NEGATIVE REGULATION OF T CELL ACTIVATION AND FUNCTION BY CIN85 ADAPTOR PROTEIN

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NEGATIVE REGULATION OF T CELL ACTIVATION AND FUNCTION BY CIN85 ADAPTOR PROTEIN

by

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TABLE OF CONTENTS

CHAPTER 2 - MATERIALS AND METHODS

CHAPTER 3 - RESULTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

APCs	antigen-presenting cells
Cbl	casitas B-lineage lymphoma
CC	coiled-coil
CIN85	Cbl-interacting protein of 85 kDa
cSMAC	central supramolecular activation cluster
dKO	doubly knockout
DCs	dendritic cells
DN	double-negative
DP	double-positive
EGFR	epidermal growth factor receptor
ERK	extracellular-signal-regulated kinase
GAH	Goat anti-hamster
GFP	green-fluorescent protein
GPI	Glycophosphatidylinositol
HRP	horseradish peroxidase
IgG	immunoglobulin G
IS	immunological synapse
ITAM	immunoreceptor tyrosine-based activation motif
JNK	c-Jun-N-terminal kinase
KO	knockout
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase.
MCC	moth cytochrome c

MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
OD	optical density
PBS	phosphate buffered saline
PE-H57	PE-conjugated anti-TCRβ antibody
PLCγ	phospholipase C-gamma
pMHC	peptide presented by MHC complex
PMA	phorbol 12-myristate 13-acetate
PR	proline-rich
PTKs	non-receptor protein tyrosine kinases
PTPases	protein tyrosine phosphatases
RTKs	receptor tyrosine kinases
RTP	room temperature
SD	standard deviation
SH3	Src homology region 3
SHP-1	Src homology region 2 domain-containing phosphatase-1
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
SP	single-positive
SR	serine-rich
Sts-2	suppressor of TCR signaling-2
Syk	spleen tyrosine kinase
TCR	T cell receptor
TCR-MCs	TCR microclusters

Teff cells	effector T cells
Tg	transgenic
Th cells	T helper cells
WT	wild-type
Zap70	ζ-chain associated protein kinase of 70 kDa
Δ	deletion

UNIT OF MEASUREMENT

Å	angstrom
a.u.	arbitrary unit
bp	basepairs
cm	centimetre
°C	degree Celsius
g	gram
Gy	gray
× *g*	relative centrifugal force
kb	kilobase
kDa	kilodalton
L	litre
M	molar
mA	milliampere
mg	milligram
µg	microgram
min(s)	minute(s)
µl	microlitre
ml	millilitre
µm	micrometre
µM	micromolar
mM	millimolar
mm	millimetre
msec	millisecond

mW	milliwatts
m/z	mass-to-charge ratio
NA	numerical aperture
ng	nanogram
nl	nanolitre
nM	nanomolar
nm	nanometer
ppm	parts per million
rpm	rotations per minute
sec	second
U	unit
V	volt
(v/v)	volume per volume
(w/v)	weight per volume
%	percentage

REGULASI PENGAKTIFAN DAN KEFUNGSIAN SEL T SECARA NEGATIF OLEH PROTEIN PENYESUAI CIN85

ABSTRAK

Pengenalpastian antigen oleh reseptor sel T (TCR) mendorong jalur isyarat intraselular yang menyebabkan pengaktifan sel T, seterusnya mendorong percambahan sel dan pelbagai fungsi efektor. Protein penyesuai memainkan peranan dalam sel T dengan mewujudkan nod untuk merekrut protein isyarat dan membentuk isyarat kompleks yang mengawal selia arah isyarat sel. Casitas B-lineage Lymphoma (Cbl)-protein interaksi 85 kDa (CIN85) ialah protein penyesuai, asalnya dikenali sebagai protein mengikat Cbl dan dicadangkan terlibat dalam internalisasi dan degradasi reseptor dan kinase. Walau bagaimanapun, fungsi fisiologi CIN85 dalam sel T tidak diketahui. Untuk memahami peranan CIN85 dalam fungsi sel T, mencit yang dihapuskan gen *cin85* (khususnya dalam sel T sahaja) (CIN85-KO) telah dijana dan diguna sebagai model untuk analisis dalam kajian ini. Sel T naïf CIN85-KO menunjukkan reaksi hyper terhadap stimulasi TCR, iaitu penghasilan interleukin-2, percambahan sel T dan pengubahsuaian perbezaan kepada T helper jenis 1 fenotip menigkat dengan ketara. Hal ini mencadangkan CIN85 menunjukkan regulasi negatif dalam aktivasi sel T. Analisa molekul isyarat menunjukkan pemfosforilan tirosine pada molekul jaluran-TCR seperti rantai-zeta yang berkaitan protein kinase 70 kDa (Zap70), protein SH2 domain mengandungi leukosit protein 76 kDa (SLP-76), fosfolipase C-gamma (PLCγ), c-Jun-N-terminal kinase (JNK) dan isyarat dikawal-luar sel kinase (ERK), telah dipertingkatkan dengan ketara dalam sel T CIN85-KO. Data ini mengimplikasikan bahawa CIN85 terlibat dalam awal isyarat

kompleks TCR untuk mengawalkan selia negatif aktivasi sel T. Mencit CIN85-KO menunjukkan jumlah penduduk sel T efektor/memori $CD44^{hi}$ yang lebih tiggi dalam perifer. Hal ini membuktikan bahawa CIN85 memainkan peranan penting dalam pemeliharaan homeostasis sel T *in vivo*. Memandangkan internalisasi TCR yang didorong oleh rangsangan tidak berubah dalam sel T CIN85-KO berbanding dengan mencit 'wild-type'. CIN85 tidak berperanan dalam proses endositosis reseptor, seperti yang dicadangkan dalam kajian dengan menggunakan jenis sel yang lain. Selepas aktivasi TCR, CIN85 didapati 'co-localize' bersama TCR mikrokluster (TCR-MCs). Kekurangan protein CIN85 dalam sel T tidak menjejaskan pembentukan TCR-MCs dan kluster-kluster SLP-76. Dalam pencarian molekul berikatan dengan CIN85, penindas isyarat-2 TCR (Sts-2) fosfatase telah ditunjukkan berinteraksi dengan CIN85 setelah rangsangan TCR, untuk aktiviti CIN85 dalam regulasi negatif terhadap aktivasi sel T. Kedua-dua domain Src homologi rantau 3 (SH3) dan rantau proline kaya (PR) kepunyaan CIN85 adalah penting untuk interaksi CIN85 dengan Sts-2. Keseluruhannya, data-data dalam kajian ini menunjukkan CIN85 mengawal aktivasi dan pembentukkan sel T secara negatif, melalui keupayaannya merekrut Sts-2 kepada isyarat kompleks supaya ia menjalankan proses difosforilasi terhadap Zap70 yang telah diaktifkan. Ini adalah penting untuk mencegah reaksi berlebihan yang berkemungkinan membawa penyakit autoimun.

NEGATIVE REGULATION OF T CELL ACTIVATION AND FUNCTION BY CIN85 ADAPTOR PROTEIN

ABSTRACT

Antigen recognition by T cell receptor (TCR) induces intracellular signaling cascades leading to T cell activation, subsequently inducing cellular proliferation and various effector functions. Adaptor proteins play important roles in T cell signaling by creating nodes to recruit signaling proteins and form signal complexes to regulate the direction of signaling. Casitas B-lineage lymphoma (Cbl)-interacting protein of 85 kDa (CIN85) is an adaptor protein, originally identified as a Cbl-binding protein, and was suggested to be involved in the internalization and degradation of receptors and kinases. Nevertheless, the physiological function of CIN85 in T cells is unknown. To understand the role of CIN85 in T cell function, T cell-specific CIN85-knockout (KO) mice were generated and analyzed in this study. CIN85-KO naïve T cells were found to exhibit hyper-responsiveness to TCR stimulation; i.e. significantly increased interleukin 2 (IL-2) production, cell proliferation and alteration of differentiation into T helper type 1 (Th1) phenotypes, suggesting that CIN85 exhibits negative regulation in T cell activation. Analysis of signaling molecules showed that tyrosine phosphorylation of TCR-downstream molecules, such as ζ-chain associated protein kinase of 70 kDa (Zap70), SH2 domain-containing leukocyte protein of 76 kDa (SLP76), phospholipase C-gamma (PLCγ), c-Jun-N-terminal kinase (JNK) and extracellular-signal-regulated kinase (ERK), were significantly enhanced in CIN85-KO T cells. This implied that CIN85 was involved in the early TCR signaling complex to negatively regulate T cell activation. As for T cell development,

CIN85-KO mice were found to display higher population of $CD44^{hi}$ effector/memory-type T cells at the periphery, suggesting an important role of CIN85 in maintaining the homeostasis of T cells *in vivo*. Since stimulation-induced TCR internalization was not altered in CIN85-KO T cells, CIN85 in T cells was unlikely to have played a role in the endocytosis as suggested in other cell types. Upon TCR stimulation, CIN85 was shown to co-localize with TCR microclusters (TCR-MCs); deficiency of CIN85 caused no impairment to TCR-MCs and SLP76-cluster formation. Suppressor of TCR signaling-2 (Sts-2) phosphatase was found to bind to CIN85 upon TCR stimulation, which is important for CIN85-mediated negative regulation of T cell activation. Both Src homology region 3 (SH3) domains and proline-rich (PR) region of CIN85 were found to be crucial for the CIN85-Sts-2 interactions. Collectively, these data showed that CIN85 negatively regulates T cell activation and development, through its ability to recruit Sts-2 to the signaling complex for its dephosphorylation activity on activated Zap70. This is important to prevent overreaction that might lead to autoimmune disease.

CHAPTER ONE

INTRODUCTION

1.1 Immune system

Despite continuous exposure to pathogenic microorganisms, we rarely fall sick. How does the body defend itself? The immune system consists of a variety of effector cells and molecules to defend the body from environmental agents, such as infectious microorganisms and harmful substances or chemicals.

Traditionally, immunity is classified into innate and adaptive immune systems with distinct properties and functions (Hoebe *et al.*, 2004). This classification is essential for understanding and compartmentalization of the immune system; nevertheless, our immune system practically functions as a single unit rather than distinct entities (Murphy *et al.*, 2008).

Innate immunity is recognized to respond rapidly and vigorously to combat a wide range of pathogens, but also can cause damage to normal healthy tissues due to loss of specificity for antigenic discrimination and lack of memory for lasting immunity (Parkin & Cohen, 2001; Hansson *et al.*, 2002). Innate immunity is also known as a phylogenetically ancient defense mechanism, which exploits a limited number of germline-encoded pattern-recognition receptors (PRRs). PRRs recognize the evolutionary conserved structures of pathogens, which are known as pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2000; Akira *et al.*, 2006; Mogensen, 2009). PAMPs include lipopolysaccharides (LPS) (a major component of the outer membrane of Gram-negative bacteria), lipoteichoic

acids (LTA) (cell wall polymer of Gram-positive bacteria), peptidoglycans (component of a mesh-like layer at outer membrane of most bacteria), mannans and glucans (fungal cell wall components), and microbial nucleic acids (e.g. bacterial DNA and viral DNA/RNA) (Medzhitov & Janeway Jr, 1997; McCance & Huether, 2010; Vincent, 2011).

In contrast, adaptive immunity induces antigen-specific immune response, which is long lasting. It is developed through adaptation when encountering specific pathogens during the individual's lifetime (Murphy *et al.*, 2008; Rimer *et al.*, 2014). Adaptive immunity is characterized as relatively delayed-response compared to innate immunity. It utilizes specific immune receptors and exhibits immunological memory that confers life-long protective immunity to reinfection with the same pathogen (Gowans & Uhr, 1966; Borghesi & Milcarek, 2007). Adaptive immunity is an immune mechanism that uniquely manifests itself in vertebrate animals (Rimer *et al.*, 2014).

Both innate and adaptive immune responses depend on the activity of leukocytes (white blood cells). Innate immunity includes neutrophils, monocytes and macrophages (phagocytosis), dendritic cells, basophils, mast cells and eosinophils (release inflammatory mediators), and natural killer cells (cytotoxic lymphocytes) (Shaykhiev & Bals, 2007; Murphy *et al.*, 2008). Physical, chemical and microbiological barriers, and molecular components, such as complement, acute-phase proteins and some cytokines, are also encompassed in innate immunity (Medzhitov & Janeway, 2000; Basset *et al.*, 2003). On the other hand, adaptive immunity utilizes B lymphocytes (B cells) and T lymphocytes (T cells) with highly

specialized surface receptors that recognize and respond to individual antigens (Hansson *et al.*, 2002).

All immune cells are derived from pluripotent hematopoietic stem cells (HSCs) that originate in the bone marrow (Domen *et al.*, 2006). These stem cells give rise to both the common myeloid progenitor cells (basophils, neutrophils, eosinophils, erythrocytes, monocytes, macrophages and mast cells) and the common lymphoid progenitor cells (T cells, B cells and NK cells). Many of these precursor immune cells develop and mature in the bone marrow. For example, B cells undergo maturation within the bone marrow. However, T cells are the only hematopoietic cells that are generated in the thymus as the precursor cells migrate from the bone marrow into the thymus and develop as thymocytes (Schultz & Grieder, 1987; Germain, 2002; Park *et al.*, 2010; Simeoni *et al.*, 2008). Both the bone marrow and the thymus are referred to as the central or primary lymphoid organs, where the lymphocytes are generated (Murphy *et al.*, 2008).

The lymphocytes then migrate out of the primary lymphoid organs via the lymphatic and blood circulating system into the secondary or peripheral lymphoid organs, i.e. spleen, lymph nodes and mucosal associated lymphoid tissue (MALT) to defend the body (Ng & Chalasani, 2008). Adaptive immune responses are initiated at the peripheral lymphoid organs, in which lymphocytes recirculate until they encounter their specific foreign antigens. This recognition induces lymphocyte activation, proliferation and differentiation into effector cells that migrate to the site of infection and launch effector responses to combat the pathogen (Alberts *et al.*, 2008).

The adaptive immune system comprised the humoral immune system and the cellular immune system, which are mediated by B cells and T cells, respectively. T and B cells use antigen-specific receptors expressed on their cell surfaces to drive targeted effector responses. Each lymphocyte bears unique variant of prototype antigen-specific receptors during its early stage of development (before exposure to antigen) (Chaplin, 2010). Random rearrangement and splicing of multiple DNA segments of genes that encode for the antigen-binding region of the receptor results in a huge repertoire of about 10^{11} possible TCR (Arstila *et al.*, 1999) and over 10^{12} antibody specificities (Alberts *et al.*, 2008; Glanville *et al.*, 2009) that enable adequate coverage of the wide variety of pathogens that an individual will probably encounter during a life time. TCR is further explained in Chapter 1.3.

1.2 T cell immunity

T cell-mediated immunity is also used as the terminology for cellular immunity that involves antigen-specific T lymphocytes, which mainly functions to eliminate bacterial, viral and parasitic infections as well as malignant cells (Broere *et al.*, 2011). T cells mount effective immune response, basically in two stages (Figure 1.1). Firstly, presenting antigen via the major histocompatibility complex (MHC) molecule on antigen-presenting cells (APCs) or infected cells recognized and bound by antigen-specific TCR, and hereafter leading to T-cell priming, activation, proliferation and differentiation into functional forms (armed effector cells) that launch immune response, either directly by killing infected cells (cytolytic activity of $CD8^+$ cytotoxic T cells) or indirectly by secreting various types of cytokines that orchestrate the responses of B cells to produce antibodies in the humoral immune response (T-cell dependent B cell response) (Delves & Roitt, 2000a; Alberts *et al.*,

2008; van den Boorn, 2006). Integration of co-stimulatory signals with TCR signaling are required for optimal TCR activation, further details are explained in Chapter 1.4. Subsets of effector T cells and TCR composition are further explained in detail in the later part of Chapter 1.2 and Chapter 1.3, respectively.

It is because B cells are unable to be activated by most antigens without "help" from helper T cells, therefore the activation of naïve T cells is indispensable in the initial stage of the adaptive immune response (Mak & Saunders, 2004). In addition to the role of T cell as an initiator of antigen-specific immunity, it also functions as an important regulator of the adaptive immune response. Hence, any aberrancy of T cell activation may lead to disease.

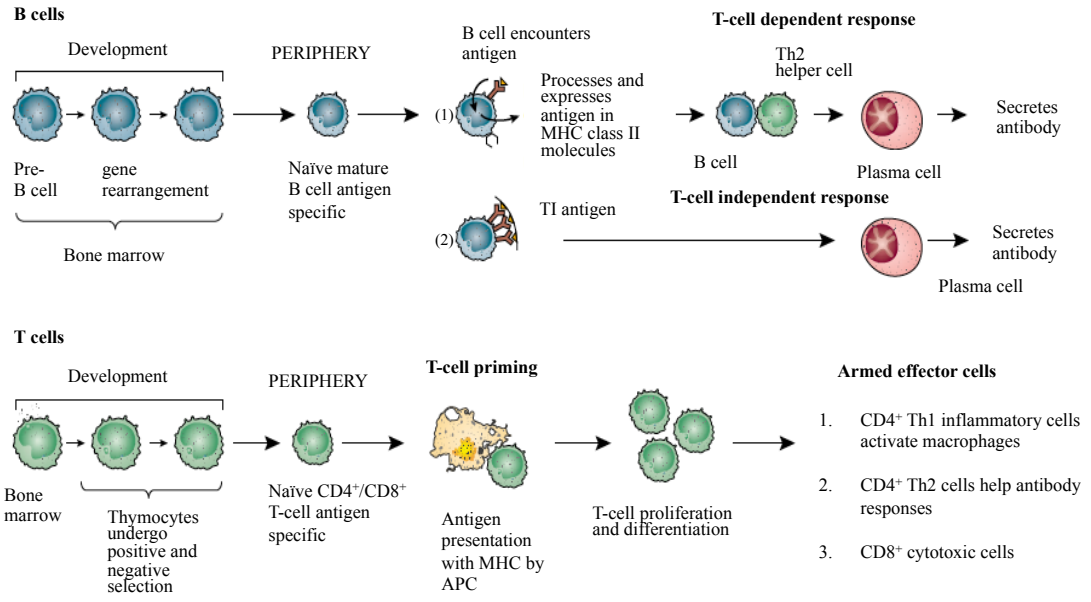

Figure 1.1: The roles and functions of T and B lymphocytes in adaptive immunity (figure modified from Parkin & Cohen, 2001).

T cell-mediated immunity plays a central role in the adaptive immune response, including primary response by naïve T cells, effector functions by activated T cells and long-lasting memory response by antigen-specific memory T cells (Warrington *et al.*, 2011). Distinct T cell populations are distinguished by surface molecules CD44 and CD62L; naïve T cells ($CD44^{lo}$ $CD62L^{hi}$), effector memory T cells ($CD44^{hi}$ $CD62L^{lo}$) and central memory T cells ($CD44^{hi}$ $CD62L^{hi}$) (Swain *et al.*, 1996; Sondel *et al.*, 2003). Naive T cells are considered mature T cells that have successfully undergone central selection (positive and negative selections) in the thymus (Bosco *et al.*, 2009). Thymic selection is further explained in Chapter 1.4. In contrast, memory T cells are T cells that are antigen-experienced with a specific set of TCR, which allows memory T cells to survive for years but allowing rapid immunological response when they are re-exposed to the same antigen subsequently (McHeyzer-Williams *et al.*, 1996; Brenchley *et al.*, 2002).

The mature T cell population can be subdivided into several categories based on their lineage markers and functional activities. The most common groups are defined by two major surface co-receptors, CD4 and CD8 (Swain, 1983), which are expressed on T cell surface membrane and differ in their MHC restriction and function (Germain, 2002; Wang *et al.*, 2009). Pathogen peptide (antigen) presentation by MHC molecules depend mainly on their origin, as typically MHC class I (MHC-I) and MHC class II (MHC-II) molecules display endogenously synthesized peptides and internalized exogenous antigens, respectively, on their cell membrane (Watts, 1997; Blum *et al.*, 2013; Mantegazza *et al.*, 2013). $CD8^{+}$ T cells are only able to recognize antigens that are associated with MHC-I molecules (Swain, 1981; Swain *et*

al., 1981; Norment *et al.*, 1988) while $CD4^+$ T cells are only able to recognize antigens that are displayed on MHC-II molecules (Doyle & Strominger, 1987).

Upon antigen recognition, naïve T cells are activated and differentiate into several functional subsets of effector T (Teff) cells with specialized activities (Figure 1.2). Naïve $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) that particularly function to recognize and kill infected cells and tumor cells. Conversely, $CD4^+$ T cells have higher flexibility in terms of repertoire of effector activities. Naïve $CD4^+$ T cells could differentiate into T helper (Th) cell subpopulations with a variety of effector functions (Anderson *et al.*, 2014; Pen *et al.*, 2014) and regulatory T (Treg) cells that have inhibitory function to limit the extent of immune activation (Sakaguchi, 2005). Th cell subsets are classified on the basis of their cytokine secretion profiles and play critical functions by coordinating the immunological activities (Luckheeram *et al.*, 2012).

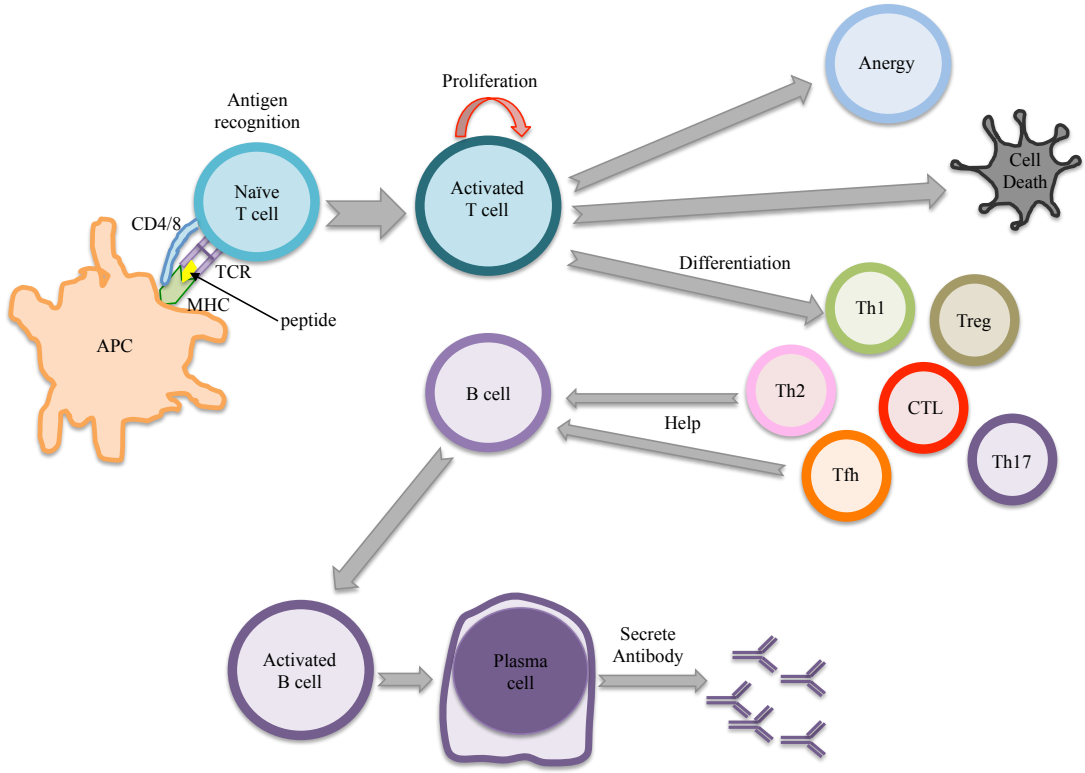

Figure 1.2: T cell fates in adaptive immunity

Currently, the main functional Th cell subsets recognized are Th1, Th2, Th17 and T follicular helper (Tfh) cells (Mosmann & Coffman, 1989; Breitfeld *et al.*, 2000; Akbar *et al.*, 2007; Peck & Mellins, 2010; Crome *et al.*, 2010). Functionally, effector Th cells are induced when naïve T cells are stimulated through TCR engagement by specific antigen under the guidance of developmental cues, such as cytokines produced by innate immune cells (Striz *et al.*, 2014). Th cells exhibit important host defense functions. Nevertheless, dysregulated or uncontrolled Teff cell responses may cause immune pathogenesis.

As shown in Figure 1.3, Th1 cells that produce IL-2 and interferon-gamma (IFN-γ) elicit delayed-type hypersensitivity responses (pro-inflammatory cell-mediated immunity), activate macrophages (phagocyte-dependent inflammation), and enhance clearance of intracellular pathogens. These cells can also be linked to various chronic autoimmune diseases, if they are involved in aberrant recognition of self-antigens. Th2 cells produce IL-4, IL-5, IL-9 and IL-13. They are vital to control certain parasitic infections and critical in allergic responses (Arthur & Mason, 1986; Mosmann *et al.*, 1986; Mosmann & Coffman, 1989; Abbas *et al.*, 1996). Th17 cells are important in regulating tissue inflammatory reactions and autoimmune disease induction via production of IL-17 and IL-22 (Harrington *et al.*, 2005; Park *et al.*, 2006). Tfh cells are localized in the follicles of secondary lymphoid organs, such as spleen, lymph nodes and the Peyer's patches, and they play critical roles in helping B cells to differentiate into plasma cells through production of IL-21 for humoral immunity (King *et al.*, 2008; Glatman Zaretsky *et al.*, 2009).

T cell stimulation not only induces proliferation, differentiation and effector T cell function for immune response activation, but also induces immune tolerance. There are several mechanisms that have been proposed to maintain immune tolerance by removing or neutralizing autoreactive T cells that have potential to recognize self-antigens as harmful substances. These immune tolerance mechanisms include immunosuppressive cytokine-producing Treg cells, T cell anergy (unresponsiveness), and activation-induced T cell apoptosis (cell death) (Van Parijs & Abbas, 1998; Xing & Hogquist, 2012) (Figure 1.2).

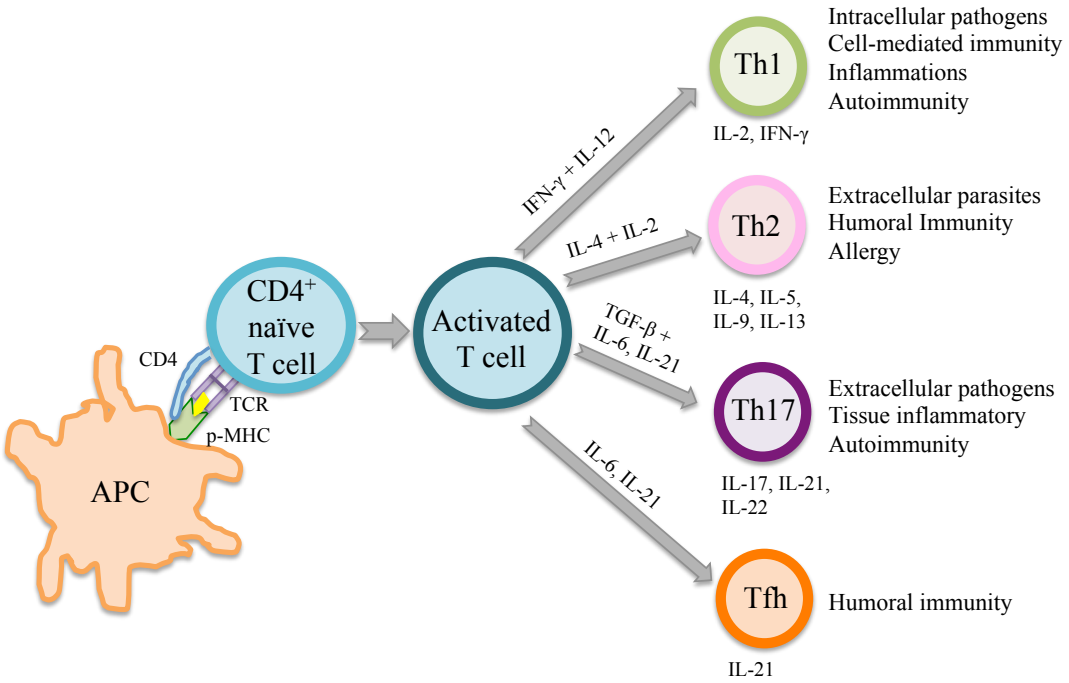

Figure 1.3: Cytokine profile and functions of $CD4^+$ helper T cells

Most of the T cells bearing TCR with specificity to thymic expressed self-antigens are deleted during development in the thymus (central tolerance), which is further discussed in Chapter 1.4. Although central tolerance is effective in eliminating autoreactive T cells, however not all self-antigens are expressed in the thymus. These extrathymic self-antigens include antigens that are only expressed at particular developmental stages, tissue-specific antigens that are exclusively expressed at the periphery, innocuous environmental peptides found in foods, and etc (Mondino *et al.*, 1996). Therefore, peripheral tolerance at the periphery is an important mechanism to tightly regulate against autoimmune disease.

1.3 T cell receptor (TCR) composition

T cells recognize antigens via a cell surface antigen receptor, known as TCR. TCR is a membrane-anchored heterodimeric protein composed of TCRα and TCRβ polypeptide chains bearing highly variable antigen-binding regions. TCR only binds to a portion of an antigen (epitope) that is displayed or presented on the surface of host cell, but not to soluble antigens (Kam & Power, 2012; Campbell *et al.*, 2015). TCR dimers can recognize and bind to specific antigenic peptides in combination with the MHC molecules (pMHC), but are unable to transduce signal into the cells (Bjorkman, 1997).

TCRα/β heterodimer is associated with the CD3 complex, which is composed of three dimers; γε, δε and ζζ that are involved in TCR-dependent signal transduction (Figure 1.4) (Irving *et al.*, 1993; Cantrell, 1996; Kirchgessner *et al.*, 2001). All the CD3 chains contain a conserved sequence motif important for signaling termed immunoreceptor tyrosine-based activation motif (ITAM) (Reth, 1989; Underhill & Goodridge, 2007). ITAMs transmit antigen-recognition signals into the interior of the cell upon antigen binding through its phosphorylation. As shown in Figure 1.4, each CD3ε, δ and γ molecule has a single ITAM, while CD3ζ molecule has three copies of ITAMs. Hence each TCR-CD3 complex contains a total of ten ITAMs (Weiss & Littman, 1994; Love & Hayes, 2010).

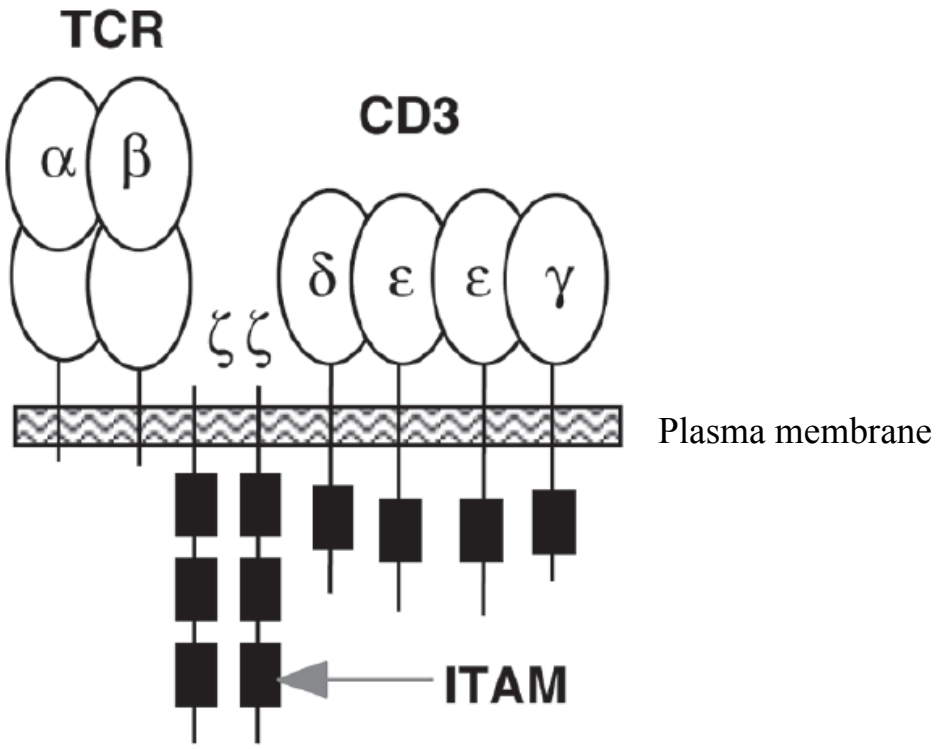

Figure 1.4: Schematic diagram of the T cell receptor/CD3 (TCR-CD3) complex expressed on T cell membrane (Broere *et al.*, 2011).

1.4 T cell development

T cell activation is tightly regulated to ensure triggering responses only occur to harmful foreign antigens but not to self-antigens. This objective is mainly achieved by thymic education (central tolerance), included positive and negative selection of thymocytes in the thymus (Berg *et al.*, 2002).

In thymic selection, CD4, CD8 and the TCR complex are important surface molecules to identify different thymocyte subsets (Erf *et al.*, 1998; Bosselut, 2004). Following generation in the bone marrow, lymphoid progenitor cells migrate to the thymus through the bloodstream to undergo development into T cells. Through interaction with the thymic stromal cells, these lymphoid progenitor cells are

triggered to proliferate and commit to T cell lineage (thymocytes), and subjected to a series of selection processes in the thymus (Kruisbeek, 1999; Anderson *et al.*, 2007).

Progenitor T cells enter the thymus at cortico-medullary junction. Since these T cell progenitors in the initial immature stage lack expression of CD4 and CD8 molecules, as well as TCR on the cell surface, they are called double-negative (DN) thymocytes (Figure 1.5) (Koch & Radtke, 2011). DN thymocytes migrate to subcapsular region of the thymic cortex and undergo the process of TCR gene rearrangements (recombination of TCRβ, γ and δ genes followed by TCRα gene rearrangement) and eventually generate two T cell lineages of mature thymocytes: the majority of thymocytes with TCRαβ and the minority with TCRγδ (Raulet *et al.*, 1991; Capone *et al.*, 1998; Livák *et al.*, 1999).

Gene rearrangement of TCRβ-chain locus occurs in αβ committed DN cells, productive in-frame β chain is then paired with pre-TCRα (pTα) surrogate chain to form pre-TCR that enables it to be expressed on the cell surface (von Boehmer & Fehling, 1997). Pre-TCR-CD3 complex assembly leads to cell proliferation, arrest further β-chain locus gene arrangement (allelic exclusion) and expresses both CD4 and CD8 on the surface of the same cell, generating double-positive (DP) thymocytes (Figure 1.5). This series of events induced by pre-TCR signal is collectively known as β-selection checkpoint (Groettrup & von Boehmer, 1993; Levelt *et al.*, 1995). Later, DP cells stop proliferating and α-chain locus starts to rearrange in these small resting DP cells. Due to somatic recombination of TCR genes, up to millions of different clonotypic variants of distinct TCRαβ with random specificities can be synthesized. DP cells then lower the expression level of TCRαβ,

and CD3 complex associated with the TCR heterodimer. Eventually, these DP thymocytes are eligible for selection (van Oers *et al.*, 1995; Schnell *et al.*, 2006).

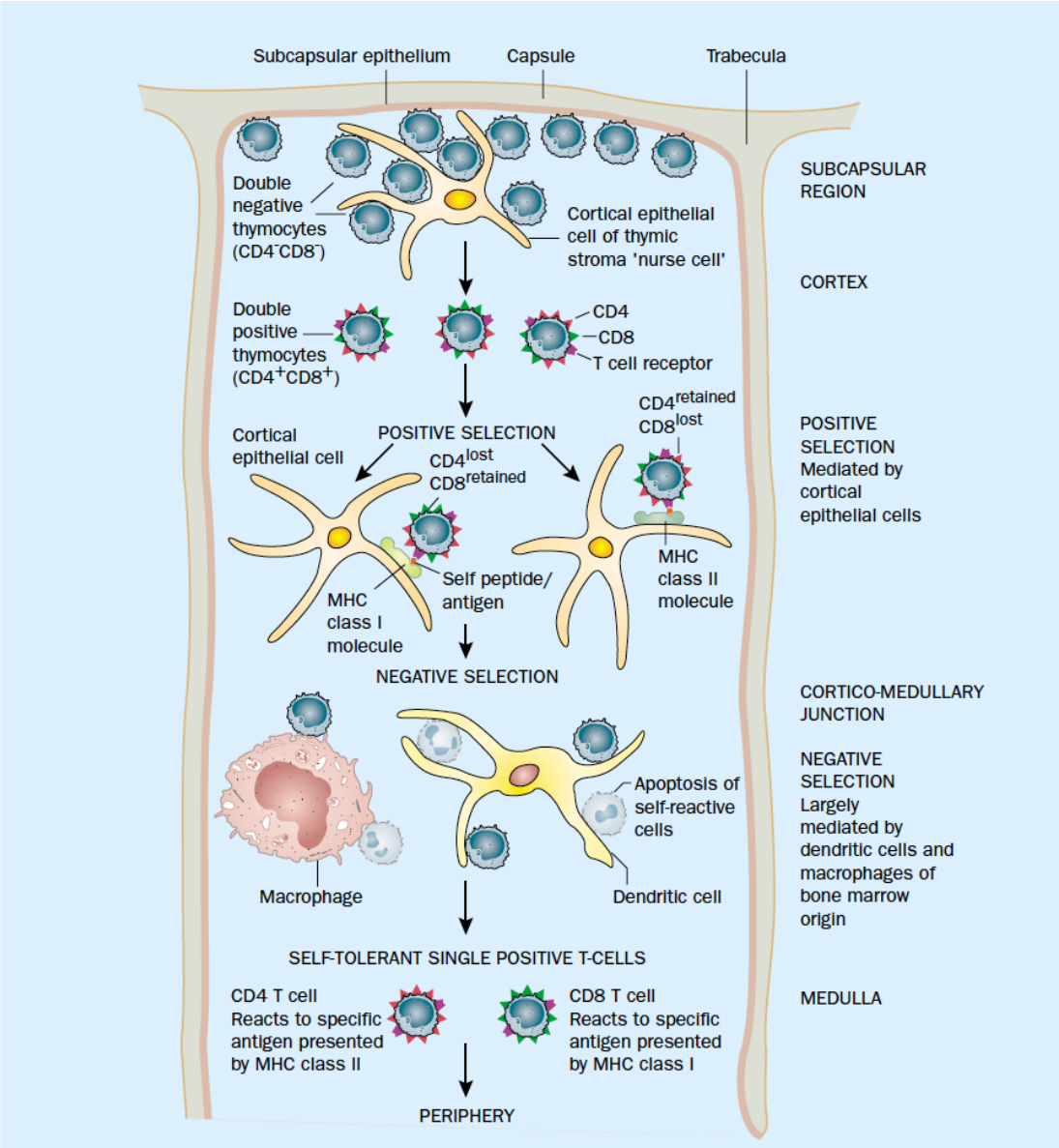

Figure 1.5: T cell maturation and development in the thymus (Parkin & Cohen, 2001).

Thymic selection is a critical event in T cell development, resulting in survival of self-restricted cells (positive selection) and death of self-reactive/autoreactive cells (negative selection) (Robey & Fowlkes, 1994; Jameson *et al.*, 1995) (Figure 1.5). DP cells that can recognize self-MHC molecules on cortical thymic epithelial cells are positively selected. But those that bear 'useless' TCR that fails to recognize self-MHC molecules will undergo programmed cell death referred to as "death by neglect" because low avidity engagement between TCR and MHC molecule could not provide enough TCR signaling to sustain cell viability (Egerton *et al.*, 1990; Anderson *et al.*, 1996). This lineage fate is determined by the restriction specificity of TCR to MHC molecules, i.e. CD8 for MHC-I and CD4 for MHC-II molecules (Singer *et al.*, 2008). Furthermore, DP cells are also undergoing negative selection in thymus to eliminate the potential threat of autoreactive cells to healthy host cells. Those cells that possess high avidity TCR binding towards self-peptide presented by MHC complex (self-pMHC) on thymic dendritic cells (DCs) and macrophages, are directed to cell death. Positively selected DP cells migrate from the cortex to the medulla after receiving cues for survival and ultimately differentiating into $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes (Murphy *et al.*, 2008; Xing & Hogquist, 2012). Mature SP cells are eventually exported to the periphery as mature naïve SP $CD4^+$ or $CD8^+$ T cells.

During T cell development, thymocytes express distinctive cell surface proteins, which are useful markers that differentiate thymocytes at different stages of development. With particular combination of these surface markers, stages of differentiation and populations of T cells can be identified. There are several surface

markers that are commonly used for mouse thymocyte development studies, including CD24, CD69, CD5, and etc.

CD69 and CD5 surface expression are commonly used as indicators of positive and negative selection for DP thymocytes. Thymocytes have 4 distinct stages of development and can be defined by the expression of CD3 and CD69 molecules. $CD3^{lo}$ $CD69^{-}$ cells represent DN cells and pre-selection DP thymocytes, $CD3^{int}$ $CD69^{+}$ cells represent thymocytes that undergoing positive selection, $CD3^{hi}$ $CD69^{+}$ cells represents post-positive-selected immature SP cells and $CD3^{hi}$ $CD69^{-}$ cells represents mature SP cells (Lesourne *et al.*, 2009; Wang *et al.*, 2012).

CD5 has been shown to negatively regulate TCR signaling (Tarakhovsky *et al.*, 1995). In a study on CD5 deficient mice, the fate of DP thymocytes was shown to shift from weak positive selection towards higher efficiency of positive selection or weaker negative selection, or to shift from strong positive selection towards low efficiency of positive selection or stronger negative selection, depending on the specificity of TCR transgenic (Tg) mice used. It was suggested that CD5-mediated signal inhibition for thymic selection was strongly dependent on the avidity of the positive selecting TCR-MHC ligand interaction (Azzam *et al.*, 2001).

CD24, also known as heat-stable antigen (HSA), expression decreased as the cells undergo maturation in SP cells (Crispe & Bevan, 1987). CD24 is used as a maturation marker and its expression level can be used to divide SP cells into two populations; $CD24^{hi}$ and $CD24^{lo}$ SP cells as mature and immature cells, respectively.

In addition to this, the members of the Bcl-2 family, i.e. Bcl-2 and Bcl-xL were shown to have function as pro-survival factors of thymocytes and peripheral T cells. Bcl-2 was shown to confer survival advantage in DP cells by inhibiting negative selection (Williams *et al.*, 1998; Siegel *et al.*, 1992), whereas Bcl-xL was suggested to salvage and elevate maturation of CD8SP cells independent of selection background (Chao & Korsmeyer, 1997).

At the peripheral lymphoid organs, naïve T cells circulate continuously until they encounter corresponding antigens presented by APCs, such as DCs and macrophages. Among APCs, DCs are the most efficient "professional" APCs due to their ability to provide co-stimulatory signals for full activation of T cells (Kooten & Banchereaut, 1997). Optimal TCR activation requires both TCR-induced signals and co-stimulatory signals. TCR engaged without co-stimulatory signals is insufficient for full activation of T cells and leads T cells to anergic state that eventually results in T cells rendered non-responsive to subsequent antigen stimulation (Mueller *et al.*, 1989; Pentcheva-Hoang *et al.*, 2009).

Co-stimulatory molecules are important to enhance TCR signaling by producing antigen-independent signals (second signal) in addition to antigen-specific signal through TCR (first signal) for full T cell activation (Macián *et al.*, 2004; Dustin, 2005). The first signal is responsible for antigen-specific immune response, while second signal is implicated in T cell adhesion and/or TCR signal enhancement (Abraham *et al.*, 1999). Requirement of co-stimulatory signals was proposed as a mechanism for maintaining peripheral T cell tolerance since co-stimulatory ligands are strictly provided by professional APCs (Chambers *et al.*, 2001).

There are a number of surface co-stimulatory molecules that are important for T cell activation, including CD80/CD86 (B7-1/B7-2) and lymphocyte function-associated antigen-1 (LFA-1) that binds to co-receptors CD28 and intracellular adhesion molecule-1 (ICAM-1) of T cells, respectively (Figure 1.6) (June *et al.*, 1990; Delves & Roitt, 2000b; Parkin & Cohen, 2001).

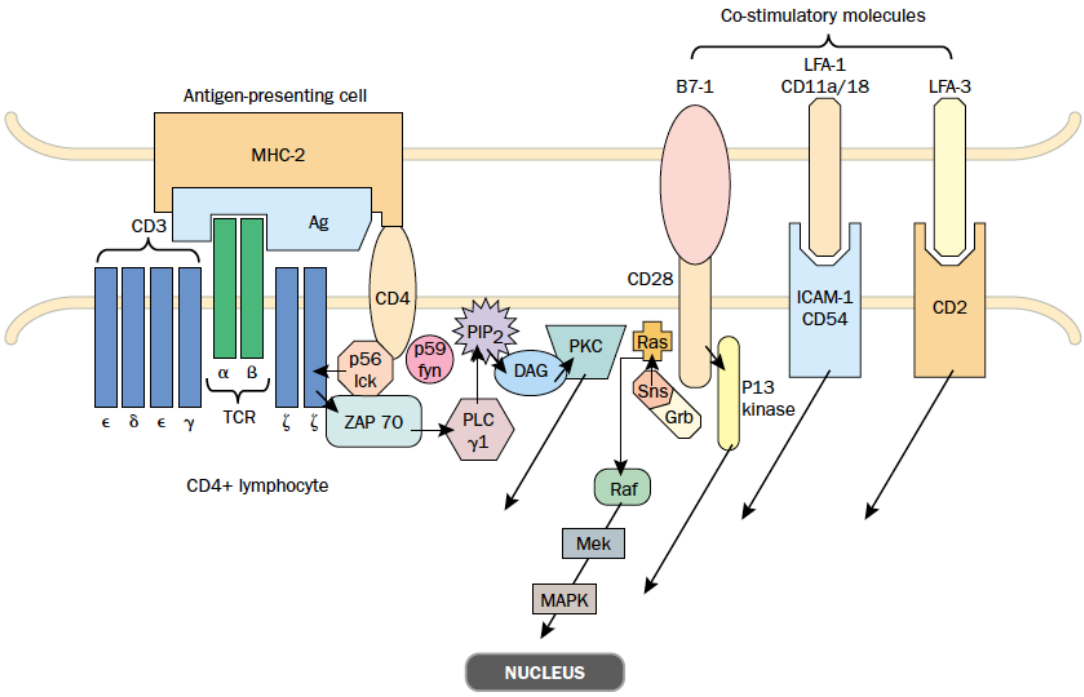

Figure 1.6: Cell surface molecules involved in T cell activation (Parkin & Cohen, 2001).

In contrast, co-receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) were found to transduce inhibitory signals that are implicated in negative regulation of T cell responses. Negative regulatory mechanisms not only function to terminate activation signals triggered by extracellular mediators, but also to set the signaling threshold for T cell activation. CTLA-4 binds to the same ligands CD80/CD86, as CD28. PD-1 has two ligands, which are PD-ligand 1 (PD-L1) and PD-ligand 2 (PD-L2). Co-inhibitory signals

(negative second signals) are feedback mechanisms triggered by T cell activation, which function to limit T cell responses, mediate peripheral tolerance and prevent autoimmunity (Chambers *et al.*, 2001; Chen, 2004; Parry *et al.*, 2005; Vigan *et al.*, 2012).

1.5 T cell response

T cells recognize antigen presented by APCs upon cell-cell contact. During contact, a specialized junction structure is generated at the interface between T cells and APCs, which is known as the immunological synapse (IS) (Dustin & Cooper, 2000; Kupfer & Kupfer, 2003; Huppa & Davis, 2003).

IS is characterized by segregated molecular rearrangements resembling a bulls-eye (Figure 1.7); a central region containing TCR, called central supramolecular activation cluster (cSMAC), which is surrounded by a ring of LFA-1 as a peripheral SMAC (pSMAC) and the most external ring enriched of large ectodomain proteins including protein tyrosine phosphatases CD45, known as a distal SMAC (dSMAC) (Monks *et al.*, 1998; Freiberg *et al.*, 2002).

As shown in Figure 1.7, the cSMAC structure (pink) contains the key molecules for T cell signaling, e.g. TCR-CD3 complex provides antigen-specific signals for T cell activation and the accessory molecules such as CD4 and CD28. Recently, cSMAC was suggested to be involved in signal termination through TCR internalization and degradation (Alarcón *et al.*, 2011). pSMAC (green), on the other hand, consists mainly of cytoskeletal or adhesion molecules, such as integrin, LFA-1 which serves to support cell adhesion and also IS maintenance (Monks *et al.*, 1998; Grakoui *et al.*,

1999). dSMAC (grey) is an active membrane movement area involve in centripetal movement of TCR-MCs. TCR-MC is further discussed later. A number of studies suggested that dSMAC corresponds to lamellipodial actin structure and pSMAC corresponds to lamellar actin structure. Both pushing force (actin polymerization) in dSMAC and pulling force (actomyosin contraction) in pSMAC coordinate TCR-MCs movement across the IS to the cSMAC (Dustin, 2007; Krummel, 2007; Sims *et al.*, 2007; Yi *et al.*, 2012).

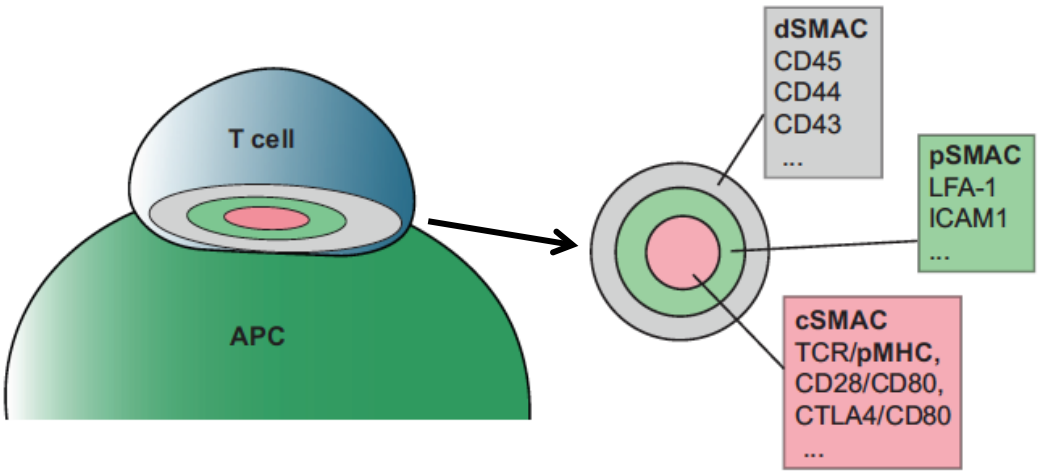

Figure 1.7: Schematic diagram of the immunological synapse (IS) formation on the interface between a T cell and an antigen-presenting cell (APC) with enriched molecules found in each SMAC zone (figure modified from Huppa & Davis, 2003).

It has been clarified that at early stages of contact, small clusters of TCR complexes accumulation termed "TCR microclusters" (TCR-MCs) are generated on the interface of T cells and APCs, which contain major upstream molecules of TCR signaling (Yokosuka *et al.*, 2005; Varma *et al.*, 2006). TCR-MCs are responsible to initiate and sustain signals for T cell activation through recruitment of kinases and adaptor proteins to form a 'signalosome' complex (Jordan *et al.*, 2003; Yokosuka *et*

al., 2005; Saito & Yokosuka, 2006). TCR-MCs continuously generated at the periphery of the IS in an actin-dependent manner and later translocate to the central of IS to form the cSMAC (Figure 1.8) (Krummel *et al.*, 2000; Varma *et al.*, 2006; Campi *et al.*, 2005).

The IS functions not only to initiate/boost TCR signaling but also attenuate strong signals by TCR endocytosis and degradation (Lee *et al.*, 2002; Lee *et al.*, 2003). TCR signaling is sustained in TCR-MCs and then terminated at cSMAC, where TCRs are sorted for degradation (Figure 1.8) (Varma *et al.*, 2006; Yokosuka *et al.*, 2008). T cell activation signals must be tightly regulated through multiple levels of positive and negative regulations, otherwise autoimmunity (excessive) or immunodeficiency (defective) might result, and TCR down-regulation is one of the principal mechanisms of negative regulation of T cell activation.

In the homeostatic state, a part of the TCR-CD3 complexes on plasma membrane of T cells constitutively undergo internalization and recycling back to cell membrane (Krangel, 1987; Minami *et al.*, 1987). Upon TCR ligation, the surface expression of TCR is rapidly reduced as the TCR-CD3 complexes are internalized/endocytosed and either rapidly recycles to the cell surface or targeted for degradation through lysosome compartments. TCR internalization was suggested to play a critical role in regulating T cell activation by attenuating TCR signaling upon stimulation (Valitutti *et al.*, 1995; Valitutti *et al.*, 1996; Valitutti *et al.*, 1997).

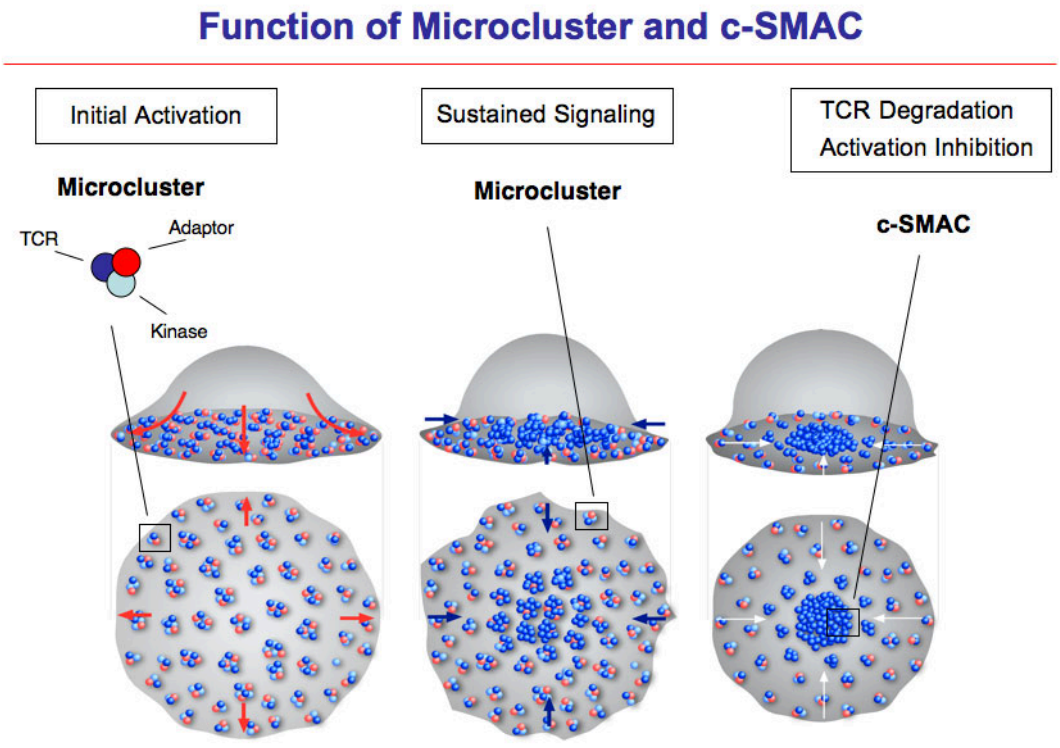

Figure 1.8: Dynamic process of TCR microclusters (TCR-MCs) and supramolecular activation cluster (cSMAC) formation on planar bilayer system (slide modified from Saito & Yokosuka, 2006).

1.6 TCR signaling cascades

In T cells, all their functions, i.e. proliferation, cytokine production and effector T cell differentiation are controlled largely by TCR signaling (Sebzda *et al.*, 1999; Singer & Koretzky, 2002). Engagement of TCR by the pMHC on the APCs initiates antigen-recognition signal, which is then translated into transmembrane signal and induces an array of intracellular signaling cascades through activation of kinases and tyrosine phosphorylation of many signaling molecules. These include the TCR/CD3 complex and also proteins assembly to form the signaling complex containing adaptors and effector enzymes. These immediate events are referred to as proximal TCR signaling (Kane *et al.*, 2000; Germain, 2001; Samelson, 2002; Jordan *et al.*, 2003). The intracellular signaling cascades ultimately determine the direction and

fate of the T cells, i.e. induction of specific functions, cytokine production, cell proliferation, transcription activation for cellular differentiation, effector functions, anergy or cell death, which are essential for the onset of a productive immune response (Unanue, 1984; Weiss *et al.*, 1987; Crabtree, 1989).

As shown in Figure 1.9, antigen recognition by TCR initially induces the phosphorylation of ITAMs within CD3 molecules, especially CD3ζ chains by lymphocyte-specific protein tyrosine kinase (Lck). Phosphorylated ITAMs provide docking sites for a spleen tyrosine kinase (Syk) family of kinases, Zap70 through its two tandem Src homology region 2 (SH2) domains and Zap70 is then phosphorylated by Lck (Chan *et al.*, 1994; Hatada *et al.*, 1995). Zap70 then acquires increased enzymatic activity to further activate several other downstream adaptor proteins, including two critical adaptor proteins, i.e. linker for activation of T cells (LAT) and SLP-76 (Chan *et al.*, 1994).

LAT is an integral membrane protein that localizes in the plasma membrane and it plays an important role to recruit numerous signaling molecules. Four key tyrosine residues within LAT are phosphorylated by Zap70 and resulted in recruitment of signaling molecules, including SLP76, PLCγ1, Vav 1 guanine nucleotide exchange factor (VAV1) and growth factor receptor-bound protein 2 (Grb2) to form a multiprotein complex, known as LAT signalosome (Zhang & Samelson, 2000). Activation of LAT and SLP76 then recruit additional signaling molecules, leading to the activation of downstream signaling pathways that branches into several distinct signal pathways (Figure 1.9) (Kane *et al.*, 2000; Brownlie & Zamoyska, 2013).

Activation of these signaling molecules then propagate the signals which branches to actin reorganization, calcium influx, the mitogen-activated protein kinase (MAPK) and the nuclear factor-κB (NF-κB) signaling pathways. All these effector cascades then lead to mobilization of transcription factors, which are critical for transcriptional activation of genes that play an essential role for cellular proliferation and differentiation. Besides, TCR signals also result in cytoskeletal reorganization and activation of integrins by inside-out signaling (Brownlie & Zamoyska, 2013).

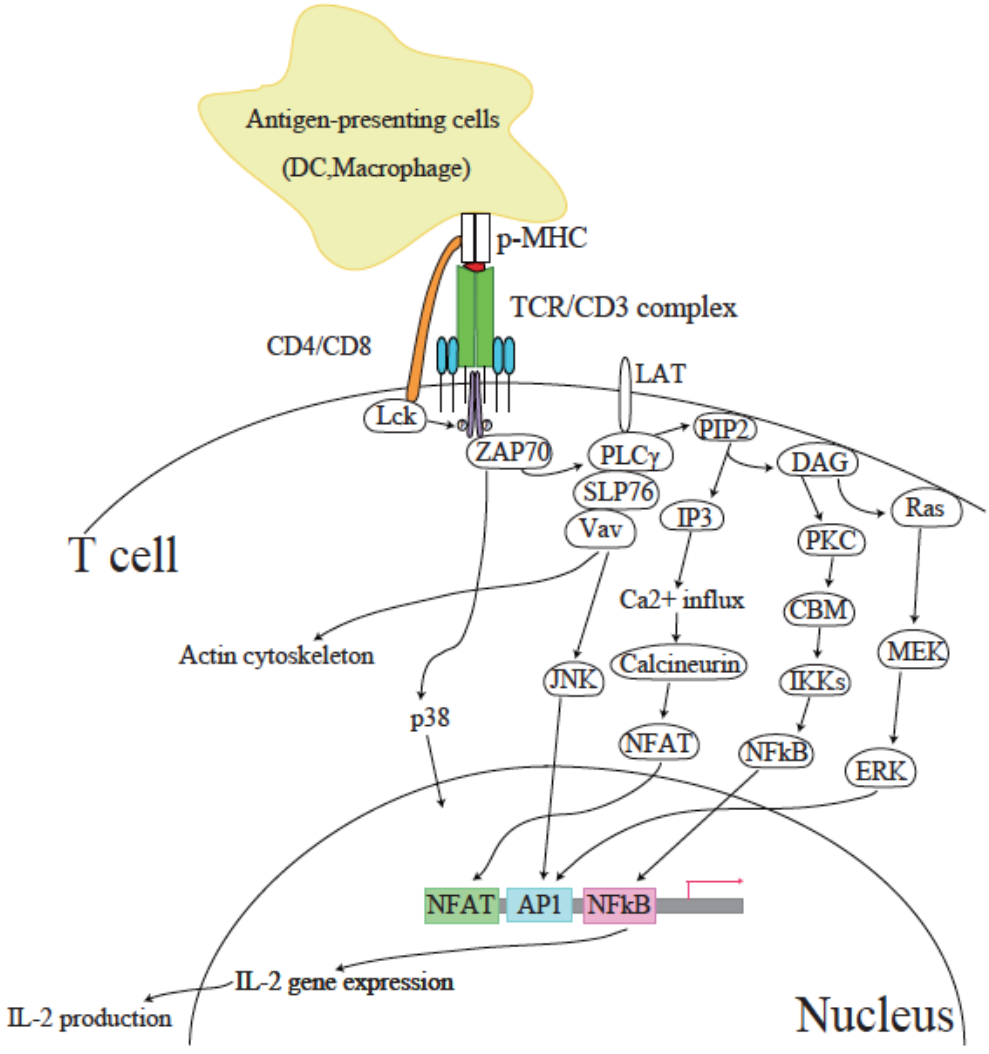

Figure 1.9: Overview of T cell receptor-dependent intracellular signaling cascades