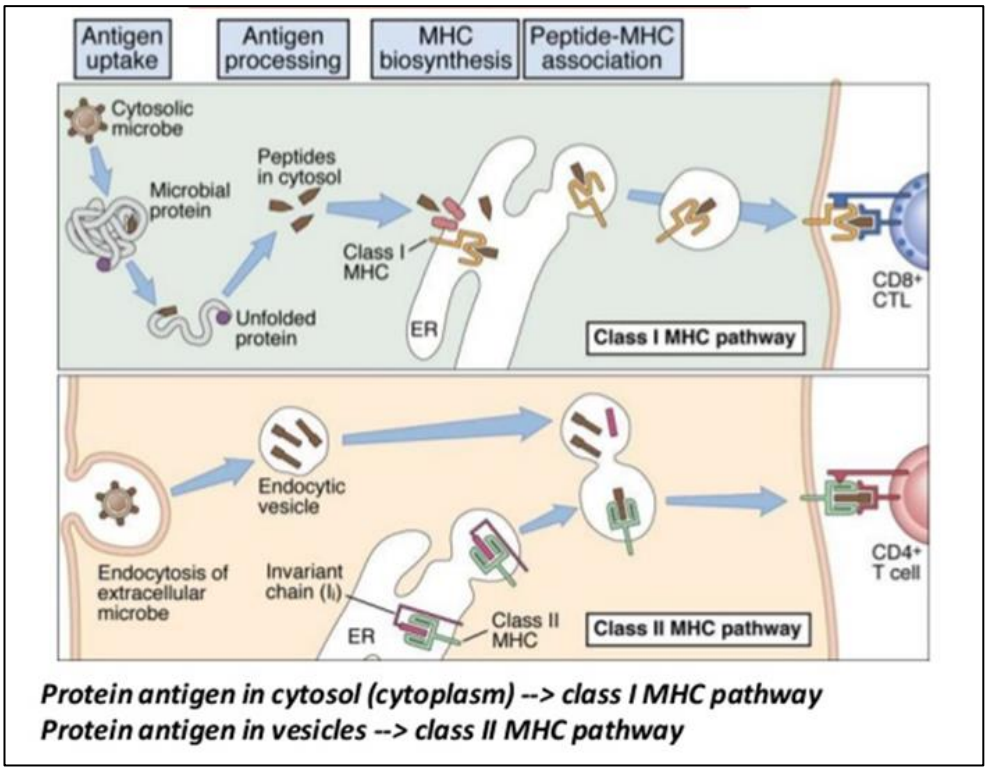

Figure 2.5 Pathways of antigen processing and presentation.
In class I MHC pathway, cytosolic protein are processed by proteasomes, peptides are transported into ER to bind with class I MHC molecules. The class I MHC complex is expressed on the cell surface to recognise by CD8+ CTL. In class II MHC pathway, extracellular microbes are processed through endocytosis vesicle bind to class II MHC from Golgi complex to form endosome. The peptide-MHC association then presented on APCs cell surface to recognise by CD4+ T cells [Adapted from Abbas, Lichtman and Pillai (2014)].

2.4 Immunological tolerance

The term immune tolerance arise from the observation of animal experiments by Sir Frank McFarlane Burnet and Frank Fenner (Burnet and Fenner, 1953). In similar, Gershon and Kondo demonstrated tolerance induction in thymic cells (Gershon and Kondo, 1970). Thus, immunological tolerance is defined as unrecognition to an antigen that is induced by previous exposure (Brent, 1997; Abbas, Lichtman and Pillai, 2014). Tolerance divided into two types mainly central tolerance and peripheral tolerance (Figure 2.6). The failure of self-tolerance mechanism results in autoimmunity. Thus, autoimmune diseases are associated with impairment of immunosuppression mechanism.

Central tolerance occurs during maturation of lymphocytes develop in the generative lymphoid organs (bone marrow and thymus) (Abbas, Lichtman and Pillai, 2014). Immature lymphoctes that recognize self antigen specifically in generative lymphoid organs are undergo negative selection process through cell death (apoptosis), change their receptors (B cells only) or develop into Treg cells (CD4+ T cells) (Abbas, Lichtman and Pillai, 2014).

Peripheral tolerance happens when the mature lymphoctes that recognize self antigen in peripheral tissues become anergy, apoptosis or suppressed by Treg. The suppression by Treg cells occurs actively in secondary lymphoid organs and non-lymphoid tissues to maintain peripheral tolerance since central tolerance is imperfect (Abbas, Lichtman and Pillai, 2014). Therefore, Treg cells are protective cells in autoimmune disorders (Sakaguchi *et al.*, 2008), but they are harmful cells in cancer (Vignali, Collison and Workman, 2008). Moreover, in chronic infectious diseases, Treg cells suppress the inflammation to reduce the tissue injury (Belkaid, 2007).

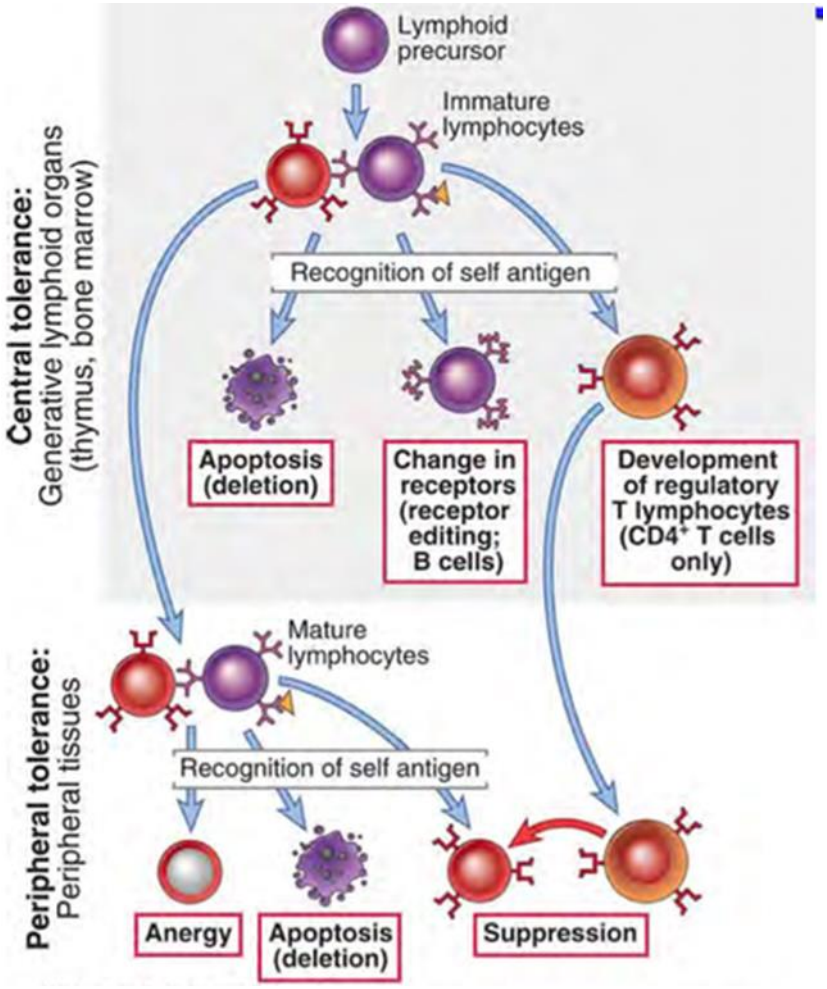

Figure 2.6 Central and peripheral tolerance.
In generative lymphoid organs, immature lymphocytes encounter self antigen are undergo deletion, receptor editing in B cells or develop into CD4+ Treg cells. Some mature self-reactive lymphocytes enter peripheral tissues become inactivated, deleted or suppressed by Treg cells in peripheral tolerance [Adapted from Abbas, Lichtman and Pillai, (2014)].

2.4.1 Heterogeneity and plasticity of Treg lineage

History of Treg cells begins in 1970 by Gershon and Kondo (Gershon and Kondo, 1970) until Sakaguchi and colleagues (1995) began the observation of adoptive transfer "suppressor" T cell as depletion of CD4+CD25+ T cells. These cells have induced multi-organ autoimmunity in recipient animals (Sakaguchi *et al.*, 1995; Asano *et al.*, 1996), thereafter these cells are now known as Treg cells.

The heterogeneity of Treg subsets have been summarized as Table 2.1. Briefly, there are two major Treg cells known as nTreg cells (Noble, Giorgini and Leggat, 2006; Miyara and Sakaguchi, 2007; Akdis and Akdis, 2009) and iTreg cells according to their cellular localization (Kretschmer *et al.*, 2005; Ray *et al.*, 2010). Despite iTreg cells mainly Th3 are developed after induction by TGF-β from CD4+CD25- Teff or naïve T cells, their suppressive function remain similar as nTreg cells (Kretschmer *et al.*, 2005; Tran, Ramsey and Shevach, 2007).

On the other hand, adapted type 1 regulatory (Tr1) exclusively produced high and potent immunosuppressive cytokine i.e. IL-10 (Bacchetta *et al.*, 1994); where its anti-inflammation has been demonstrated in mouse model to prevent colitis (Groux *et al.*, 1997). The co-expression of CD49b and lymphocyte-activation gene 3 (LAG-3) has become specific biomarkers for Tr1 cell in mice and human despite its specific transcription factor has not determined (Gagliani *et al.*, 2014). Also, Tr1 controls allergy inflammation through inhibition on Th2, mast cells, basophils and eosinophils (Wu *et al.*, 2007) other than transplantation and autoimmune (Pot, Apetoh and Kuchroo, 2011).

Oral tolerance investigation has been carried out on gut-associated lymphoid tissue (GALT), TGF-β secreting Treg cells, Th3 has been found work simultaneously with IgA in mucosal immunity (Chen *et al.*, 1994) which is able to suppress both Th1