THE INFLUENCE OF PPARγ LIGANDS ON THE EXPRESSION OF FOXP3 IN NATURAL T-REGULATORY CELLS IN BALB/c AND TYPE 1 DIABETES MOUSE MODEL

NOR EFFA SYAZULI BT ZULKAFLI

UNIVERSITI SAINS MALAYSIA

2015

THE INFLUENCE OF PPARγ LIGANDS ON THE EXPRESSION OF FOXP3 IN NATURAL T-REGULATORY CELLS IN BALB/c AND TYPE 1 DIABETES MOUSE MODEL

BY

NOR EFFA SYAZULI BT ZULKAFLI

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

MARCH 2015

ACKNOWLEDGEMENT

الحمد لله

Praise and gratification are due to Allah for His continuous guidance and love that gave me the ingredients of success towards completing this study.

First of all, I would like to thank my supervisor, Prof Norazmi Mohd Nor and my co-supervisor, Prof Nik Soriani Yaacob for their constant support, efficient supervision and wisdom throughout this study.

I would like to express my gratitude to my sponsor, Ministry of Education, Malaysia and Universiti Sains Malaysia for awarding me the Academic Staff Training Scheme. Special thanks are due to colleagues from NMN's, NSY's and SS's research group for their friendship and support. I am indebted with Mr Jamaruddin Mat Asan from Immunology Lab, PPSP for his helpful suggestions and discussion. I would like to thank Mr Nor Hisyam Yaakob, Miss Nor Hidayati and lab technicians of Molecular Biology and Cell culture Labs from PPSK.

My deepest appreciation is honored to my parents and siblings for their unwavering support and love. This thesis is dedicated to my dear spouse, Mr. Mohd Firdaus, and little supporters, Adib Rayyan and Ainur Husna for their endless support and understanding. Lastly, this thesis would not have been completed without the support of many people. May Allah reward all of your sacrifice you deserved. Thank you for being supportive.

Content **Page**

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOL AND ABBREVIATIONS

ANOVA	One-Way Analysis of Variance
APC	Antigen presenting cells
AP-1	Activated protein-1
ATF	Activating transcription factor
Bm12	$H2^{bm12}$; Histocompatibility 2, class II antigen A, beta 1; b haplotype mutation 12
CARMA	Caspase-recruitment domain + membrane associated guanylate kinase
CARD	Caspase-recruitment domain
cAMP	Cyclic adenosine monophosphate
CCR7	C-C Chemokine receptor
CFSE	Carboxyflourescein Succinimidyl ester
CNS	Conserved Non-coding Sequence
COX	Cyclooxygenase
CpG	Cytosine-phosphate-Guanine
CREB	cAMP response element binding protein
CTS	Cataract prone subline
CTL	Cytotoxic T-lymphocytes
CTLA-4	Cytotoxic T-lymphocytes antigen 4
DNMT	DNA methyltransferase
DAG	Diacylglycerol
DBD	DNA-binding domain
DC	Dendritic cell
DN	Double negative
DP	Double positive
DR	Direct repeat
EAE	Experimental autoimmune encephalomyelitis
ERK	Extracellular signal-regulated kinase
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead Box P3
GAD65	Glutamic acid decarboxylase
GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GITR	Glucocorticoid inducible TNF receptor
GPR40	G-protein associated receptor 40
Grb-2	Growth factor receptor-bound protein
GTP	Guanine triphosphate
HIF-1	Hypoxia-inducible factor-1
HLA	Human Leukocyte Antigen
HSP60	Heat-shock protein 60
IA-2	Islet-antigen 2
IBD	Inflammatory bowel disease
IDO	Indolamin 2,3-dioxygenase
IGRP	Glucose-6 phosphatase catalytic subunit related protein
IκB	Inhibitor of κB
IKK	IκB kinase

iNOS	Inducible nitric oxide
IPEX	Immuno dysfunction, polyendocrinopathy, enteropathy X-linked
IP3	Inositol-1,4,5-triphosphate
iTreg	Induced T-regulatory
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun NH-terminal kinase
LAT	Linker for the activation of T cells
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation gene 1
MAPK	Mitogen activated protein
MHC	Major Histocompatibility Complex
NCoR	Nuclear co-repressor
NFAT	Nuclear Factor of activated T cells
NF-kB	Nuclear Factor kappa B
NOD	Non-obese diabetic
NOR	Non-obese resistant
NK	Natural killer
NSB	Non specific binding
nTreg	Natural T-regulatory
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD-1	Programmed cell-death 1
PE	R-phycoerithin
PIP2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLN	Peripheral lymph node
PPAR	Peroxisome proliferator activated receptor
PPARγ	Peroxisome proliferator activated receptor gamma
PPRE	Peroxisome proliferator activated receptor response element
PTK	Protein tyrosine kinase
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCID	Severe combined immuno-deficient
SH2	Src homology 2
SLE	Systemic Lupus Erythematosus
SLP-76	SH2 domain-containing leukocyte phosphoprotein of 76 kDa
Smad	Small ‘mothers against’ decapentaplagic
SP	Single positive
STAT	Signal Transducer and Activators of Transcription
SUMO	Small ubiquitin-like modifier
TCR	T cell receptor
Teff	T effector
TGF-β	Transforming growth factor-beta
Th1	T helper-1
Th17	T helper-17

TIGIT	T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif
TRAIL-DR5	Tumour necrosis factor related apoptosis inducing ligand-death receptor 5
TSDR	Treg-specific demethylated region
TZD	Thiazolidinediones
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Ubc5	Ubiquitin-conjugating enzyme 5
VAT	Visceral adipose tissue
WT	Wild type
ZAP-70	ζ-associated protein of 70 kDa phosphoprotein
15d-PGJ2	15-Deoxy-Delta-12,14-prostaglandin2

PENGARUH PPARγ LIGAN KE ATAS EKSPRESI FOXP3 DALAM SEL T-ATURAN SEMULAJADI DALAM TIKUS BALB/c DAN TIKUS MODEL DIABETES JENIS 1

ABSTRAK

Sel $CD4^{+}CD25^{+}Foxp3^{+}$ T-aturan (nTreg) adalah subset sel T-aturan yang berasal dari timus. Fungsi pengurang-aturan-imun oleh sel T-aturan adalah kritikal dalam proses toleransi-diri periferal. Selari dengan itu, sifat-sifat anti-radang reseptor-teraktif pemproliferator peroksisom- γ (PPARγ) telah banyak dikaji dengan mendalam sejak kebelakangan ini. Memandangkan peranan penting mereka dalam pengawalaturan imun, kajian semasa telah dijalankan untuk mentafsirkan mekanisme pengubah-aturan oleh PPARγ ligan dalam sel T-aturan daripada tikus jenis BALB/c, dan model Diabetes Jenis 1 (T1D), Non obese Diabetic (NOD) dan juga dalam Non obese Resistant (NOR). Pada mulanya, tahap optimum titik masa dan kepekatan IL-2 ditentukan pada kultur sel T-aturan *in vitro* dalam tikus BALB/c. Kemudian, analisis fungsi sel T-aturan yang telah diasingkan daripada tikus BALB/c telah diukur. Tahap ekspresi konstitutif dan aruhan isofom PPARγ dalam sel T-aturan daripada tikus BALB/c telah diperiksa untuk menilai hubungan antara PPARγ dan sel T-aturan. Aktiviti penggabungan unsur-unsur tindak balas PPARγ, PPRE, telah diperiksa dalam sel T-aturan yang dirawat dan tidak dirawat untuk menentukan mekanisme bebas- atau bergantung- PPARγ oleh ligan PPARγ. Intervasi daripada ligan PPARγ dalam komponen isyarat sel nTreg diperiksa dengan menganalisa komponen-komponen ZAP-70 dan STAT-5 dalam sel-sel T-aturan daripada tikus NOD dan NOR. Dengan menggunakan PCR array, tahap ekspresi gen sasaran yang terlibat dalam laluan isyarat di dalam sel T-aturan juga diukur. Dengan menggunakan aliran

sitometri, titik masa optimum untuk pengkulturan *in vitro* sel T-aturan ditentukan pada hari ketiga, dengan kehadiran 5 ng/mL IL-2. Didapati juga bahawa sel-sel T-aturan yang dikultur merencat sel-sel T-efektor teraktif (Teff). Dengan menggunakan real-time PCR, didapati bahawa sel-sel ini secara konstitutif, mengekspresi PPARγ1 dan PPARγ2 pada tahap yang rendah dan ciglitazone telah mengaruh ekspresi PPARγ1 dalam sel T-aturan melalui mekanisme bergantung-PPARγ ($P < 0.01$), tetapi tiada kesan ke atas ekspresi PPARγ2. Kedua-dua ligan PPARγ menurunkan ekspresi Foxp3 dalam sel T-aturan daripada tikus BALB/c, NOD dan NOR melalui mekanisme bebas-PPARγ berbanding dengan sel T-aturan tidak dirawat ($P < 0.01$). Disamping itu, tiada perbezaan yang ketara antara aktiviti penggabungan PPARγ dan PPRE dalam sel T-aturan dirawat dan tidak dirawat daripada tikus BALB/c, NOD dan NOR. Tambahan pula, tahap pemfosforilan ZAP-70 dan STAT-5 dalam sel T-aturan dirawat daripada tikus NOD dan NOR tidak dimodulasi oleh ligan PPARγ. Di samping itu, berlaku penurunan ekspresi pada kebanyakan gen sasaran yang berkait dengan laluan isyarat di dalam sel T-aturan daripada tikus NOD selepas rawatan dengan PPARγ ligan. Dalam tikus NOR, ekspresi gen sasaran yang terlibat dalam isyarat laluan TGF-β, p53, NF-κB, NFAT dan Ca^{2+} & PKC telah meningkat dalam sel-sel T-aturan yang dirawat dengan ciglitazone, disamping itu, penurunan ekspresi juga berlaku pada beberapa gen. Penurunan ekspresi Foxp3 oleh ligan PPARγ dalam sel T-aturan mencadangkan pengurang-aturan fungsi pengawalaturan sel T-aturan dalam keadaan normal dan T1D. Mekanisme pengurang-aturan oleh PPARγ ligan pada sel T-aturan ini mungkin berlaku terutamanya melalui laluan bebas-PPARγ. Ianya berkemungkinan bahawa ligan PPARγ merencat ekspresi Foxp3 dalam sel T-aturan melalui mekanisme trans-represi bergantung-PPARγ ligan.

THE INFLUENCE OF PPARγ LIGANDS ON THE EXPRESSION OF FOXP3 IN NATURAL T-REGULATORY CELLS IN BALB/c AND TYPE 1 DIABETES MOUSE MODEL

ABSTRACT

Natural $CD4^+CD25^+Foxp3^+$ T regulatory cell (nTreg) is a subset of regulatory T cell that is derived from the thymus. The immunodownregulatory function of nTreg cells is critical in mediating peripheral self-tolerance. Concordantly, the anti-inflammatory properties of peroxisome proliferator-activated receptor γ (PPARγ) have been intensely studied in recent years. Given their crucial role in immune regulation, the current study was conducted to decipher the modulatory mechanism by PPARγ ligands in nTreg cells of BALB/c, and the Type 1 Diabetic (T1D) model, Non Obese Diabetic (NOD) as well as in the Non Obese Resistant (NOR) mice. Initially, we optimized the time-points and the concentrations of IL-2 for *in vitro* culture of nTreg cells in BALB/c mice. Subsequently, the functional analysis of isolated nTreg cells of BALB/c mice was measured. The constitutive and induced levels of PPARγ isoforms in nTreg cells of the BALB/c mice were examined to evaluate the correlation between PPARγ and nTreg cells. PPARγ binding activity to its response elements, PPRE, was examined in treated and untreated nTreg cells to determine the PPARγ-dependant or –independant pathway of PPARγ ligands. The possible intervention of PPARγ ligands in signaling components of nTreg cells was examined by analyzing ZAP-70 and STAT-5 signaling components in nTreg cells of NOD and NOR mice. In addition, the expression levels of pathway-related target genes of nTreg cells were also measured. By using flow cytometry, the optimized time-point for *in vitr*o culture of nTreg cells was determined on day three, in the

presence of 5 ng/mL of IL-2. It was also found that cultured nTreg cells were suppressive towards activated T effector (Teff) cells. By using real-time PCR, the constitutive and induced expression levels of PPARγ isoforms in stimulated and unstimulated nTreg cells of BALB/c mice were measured. It was found that these cells constitutively expressed low levels of PPARγ1 and PPARγ2 and treatment with ciglitazone induced PPARγ1 expression in nTreg cells via PPARγ-dependant pathway ($P < 0.01$), but not PPARγ2 expression. Both PPARγ ligands downregulated the expression of Foxp3 in nTreg cells of BALB/c, NOD and NOR mice via PPARγ-independant pathway compared to untreated group ($P < 0.01$). In addition, it was shown that there is no significant difference of PPARγ and PPRE binding activity between treated and untreated nTreg cells of BALB/c, NOD and NOR mice. Furthermore, the phosphorylation levels of ZAP-70 and STAT-5 in treated nTreg cells of NOD and NOR mice were not modulated by PPARγ ligands. In addition, it was found that most of pathway-related target genes were downregulated in NOD nTreg cells following treatment with PPARγ ligand. In NOR mice, target genes involved in TGF-β, p53, NF-κB, NFAT and Ca^{2+} & PKC signaling pathways were upregulated in ciglitazone-treated nTreg cells, while a few genes were downregulated. The downregulation of Foxp3 expression by PPARγ ligands in nTreg cells may suggests the downregulation of immuno-downmodulatory function of nTreg cells in normal and T1D conditions. The downregulatory mechanism of PPARγ ligands on nTreg cells may occur primarily via PPARγ-independant pathway. It is possible that PPARγ ligands suppress Foxp3 expression in nTreg cells via PPARγ ligand-dependant transrepression mechanism.

CHAPTER 1

INTRODUCTION

1.1 Study background

Over the past twenty years since the identification of naturally-occurring $CD4^+CD25^+Foxp3^+$ Regulatory T (nTreg), there have been intense research in delineating the immunobiology of nTreg cells in physiological and pathological conditions (Bettini & Vignali, 2010). The master regulator in nTreg cells is the transcription factor Forkhead box P3 (Foxp3) which plays an important role in the development and function of nTreg cells (Fontenot et al, 2003). Foxp3 is expressed in the thymus by nTreg and transiently expressed by $CD4^+CD25^-$ conventional T cells (iTreg) peripherally. In pathological conditions such as autoimmune disorders, the recognition of self-tissues by auto-reactive T cells leads to the destruction of host tissues or organs. The immunosuppressive role of nTreg cells prevents such destruction from occurring by establishing peripheral self-tolerance toward auto-reactive T cells. This will thus hinder the development of debilitating autoimmune diseases from occurring. Mutation of Foxp3 gene in mouse models results in the loss of immunoregulatory function of nTreg cells, predisposing the hosts towards autoimmune responses.

Autoimmune disorders are a group of multiple disorders related to aberrant immune responses in the host system. The onset of each autoimmune condition may exist simultaneously. For example, celiac disease co-occurs as an extra-pancreatic

manifestation in children with Type 1 Diabetes (T1D) (Vitoria et al, 1998). Similarly, rheumatoid arthritis and nephritis are simultaneously developed in patients with systemic lupus erythematosus (SLE) (Tan et al, 1982). Although individually, autoimmune disorder is not a common disease, collectively, it is the third leading cause of morbidity among more than 23.5 million Americans (Nakazawa, 2008). Mortality rate caused by autoimmune disorders has increased among the young and middle aged women in the United State (Walsh & Rau, 2000). The worldwide prevalence of T1D among children is alarming. There has been a steady rise in T1D incidence in many parts of the world within genetically stable population (Gale, 2002). According to The American Diabetes Association, T1D incidence has increased by 23 % between 2001 and 2009 (Rattue, 2012). In Malaysia, approximately 0.1 % of 657,839 diabetic patients are T1D individuals (National Diabetes Registry, 2012).

It is fairly well-established that PPARγ activation is capable of inducing immunodownregulatory responses (Li et al, 2000; Rotondo & Davidson, 2002; von Knethen et al, 2007; Sauter et al, 2012). Therefore, the putative role of PPARγ in inducing anti-inflammatory responses in immune cells has been put forth. The activation of PPARγ by their ligands has been shown to downregulate the clonal expansion of activated T effector (Teff) cells (Clark et al, 2000). The use of PPARγ ligands such as thiazolidinediones (TZDs) class of drugs, including pioglitazone, ciglitazone, and rosiglitazone has positively alleviated adverse autoimmune conditions in allergic reactions, multiple sclerosis and inflammatory bowel disease (IBD) (Hammad et al, 2004; Klotz et al, 2005; Hontecillas et al, 2011; Bertin et al, 2013). In addition, researchers have shown that these ligands may act via PPARγ-dependant or -independant mechanisms in modulating the immune response

(Yamashita et al, 2008), which may result in the genomic or non-genomic signaling of PPARγ activation. Despite this, very few studies have investigated the relationship between immunomodulatory properties of PPARγ and nTreg cells in healthy and T1D conditions. The study on the interaction between these two components may enhance our understanding on the immunomodulatory mechanisms involved in T1D.

The purpose of the current study is to understand the modulation of Foxp3 expression and the possible crosstalk by PPARγ ligands in nTreg cells in healthy and T1D mouse models. Therefore, the current study examined the immunomodulatory properties of PPARγ ligands on Foxp3 expression in nTreg cells of BALB/c and the T1D mouse model, NOD mice. The use of spontaneous T1D NOD mouse has greatly enhanced our understanding on the pathological mechanisms involved in this disease (Candon et al, 2007). The NOR mouse model was used along with NOD mice as the control group. In the current study, ciglitazone and the natural PPARγ ligand, 15d-PGJ_2, were used as PPARγ ligands to activate PPARγ. In addition, PPARγ inhibitor, GW9662 was added to determine the PPARγ-PPRE-dependant or –independant mechanisms of these ligands.

Therefore, this study may provide fundamental information on the potential role of PPARγ ligands in nTreg cells as an immunomodulator in healthy and T1D conditions. This information may be applied in cell-based therapy for immune-related diseases such as T1D, lupus and multiple sclerosis. However, this study did not examine the specific mechanisms adopted by PPARγ ligands in suppressing Foxp3 expression. This is due to time constraints and limited specific antibodies available. Future studies may explore the suppressive mechanisms of PPARγ ligands on Foxp3 expression at molecular levels.

1.2 Study objectives

This study attempts to test the hypothesis that the immunoregulatory properties of PPARγ may synergistically act with Foxp3 in the function of nTreg cells. Figure 1.1 and Figure 1.2 illustrate the schematic workflow of the current study. The objectives of the current study are:

1. To determine the optimal culture conditions for nTreg cells from BALB/c mice.
2. To quantify the optimal levels of PPARγ expression in nTreg cells from BALB/c mice.
3. To examine the influence of PPARγ ligands on PPARγ and Foxp3 expressions in nTreg cells of healthy and T1D mouse models.
4. To determine the influence of PPARγ ligands in nTreg cells occurs either via PPARγ-dependant or -independant pathways.
5. To determine the correlation between PPARγ and relevant signaling pathways in nTreg cells of NOR and NOD mice.

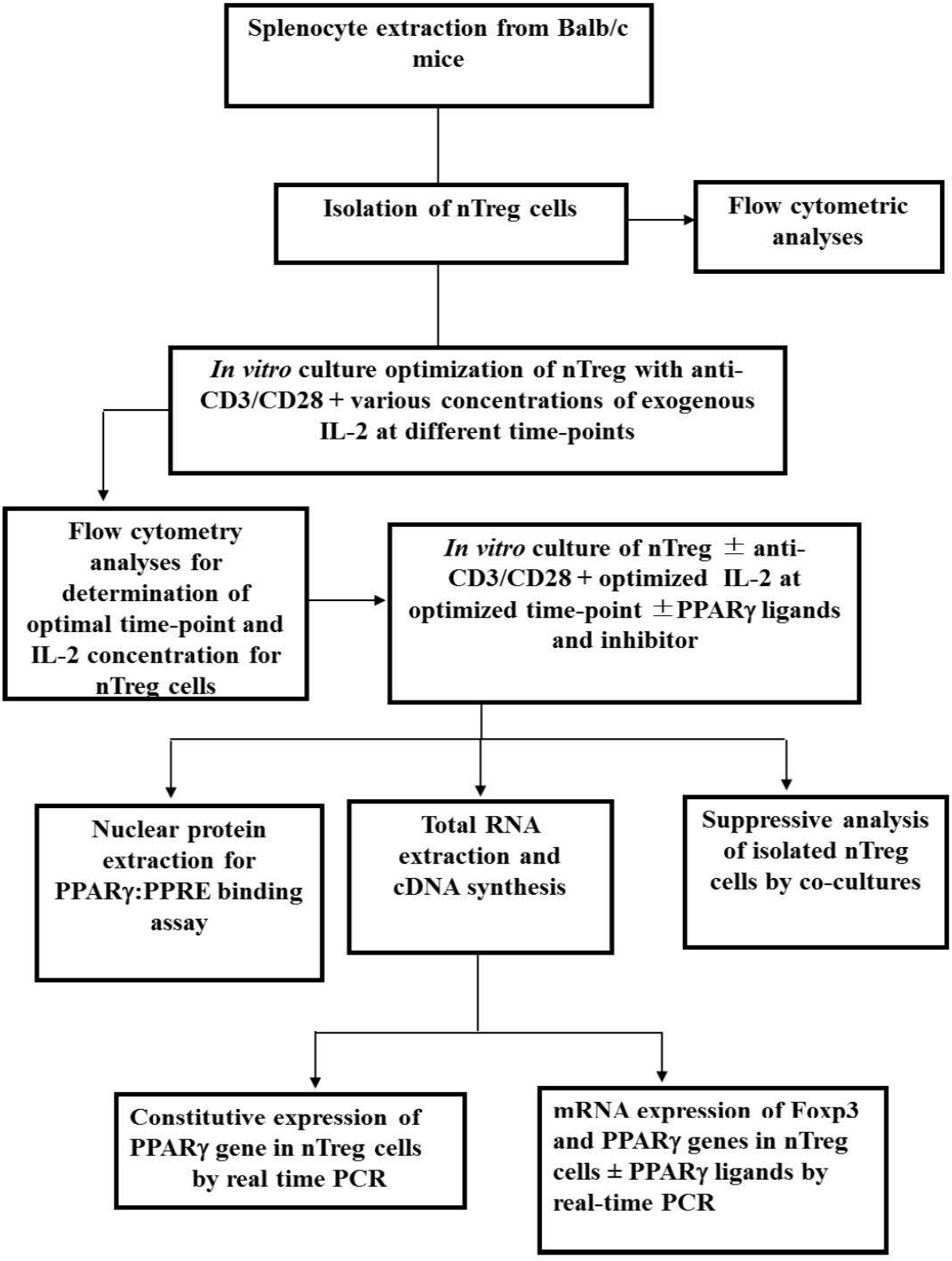

Figure 1.1 Schematic workflow of *in vitro* study in BALB/c mice.

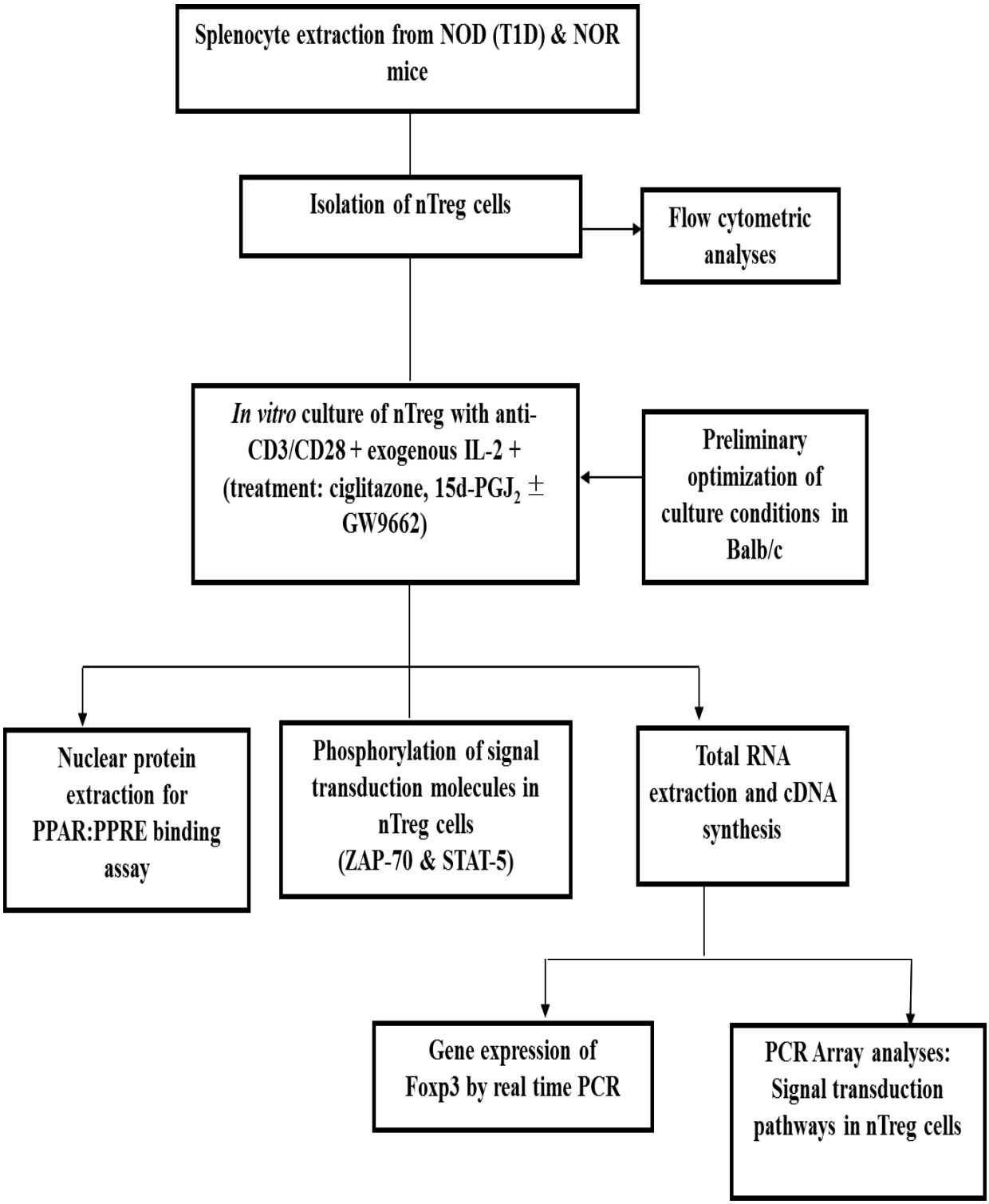

Figure 1.2 Schematic workflow of *in vitro* study in NOD and NOR mice.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to the immune system

Generally, the immune system can be classified into two main components i.e. innate immunity and adaptive immunity. Host defence against microbes is mediated by the early reactions of innate immunity and later, response of adaptive immunity. If an organism enters the body by breaking through the barrier of the body, it will encounter with phagocytic or natural killer (NK) cells as well as the complement system. In addition, breaching into the host body also initiates other vital components of the innate immunity, such as inflammation and fever (Tortora & Grabowski, 2003).

Innate immunity provides an early line of defense against microbes. It comprises (1) physical and chemical barriers (2) phagocytic cells (neutrophils, and macrophages) (3) the complement system and (4) cytokines (Hoffman et al, 1999). The adaptive immunity will be triggered when the exposure to infectious agents increased in magnitude that overcomes the protection by innate immunity (Medzhitov et al, 1997). Two cardinal properties distinguish adaptive immunity from innate immunity, which is specificity and the ability to "remember" previously encountered antigens (Tortora & Grabowski, 2003). The adaptive immune response consists of two types of response, called humoral immunity and cell-mediated immunity. While the former type refers to the response mediated by antibodies, the latter pertains to T-cells mediated response (Silverstein, 2003).

2.2 Antibody-mediated immunity

2.2.1 Antibody production by B cells

The activation of B cells to differentiate into antibody-secreting plasma cells is triggered by antigens that are recognized by T helper cells. The recognition of antigens by specific B and T cells is preceded by the antigen-presentation by antigen-presenting cells (APC) to T helper cells. Upon recognition of antigen, T helper cells become activated and induced to express CD40L and secrete IL-2, IL-4 and IL-21. The ligation of CD40 and CD40L act in concert with these cytokines to stimulate B lymphocyte proliferation and differentiation into antibody-secreting plasma cells. Both stimuli activate transcription factors for immunoglobulin (Ig) synthesis to increase the production and class switching of Ig by B cells (Davies & Metzger, 1983).

Plasma cells are terminally differentiated B cells that mainly reside in extrafollicular sites such as the medulla of lymph nodes. The plasma cells can be divided into short-lived and long-lived plasma cells. Short-lived plasma cells refer to plasma cells that reside in secondary lymphoid organs and peripheral non-lymphoid tissues such as liver, lung and peritoneal cavity (Kapoor, Kang, & Welsh, 2014). On the other hand, the long-lived plasma cells mainly reside in the bone marrow and continue to secrete antibodies after the antigen is no longer present (Abbas et al, 2009). These antibodies serve as an immediate protection against previously encountered antigen. In addition, the Fc region of antibody serves as a binding site for phagocytes. The binding between antibody and phagocytes will enhance the phagocytosis process of phagocytes (Davies & Metzger, 1983; Abbas et al, 2009).

2.3 T cell mediated-immunity

2.3.1 T cell development in the thymus

T lymphocytes undergo complex maturation stages in the thymus, which determine their functional and phenotypic characteristics of mature T cells before they reside in the peripheral lymphoid organs (Schmitt & Zuniga-Pflucker, 2002). Anatomically, the thymus is divided into two regions; a peripheral cortex and a central medulla (Figure 2.1). There are three distinct processes in T cell development in this organ, which comprises lymphopoiesis, T cell receptor (TCR)-mediated selection and functional maturation (Lind et al, 2001).

The lymphoid progenitor cells migrate from the bone marrow into the cortico-medullary junction and differentiate into T cell lineage, the double negative (DN) thymocytes. These DN thymocytes will move through the cortex and progress into four different stages (Figure 2.2). The first stage of development is characterized by lineage double negative stage, $CD4^-CD8^-CD25^-CD44^{hi}$ (DN1), followed by $CD4^-CD8^-CD25^+CD44^{hi}$ (DN2), and finally the committed T cell lineage, $CD4^-CD8^-CD25^+CD44^{lo}$ (DN3). The final lymphopoiesis stage during DN3 is marked by downregulation of CD25 expression to become double-negative $CD4^-CD8^-CD25^-CD44^-$ thymocytes (DN4). The development progresses into TCR gene rearrangement and upregulation of CD4 and CD8 expression to become committed double positive $CD4^+CD8^+$ $\alpha\beta$ TCR and $\gamma\delta$ TCR thymocytes (DP) (Ardavin et al, 1993; Wu et al, 1996).

DP thymocytes undergo positive selection to allow only thymocytes with functional TCR that have no reactivity towards self-peptide/self-MHC complexes to

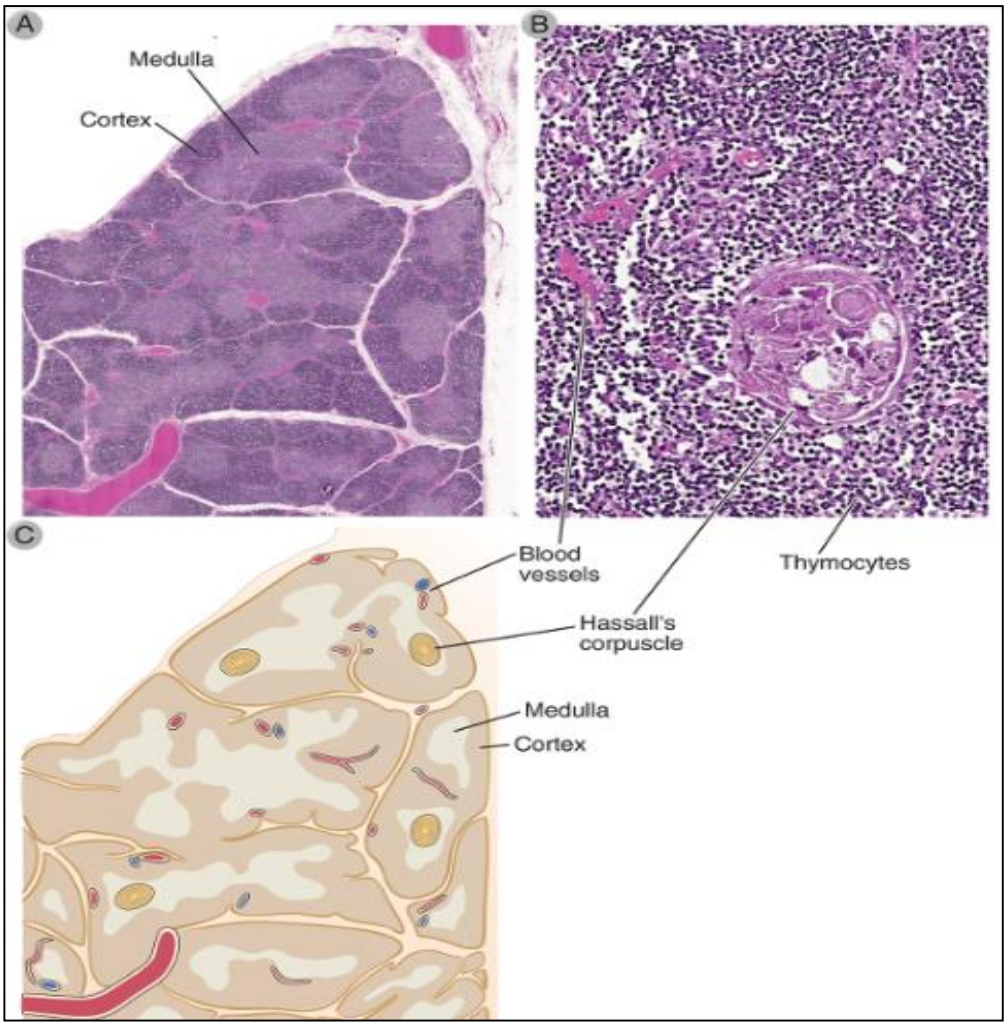

Figure 2.1 Anatomy of the thymus. The migration of lymphoid progenitor cells into the thymus results in the development of thymocytes. Thymocytes reside mainly in the cortex of the thymus. After further maturation these thymocytes migrate to the central medullary region. In the medulla, thymocytes undergo further differentiation processes to become mature T lymphocytes before migrate into the periphery (Adapted from Abbas et al (2012) via studentconsult.com).

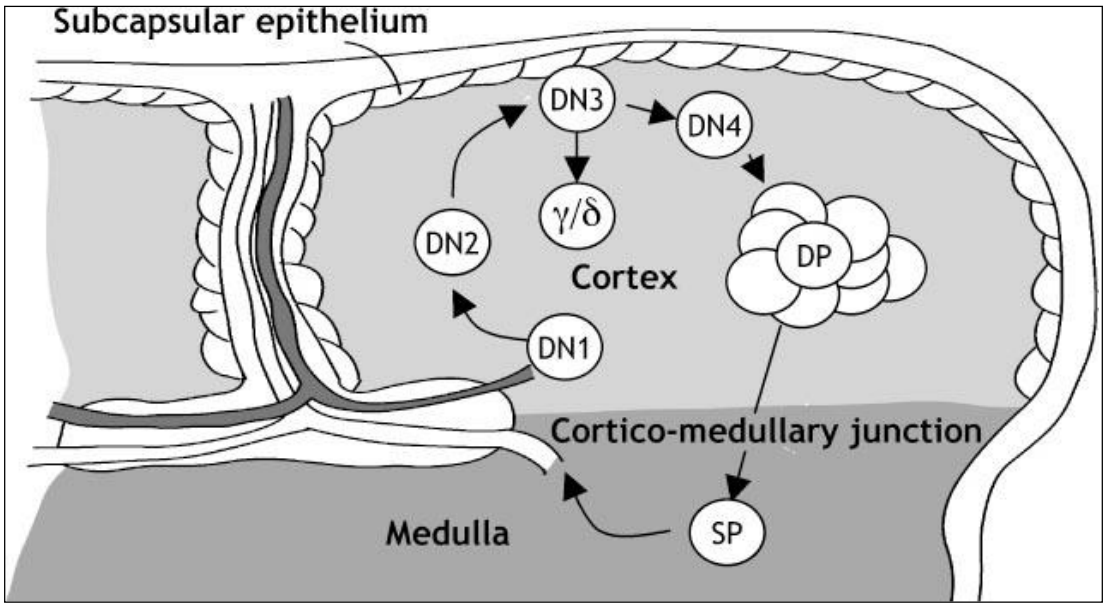

Figure 2.2 Development stages of lymphoid progenitor cells. The lymphoid progenitor cells differentiate into double negative 1 (DN1) thymocytes. As these cells migrate towards the cortico-medullary junction, they upregulate CD25 to develop into DN2 cells. DN2 cells downregulate CD44 to develop into DN3 cells. DN3 cells downregulate CD25 to become DN4 cells. DN4 cells undergo TCRβ re-arrangement and upregulate CD4 and CD8 to become committed DP cells. DP cells undergo positive and negative selection. Cells that pass these selections (SP) will migrate through medulla region and enter the periphery (Adapted from Starr et al, 2003).

pass the selection. During this selection, DP thymocytes are exposed to a wide range of self-peptides that bind with MHC class I or II. DP thymocytes with TCRs that bind with low-affinity to self-peptides on class I self-MHC will downregulate CD4 expression and become $CD8^{+}\alpha\beta^{+}$TCR, while thymocytes with TCRs that bind with low-affinity to self-peptide/self-MHC class II will downregulate CD8 and become $CD4^{+}\alpha\beta^{+}$TCR thymocytes (von Boehmer et al, 2003). Only a small fraction (5 %) of DP thymocytes that pass the positive selection proceeds to the medulla region for the next maturation stage, while the majority (95 %) of DP thymocytes undergo apoptosis (Surh & Sprent, 2005). Subsequently, these single-positive (SP) $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ thymocytes migrate from the cortex to the medulla for negative selection. This selection process adds another layer of tolerance in the immune system whereby thymocytes with high affinity TCRs towards self-peptides will be eliminated by apoptosis (Sprent et al, 1995; Starr et al, 2003).

The αβ T cells that express $CD4^{+}$ class II-MHC-restricted or $CD8^{+}$ class I MHC-restricted T lymphocytes represents the mature repertoire of T lymphocytes. The migration of thymocytes through the anatomic arrangement of the thymus allows physical interactions between thymocytes and the other cells of the thymus such as epithelial cells, bone marrow-derived dendritic cells and macrophages within the medulla. These mature T lymphocytes are known as naive T lymphocytes. Once these naive cells encounter their specific antigens, they become activated and differentiate into effector T lymphocytes that are able to remove antigens (Abbas et al, 2009). Another T cell lineage, the $\gamma\delta^{+}$ TCR thymocytes do not express CD4 or CD8 receptors and thus they skip the positive and negative selection processes. These $\gamma\delta^{+}$ TCR thymocytes are abundant in epithelial tissues such as in the small intestines. They recognize exogenous and endogenous peptides such as viral and heat

shock proteins (in mice) and phosphorylate bacterial metabolites (in human). In the context of MHC class I or class II presentation, γδ T lymphocytes recognize peptides that are different from αβ T lymphocytes (Kabelitz & Wesch, 2003).

2.3.2 Subsets of T cells

The two major T cell subsets are $CD4^+$ αβ T helper lymphocytes (Th cells) and $CD8^+$ αβ T cytotoxic lymphocytes (CTLs) whilst regulatory T-lymphocytes (Treg cells) and γδ T-lymphocytes are of lesser numbers (Mosmann & Sad, 1996). $CD4^+$ Th cells will bind to its cognate MHC Class II expressed by APC while CTLs bind to peptide-loaded MHC Class I presented by most nucleated somatic cells. With the ability to act specifically towards their cognate antigens, these cells are responsible to initiate the cell-mediated immunity and switch on humoral immunity. $CD4^+$Th cells can be further differentiated into effector Th1 cells that secrete IFN-γ to mediate phagocytosis activity of macrophages, or into Th2 cells which recognize antigens of extracellular microbes and helminthic infections (Farrar, Asnagli, & Murphy, 2002).

Another subset of $CD4^+$ cells, which is known as Th17 cells, are characterized by the secretion of IL-17. These cells are essential in mediating neutrophilic inflammatory responses and responsible for tissue damage during inflammation. Th17 cells are induced from naïve $CD4^+$ cells in the presence of TGF-β, IL-6 and IL-21. In addition, IL-23 help to maintain Th17 cell differentiation and survival (Weaver et al, 2006). On the other hand, Treg cells are part of the T cell subsets which also express CD4 molecules on their surfaces, along with CD3 and CD25 receptors. This subset of T cells play a role in regulating the immune system

as immune response triggered by specific antigens may become overwhelmed and detrimental to the host.

2.3.3 TCR activation and signaling of $CD4^+$ T lymphocytes

The activation of naive $CD4^+$ T lymphocytes requires the interaction between TCR with peptide-MHC II complex (Figure 2.3). The TCR complex consists of αβ TCR, CD4 and co-receptor proteins CD3 and zeta chains (ζ chains). This complex requires the co-stimulatory component i.e. CD28 molecules for T cell activation (Borst et al, 1984; Nunes et al, 1996; Wang et al, 2001; Abraham & Weiss, 2004). The engagement of TCR complex with peptide-MHC II complex triggers a cascade of intracellular signalling involving phosphorylation of protein tyrosine kinases. This activation results in transcriptional activation of proinflammatory cytokines and cell proliferation. Unlike αβ TCR molecules, CD3 and especially the ζ chains have long cytoplasmic tails that associate with tyrosine residues known as immunoreceptor tyrosine-based activation motifs (ITAM). Each ITAM molecule has two tyrosine sequence elements (Tyr-X-X-Leu) that serve as phosphorylation sites for protein tyrosine kinases (PTK). Phosphorylation of ITAM by Lck, i.e. a member of the PTK, will initiate signal transduction and activation of other tyrosine kinases (Wegener et al, 1992).

The phosphorylated ITAM serves as a docking site for ζ-associated protein of 70 kDa phosphoprotein (ZAP-70) (Figure 2.3). As a member of the Syk kinase PTK family, ZAP-70 has two Src-homology 2 (SH2) domains that determine their active/inactive state (Chan et al, 1992). Binding of these domains to phosphorylated ITAM will initiate ZAP-70 activation, which in turn will initiate sequence of phosphorylation cascade of adaptor proteins including linker for the activation of

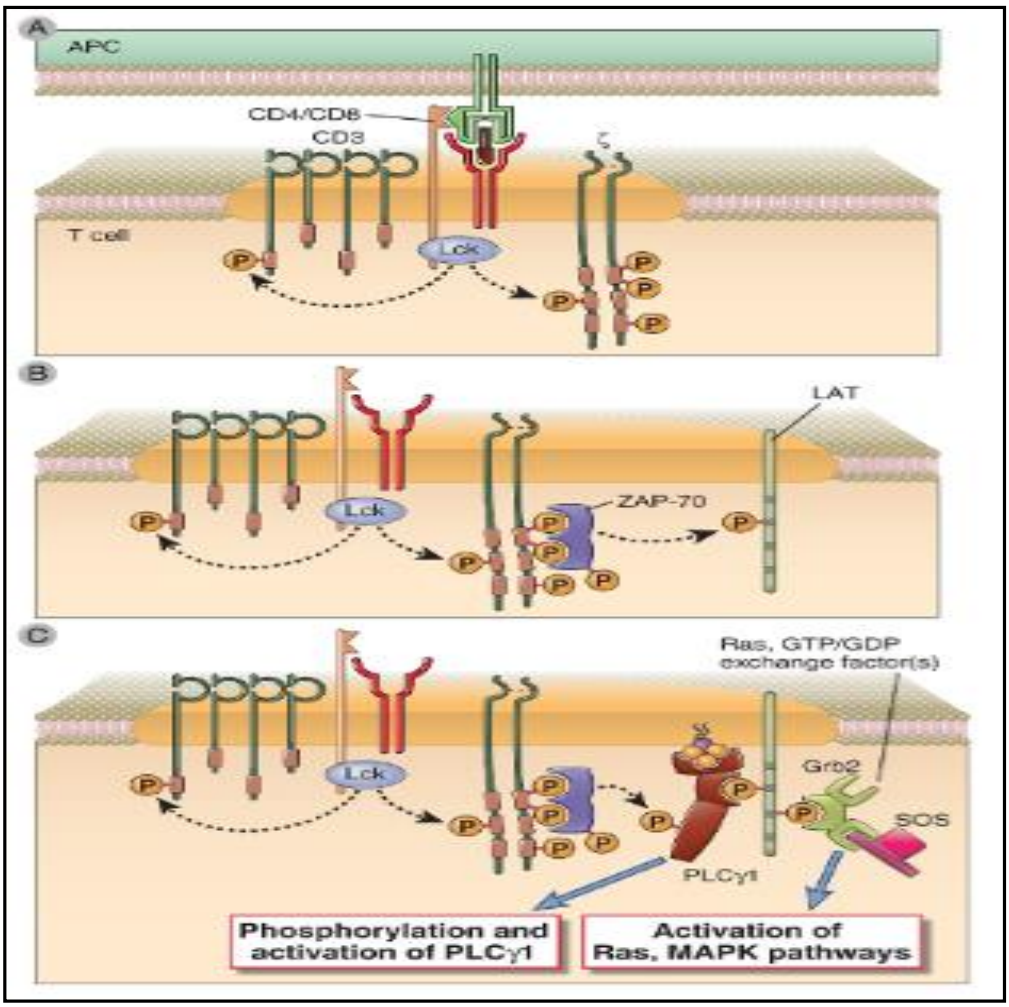

Figure 2.3 Early phosphorylation events upon TCR activation. A) TCR complex and co-receptors are clustered together to initiate phosphorylation of cytoplasmic tail-containing ITAMs B) Phosphorylated ITAMs serve as the docking site for ZAP-70 molecules, which triggers the activation of adaptor protein, LAT C) Activation of adaptor proteins allow the binding of PLCγ1 and other Ras GTP/GDP molecules which in turn catalyse various intracellular signaling molecules (Adapted from Abbas et al (2012) via studentconsult.com).

T cells (LAT) (Zhang et al, 1998), SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) (Wardenberg et al, 1996) and growth factor receptor-bound protein 2 (Grb-2) (Buday et al, 1994). The phosphorylation of these adaptor proteins involves two distinct signalling pathways. These pathways are the phospholipase C-γ1-dependant (PLCγ1) and diacylglycerol-dependant (DAG) pathways, in which each pathway is responsible for the activation of specific transcription factors for target gene expression (Abbas et al, 20012) (Figure 2.4).

SLP-76 mediates PLCγ1 activation by direct interaction with SH3 domains of PLCγ1 molecules, causing hydrolysis reaction of phosphatidylinositol-4,5-biphosphate (PIP_2). This reaction produces inositol-1,4,5-triphosphate (IP_3) and DAG, which respectively trigger calcium flux and protein kinase C (PKCθ) activation (Yablonski et al, 2001). The presence of IP_3 in the cytosol triggers Ca^{2+} depletion in the endoplasmic reticulum, causing high extracellular Ca^{2+} influx. This process will subsequently activate calcineurin, an enzyme that will de-phosphorylate nuclear factor of activated T cells (NFAT) which causes the translocation of NFAT into the nucleus to bind to the IL-2 promoter region (Figure 2.4) (Northrop et al, 1993; Woodrow et al, 1993).

The other product of hydrolyzed PIP_2 , DAG, is required for the activation of mitogen-activated protein (MAP) kinases and the PKCθ pathway. The MAP kinase pathway comprises several components including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH-terminal kinase (JNK) and p38 MAP kinases. DAG activates the MAP kinase pathway by recruiting Ras-GTP ligation, which in turn activates serine-threonine residues of Raf-1. Raf-1 will induce phosphorylation and activation of MAPK Kinases (MAPKKs). Activated MAPKKs will phosphorylate

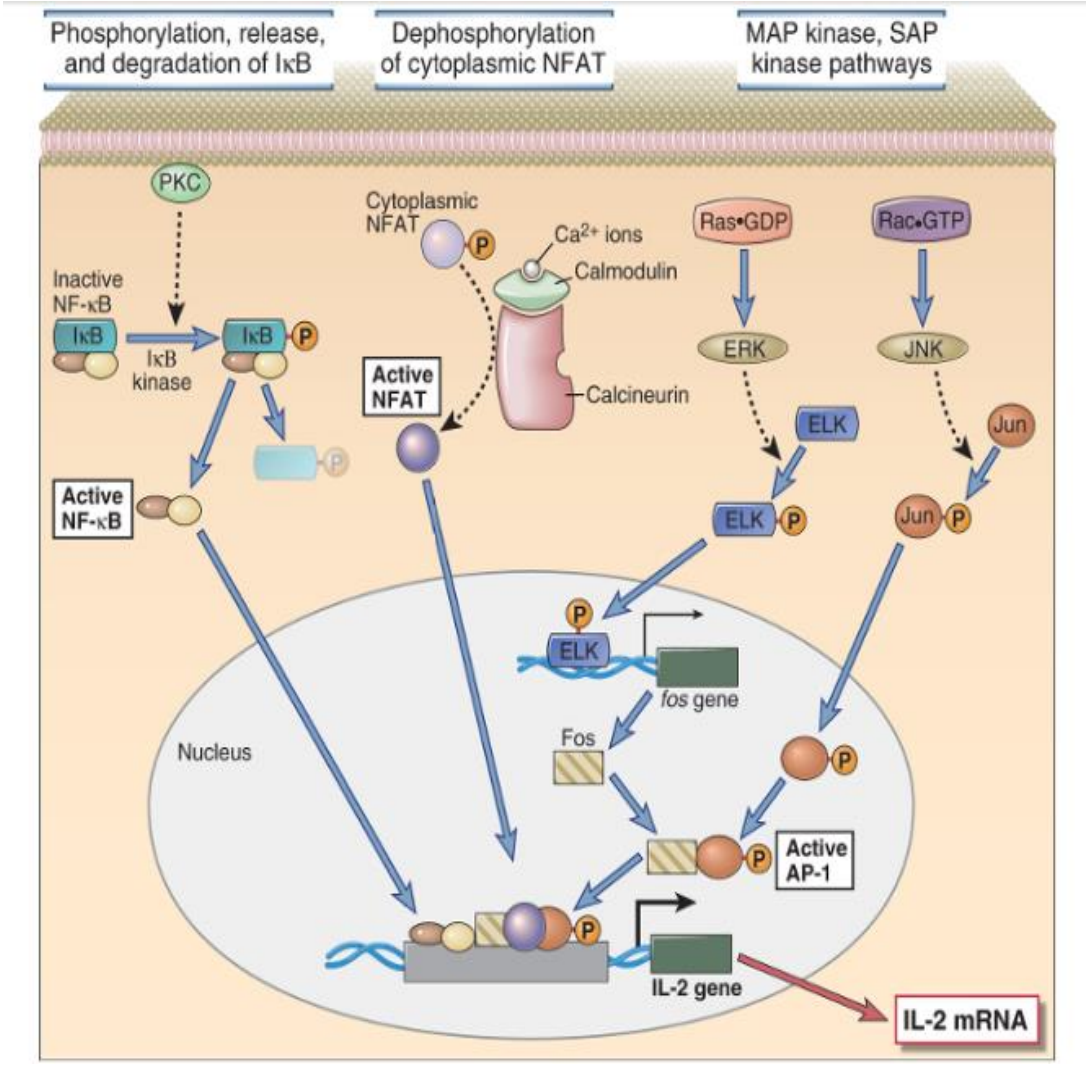

Figure 2.4 Activation of multiple upstream signaling pathways converge downstream to initiate transcription of target genes. The TCR complex ligation initiates the phosphorylation of cytoplasmic tails of surface receptors. This phosphorylation event will trigger phosphorylation cascades and activates multilayer signaling pathways in the cytoplasm. The activation of upstream signaling pathways will eventually activate transcription factors such as NFAT, NF-κB and AP-1. These transcription factors will translocate into the nucleus and bind to the transcriptional complex at the promoter region of genes encoding proinflammatory cytokine, such as IL-2 (Adapted from Abbas et al (2012) via studentconsult.com).

tandem tyrosine and threonine residues of ERK1/2, thus activating ERK molecules resulting in their translocation into the nucleus (Crew et al, 1992; Roose et al, 2005). In the nucleus, ERK continues to phosphorylate Elk-1 nuclear transcription factor and allow its binding to c-Fos promoter region (Babu et al, 2000). The presence of c-Fos as a transcriptional factor at the promoter region of AP-1 is critical for its expression (Angel & Karin, 1991). Parallel to this pathway, Ras-GTP also initiates JNK to activate c-Jun molecule by phosphorylation on the Ser 63- and Ser-73 residues, located within its NH_2-terminal transactivation domain, (Adler et al, 1992). Activated c-Jun forms heterodimer with c-Fos and binds to AP-1 complex at gene promoters that regulate cell differentiation and proliferation (Figure 2.4) (Whitmarsh & Davis, 1996).

The activation of PKCθ molecules is important to initiate the activation of nuclear factor kappa B (NF-κB) in the cytosol. Inactive NF-κB associates with its inhibitor IκB, which prevents its translocation into the nucleus. Thus, degradation of this inhibitory molecule is necessary to activate NF-κB. The production of DAG will also activate membrane-associated PKCθ by inducing its translocation, allowing binding to DAG-specific-binding domain of PKCθ (Villalba et al, 2002). PKCθ initiates degradation of IκB by IκB kinase (IKK) through an array of phosphorylation and activation of protein complexes that are formed by caspase-recruitment domain + membrane-associated guanylate kinase (CARMA1), mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) and CARD-containing adaptor protein Bcl10 (Gaide et al, 2001; Pomerantz et al, 2002; Schulze-Luehrmann & Ghosh, 2006).

PKCθ phosphorylation will activate CARMA1 site, allowing the association of CARMA1, MALT1 and Bcl10-associated adaptor protein to form a tri-molecular complex. The binding of MALT1 to Bcl10 activates IKK from its inactive IKKγ state (Schulze-Luehrmann & Ghosh, 2006). The active form of IKK will then cleave the binding of NF-κB and IκB, allowing migration of NF-κB into the nucleus where it binds to transcription complex of genes encoding cytokines for T cell proliferation and function (Figure 2.4) (Schulze-Luehrmann & Ghosh, 2006).

2.4 Immunological tolerance

Immunological tolerance is a well-regulated process that involves central and peripheral mechanisms. Immunological tolerance is important to limit recognition of self-antigens by actived T-lymphocytes, hence regulating the immune response. This self-tolerance of T lymphocytes starts in the central thymus and is maintained in the periphery.

Central tolerance occurs during T cell development in the thymus. Several mechanisms are involved before T-lymphocytes are able to induce self-tolerance. During negative selection in the thymus, T lymphocytes with potential to recognize self-antigens will be deleted from the $CD4^+$ cell pool by clonal deletion. As described in section 2.2.1, immature T lymphocytes with TCR that produce strong intracellular signalling towards self-antigens/MHC complex will be triggered to undergo clonal deletion via apoptosis (Sprent et al, 1995; Starr et al, 2003). However, a small portion of cells that recognize self-antigens are not deleted by clonal deletion, instead, they differentiate into subsets of $CD4^+$ cells, known as natural T regulatory (nTreg) cells (Sakaguchi et al, 1995; Von Boehmer et al, 2003; Fontenot et al, 2005a). These cells exhibit suppressive function in the periphery by expressing high

levels of cytotoxic T-lymphocyte antigen (CTLA-4) and glucocorticoid inducible TNF receptor (GITR) molecules on their surfaces (Von Boehmer et al, 2003).

Due to diverse T cell repertoires, some mature T lymphocytes in peripheral tissues become auto-reactive and recognize self-antigens, causing destruction of self-tissues. Therefore, peripheral tolerance is required to render inactivation of self-reactive T lymphocytes. Peripheral tolerance requires the engagement of inhibitory receptors such as CTLA-4 and programmed death-1 (PD-1) molecules that induce anergy, and suppressive nTreg cells that inhibit self-reactive T lymphocytes.

The engagement of CTLA-4 will result in degradation of TCR signaling proteins, thus inducing T lymphocyte anergy (Walunas et al, 1994; Tivol et al, 1995). Anergy of self-reactive T lymphocytes results in the inability of these cells to respond to self-antigens, by inhibiting their proliferation and cytokine production (Miller & Morahan, 1992). On the other hand, engagement of PD-1 expressed on self-reactive T lymphocytes with its ligand will inhibit activation of these cells via inducible Treg-dependant (iTreg) pathway (Qiao et al, 2012). Induced Treg (iTreg) are $CD4^+CD25^+Foxp3^+$ cells that induced Foxp3 expression at the periphery and are responsible to regulate inflammatory response (Sakaguchi et al, 2009). It is also important to note that peripheral tolerance also involves nTreg cells that function as a suppressor for auto-reactive T lymphocytes (Sakaguchi et al, 1995). These mechanisms maintain self-tolerance and control the immune homeostasis.

2.4.1 Natural $CD4^+CD25^+$ T-Regulatory (nTreg) cells

In 1995, Sakaguchi and colleagues first discovered that CD25 is the molecular component of immunosuppressive T cells. This subset of $CD4^+$ cells is

now known as $CD4^+CD25^+$ T regulatory cells (Treg). Depletion of $CD4^+CD25^+$ Treg cells is associated with autoimmune diseases (Sakaguchi et al, 1995; Balandina et al, 2005; Candon & Chatenoud, 2005).

Natural Treg cells are derived from the thymus, and later migrate to the periphery, where they constitute approximately 8% to 10 % of peripheral $CD4^+$ T cells (Sakaguchi et al, 1995; Papiernik et al, 1998). The cardinal feature of nTreg cells is the constitutive expression of CD25 molecules (IL-2 receptor α-chain) on the surface and intracellular Foxp3 molecules (Sakaguchi et al, 2005). Owing to its natural capacity to suppress the auto-reactive immune cells, nTreg cells become the prominent subtype of regulatory T cells in many auto-immune related studies such as inflammatory bowel disease (IBD), Type 1 autoimmune diabetes (T1D), multiple sclerosis and allergic reactions.

2.4.1.1 Mechanism of suppression by T-regulatory cells

The mechanism of action of nTreg cells *in vivo* has been reported by Vignali et al (2008) (Figure 2.5). Regulatory mechanisms comprise inhibitory cytokines (TGF-β, IL-10, and IL-35), cytolysis (granzyme and perforin), IL-2 deprivation and cell contact-dependant manner (LAG-3, CTLA-4, CD39, and CD73). Similarly, the same mechanisms may also be used by nTreg *in vitro*, although TGF-β-independant and IL-4 cytokine mediation were also observed in these cells (Shevach et al, 2006).

2.4.1.1.1 Metabolic disruption

The first regulatory mechanism imposed by nTreg cells was by IL-2-mediated pathway as suggested by Thornton and Shevach in 1998. They suggested that Treg cells could inhibit the production of IL-2 cytokines by effector T cells,

causing anergy in these cells. This mechanism was later supported by de la Rosa et al (2004) whereby IL-2 consumption by nTreg cells induced suppression towards Teff cells. This suppressive effect was reversible in the presence of IL-2R blocker. Finally, by using the same *in vitro* co-culture setting, Pandiyan and colleagues (2007) showed that *in vitro* IL-2 deprivation by Treg cells induce apoptosis in effector T cells, thus reduces the effector T cell function.

Natural Treg cells are capable of suppressing Teff cells via transfer of cyclic adenosine monophosphate (cAMP) in a contact-dependant manner. The accumulation of cAMP in Teff cells inhibits their proliferation (Bopp et al, 2009). In addition, the expression of ectonucleotidase CD39 and CD73 on nTreg cells catalyses extracellular nucleotides will results in the generation of pericellular adenosine (Deaglio et al, 2007). The accumulation of pericellular adenosine will block IL-2 suppression by Teff cells via A2A adenosine receptor (Huang et al, 1997).

2.4.1.1.2 Inhibitory cytokines

Anti-inflammatory cytokines such as TGF-β and IL-10 play an important role as inhibitory mediators to curtail excessive inflammatory response (Abbas et al, 2007). Murine *in vivo* studies showed that transfer of nTreg cells into CD25-deficient mice requires IL-10-mediated suppression (Pontoux, Banz & Papiernik, 2002). Similarly, transfer of nTreg cells from wild type (WT) mice into SCID mice protected the latter from colitis while administration of TGF-β antibody resulted in loss of suppressive function of Treg cells. On the other hand, transfer of IL-10-deficient nTreg cells failed to protect SCID mouse model from colitis (Asseman et al, 1999). In addition, allergy and asthmatic reactions in mouse models are also reduced upon nTreg cell transfer (Hawrylowicz & O'Garra, 2005), and synergistic

effect by TGF-β is required by these cells to alleviate these reactions (Joetham et al, 2007; Brandenburg et al, 2008).

However, the role of IL-10 in the nTreg cell mediated-suppression is still debatable. For example, previous *in vitro* studies showed that IL-10-deficient nTreg cells were suppressive towards Teff cells (Thornton & Shevach, 1998). In addition, the deletion of IL10 allele in nTreg cells only caused the development of localized tissue inflammation but not systemic autoimmune reaction, in mouse model (Rubstov et al, 2008).

IL-35 is a new member of the heterodimeric IL-12 cytokine family that was discovered by Collison et al (2007). IL-35 is formed by the pairing of Epstein-Barr Virus-induced gene 3 (*Ebi*) with *il12a* gene (Collison et al, 2007). In normal condition, *Ebi* pairs with p28 to encode IL-27 while p35 pairs with p40 to encode IL-12 (Collison et al, 2007). The expression of *Ebi* and *il12a* genes are highly increased in mouse Foxp3^{+} nTreg cells, and are significantly upregulated in activated nTreg cells, but not in Teff cell population (Collison et al, 2007). The ability of IL-35-secreted Treg cells to regulate immune homeostasis is significantly lowered in *Ebi*$^{-/-}$ and *il12a*$^{-/-}$ *in vitro* and failed to control IBD *in vivo* (Collison et al, 2007). Furthermore, IL-35-secreting nTreg cells could inhibit the development of pro-inflammatory Th17 cells, both *in vitro* and *in vivo* (Niedbala et al, 2007).

2.4.1.1.3 Dendritic cells (DC) modification

Natural Treg cells has been postulated to act on dendritic cells (DC) as one mechanism to regulate immune homeostasis, mainly via CTLA-4 and its ligands CD80/86 (Tang et al, 2004; Oderup et al 2006; Tadokoro et al, 2006). Fallarino and

co-workers (2003) reported that the interaction between CTLA-4 and CD80/86 molecules induced DC to secrete indolamin 2,3-dioxygenase (IDO), a potent regulatory molecule known to induce pro-apoptotic metabolites, as a result of tryptophan catabolism, leading to immunosuppressive activity in T effector cells.

2.4.1.1.4 Suppression by cytolysis

Natural Treg cells also mediate the immune response via granzyme by perforin-dependant mechanism (Grossman et al, 2004). It was shown that nTreg cells of granzyme and perforin knockout mice are less effective in suppressing Teff cells (Gondek et al, 2005). In addition, previous reports have suggested that murine nTreg cells are capable of inducing apoptosis in Teff cells via the tumour-necrosis-factor-related-apoptosis-inducing-ligand-death-receptor 5 (TRAIL-DR5) pathway *in vitro* (Ren et al, 2007). The prolonged protection of allogeneic skin graft from rejection by nTreg cells was overcomed by addition of death-receptor-5 (DR-5) blocking antibodies, indicating the same mechanism may be operative *in vivo* (Ren et al, 2007). Nevertheless, it was reported that TRAIL-DR5 pathway is the default mechanism of suppression in nTreg cells of Balb/c mice, while inhibitory cytokine-mediated suppression is probably the primary mechanism employed in nTreg cells of C57BL/6 mice (Pillai et al, 2011).

2.4.1.2 IL-2 and Jak/STAT signalling

The high-affinity IL-2 receptor comprises 3 subunits: the α-chain of the IL-2R (CD25), β-chain of the IL-2R (CD122) and the common γ-chain of the IL-2R (CD132) (Malek et al, 2004). Figure 2.6 depicts the binding of IL-2 to IL-2R α-chain